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Improvement of new and traditional industrial crops by induced mutations and related biotechnology

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FOREWORD

Industrial crops are an important source of income for many small landholders in developing countries and contribute directly or indirectly to food security in rural areas. Crop diversification, finding alternative crops, development of new uses for existing crops and introduction of new crops are important components in the efforts to meet the demand for food, pharmaceuticals, chemical raw materials, fibres and fuel in developing and developed countries. Plant breeding efforts combining genetic resources and induced mutations using classical, in vitro and innovative molecular approaches have been responsible for much of the development of industrial crops.

This co-ordinated research project (CRP) was initiated in 1994. It focused on developing mutagenesis approaches for previously unstudied species, screening procedures for agricultural and industrial requirements and suitable genotypes of traditional industrial crops adapted to new areas and for new needs. The industrial crops selected for improvement under this CRP were oilseeds and fibre plants. The potential of induced mutations to affect critical steps in various biosynthetic pathways leading to oil quality and other metabolic modifications was investigated.

The success of this CRP is evidenced by the application of mutation techniques, in combination with in vitro and molecular techniques in genetic improvement of oilseed crops such as soybean, rapeseed, sunflower, linseed, cuphea, meadowfoam and fibre plants such as cotton and jute. As a result, improved breeding lines are available in all the industrial crops that the CRP focused on. Novel oil types were developed in cuphea with potential use as a renewable, economical and safe energy source and in linseed with increased levels of saturated fatty acids. Genes of fatty acid synthesis were isolated from one species and used for modification of quality of other oilseeds. Disease and pest resistance was improved in oilseeds and fibre crops through transgenesis and introgression of desired genes from related species. It is expected that many of the breeding lines developed under this CRP will be released in the near future as official varieties. They will contribute to the increased crop options of farmers, sustaining the biodiversity of industrial crops, providing higher income at the farm gate, and improving availability of renewable resources and raw materials for the chemical industry. Industrial crop varieties with improved resistance to diseases and pests will lead to a more sustainable and environmentally friendly agriculture.

This publication summarizes the results presented at the third and final research co-ordination meeting of the CRP, which was held in Corvallis, Oregon, USA, 2–6 August 1999. The IAEA officers responsible for this publication are K. Nichterlein of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, and former IAEA officer A. Ashri.

EDITORIAL NOTE

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SUMMARY

1. INTRODUCTION

There is an urgent need for crop diversification to add new uses to existing crops and to introduce new crops to meet new demands for food, fibres, fuel, pharmaceuticals and chemical raw materials. This need is recognized in both the developing countries (to give more options to their farmers) and developed ones (to overcome surplus problems). It is driven by efforts to reach a more sustainable and environmentally friendly agriculture. These efforts call also for renewable resources, new and more specialized raw materials for the chemical industry, vegetable oils for fuel, disease and pest resistance sources which will reduce the use of pesticides, products with modified qualities and adaptation to stress conditions.

Industrial crops can fill these needs very well, especially since many are also food crops and they satisfy essential dietary needs. The crops grouped under the term 'industrial' have to be processed before use. Their products include oils, sugars, protein sources, food additives, beverages, fibres, gums, starches, elastomers, medicinal substances and stimulants. Furthermore, crops that have been traditionally grown for one purpose can be modified for other uses. Today many developing countries' economies and millions of smallholders depend heavily on their farm gate income from industrial crops.

Plant breeding efforts combining genetic resources and induced mutations using classical, in vitro and innovative molecular approaches have been responsible for much of the intensified development of industrial crops in recent decades. In addition, these efforts have changed quality characteristics, which are more exacting in industrial crops. Many of the critical steps in the relevant biosynthetic pathways are controlled by one or a few major genes, which can be modified by induced mutations. Similarly, many of the important characteristics distinguishing cultivated, domesticated plants from their undomesticated wild relatives are controlled by one or very few major genes. These lend themselves to modification by induced mutations and breeding manipulations.

It was against this background that the Joint FAO/IAEA Division initiated a co-ordinated research project on industrial crops in 1994, bringing together 9 institutions in developing and 5 institutes in developed countries to form a network and share approaches, methods, germplasm, etc. The first research co-ordination meeting (RCM) was held in Vienna, Austria in 1995 to discuss project work plans for the improvement of oil and fibre crops to be implemented during this CRP. The second RCM was held in Giessen, Germany in 1997 to report and review preliminary research results and discuss future activities. The results presented at the third and final RCM, held in Corvallis, Oregon, USA, in 1999 are presented in this TECDOC.

2. ACHIEVEMENTS

On the basis of the research conducted through this CRP, it is clear that induced mutations were used to generate mutated germplasm and genetic diversity in oil and fibre crops in Bangladesh, Brazil, Canada, China, Germany, Greece, Hungary, India, Pakistan and the USA. Mutation techniques in combination with interspecific hybridization, doubled haploid and molecular markers techniques as well as genetic transformation techniques were applied to modify agronomic and quality traits of oilseed crops such as soybean, rapeseed, sunflower, linseed, cuphea, meadowfoam and *Oenothera*, and fibre plants such as cotton and jute. New mutation and biotechnological methods have been developed for improvement of these crops.

Improved germplasm, isolated genes and DNA sequences for modification of fatty acid profiles have been exchanged among the participants.

2.1. New methodologies/approaches

2.1.1. Oilseed crops

Genetic transformation of soybean remains difficult and has been successful in only very few genotypes. The Chinese commercial variety of soybean, Hei Nong 35, developed from a cross with the mutant Heinong 16, was identified as being very suitable for genetic transformation and a RAPD marker for resistance to race 4 of the soybean cyst nematode (*Heterodera glycines)* was developed.

Microspore culture from rapeseed plants that were irradiated with 50 Gy led to the development of doubled-haploid lines with double zero quality ("Canola quality"). This indicates that the combination of radiation and doubled-haploid techniques can speed up the development of improved rapeseed germplasm. Doubled-haploid plants developed from mutant hybrids of rapeseed indicate a potential for fixation of mutant heterosis in rapeseed.

A transformation protocol for rapeseed was modified for use in the development of medium-chain fatty acids in new genotypes. Transformed rapeseed plants with increased levels of saturated fatty acids were developed.

Fluorescence in situ hybridisation (FISH) and Genomic *in situ* hybridisation (GISH) analysis techniques which were developed for Brassica oilseeds facilitated the localization of repetitive DNA sequences and characterisation of donor DNA from *Raphanus sativus*, *Sinapis arvensis* and *Coincya monensis* in rapeseed hybrids.

The regeneration potential of interspecific hybrid lines of sunflower was evaluated from *in vitro* cultured apical meristems: hybrid combination of cultivated sunflower with *H. decapetalus*, *H. giganteus*, *H. mollis* and *H. strumosus* showed high shoot regeneration. Hybrids with *H. decapetalus* and *H. strumosus* have acceptable agronomic performance, therefore they were identified as ideal breeding material for further improvement through biotechnological methods.

Over 100 simple sequence repeats (SSRs) molecular markers were developed in sunflower and will be in the public domain. Genes controlling steps in the biosynthesis of fatty acid synthesis of sunflower were cloned.

Mutation techniques were successfully used to create novel oil types in cuphea with increased content of short and medium-chain fatty acids. Mutant lines with short-chain fatty acids have potential use as a renewable, economical and safe energy source.

In meadowfoam (*Limnanthes* spp.) mutation techniques were successfully used to reduce the erucic acid content of the seed oil from about 9 to 2%, opening the way to develop new industrial and food market niches for meadowfoam oil.

A mutation protocol for gamma treatment of seeds was developed for the domestication of *Oenothera erythrosepala* as a new oil crop providing gamma linolenic acid as raw material for the medical and health products industries. Cultural practices for the best early maturing mutant line were established (sowing date, planting density, fertilizer treatment).

TABLE I. IMPROVED BREEDING LINES OF VARIOUS OIL AND FIBRE CROPS WITH USEFUL TRAITS PER COUNTRY

Crop	**Country**	**Lines developed**	**New useful traits**
Soybean	China	5 mutant lines	Increased yield
	Hungary	2 mutant lines	Increased protein/oil content
Rapeseed/mustard	China	15 doubled-haploid mutant lines of *Brassica napus*	Improved oil and meal quality ('double zero')
	India	7 mutant lines of *B. napus*	Improved oil and meal quality ('double zero')
	Germany	Intergeneric lines from *B. napus* x *Raphanus sativus*	Nematode resistance
	Pakistan	14 advanced mutant lines of *B. juncea*	Increased seed yield, early flowering
		3 DH lines from *B. napus* mutant hybrids	Increased yield potential
		More than 50 mutants and recombinants from various *Brassica* species	Improved oil quality (reduced erucic acid content)
Sunflower	Brazil	50 early mutants	Putative tolerance to Alternaria
	Germany	29 interspecific hybrids	Sclerotinia resistance
		9 interspecific hybrid progenies	Improved *in vitro* response useful for transgenesis
	India	9 mutant lines	Consumer-preferred seed colour (black seed) and increased yield
Linseed	Canada	1 stable recombinant mutant line	High palmitic and low linolenic acid for specialised oil with improved shelf life, brown seeds
Cuphea	USA	Mutant germplasm collection	Non-stickiness, changed oil quality for various industrial purposes (high capric, high caproic, high myristic acid)
Meadowfoam	USA	2 elite populations with mutant introgressions	Low erucic acid content with improved nutritional quality
Oenothera	China	2 mutant lines	Early maturity
		1 interspecific line	High capsule density
Cotton	Bangladesh	2 mutant lines	Earliness
		12 mutant lines	Earliness, increased fibre yield and quality
	Greece	2 mutant lines	Earlier maturity
	Pakistan	*G. hirsutum* lines from DNA-mediated trans-formation with other *Gossypium* species	Virus resistance or improved yield
	USA	Various genotypes of *G. arboreum* and *G. longicalyx*	Good economic yield or resistance to reniform nematode
		Transgenic lines with lectin genes	Increased tolerance to boll worm and aphids
Jute	India	One mutant line of *Corchorus olitorius*	Photoperiod insensitivity suitable for new cropping systems

TABLE II. BREEDING LINES, GERMPLASM, DNA SEQUENCES AND GENES EXCHANGED AMONG PARTICIPATING COUNTRIES

Crop	**Donating country**	**Receiving country**	**Exchanged material**
Rapeseed/mustard	Germany	Pakistan	Resynthesized rapeseed with high erucic acid
	Germany	China	Canola type rapeseed based on resynthesized material
	Germany	India	Canola types of brown mustard
Sunflower	Germany	Brazil	Interspecific hybrid progenies as potential source for disease resistance
	India (recommended)	Brazil	True-breeding mutants as potential source for disease resistance
	USA	Germany	Simple sequence repeats (SSR, micro-atellites) used in molecular marker development for disease resistance, oil quality and other agronomic traits Genes for fatty acid biosynthesis (oleoyl desaturase, OLD7; stearyl ACP desaturase, SAD7 and SAD17) for mapping and use in transformation experiments
Linseed/flax	Germany	Canada	Doubled-haploid lines
	Canada	Germany, India, USA	Low linolenic lines (solin types)
	USA	Canada, Germany, Netherlands	Recombinants between solin types and fibre flax
Cuphea	USA	Canada	Cuphea gene for synthesis of medium-chain fatty acids (acyl-ACP thioesterase, FATB) for transformation of linseed (solin)
Cotton	USA	Pakistan	Three-species cotton material with virus resistance, wild cotton species, molecular marker literature and software Genomic DNA of twenty *Gossypium* species
	Greece	Bangladesh	Two early cotton cultivars
Oenothera	USA	China	Local germplasm

Novel oil types were developed in linseed with increased levels of saturated fatty acids and medium chain fatty acids by transferring the medium chain thioesterase gene (*Fat B*) from *Cuphea wrightii* to mutant low linolenic acid lines of linseed.

2.1.2. Fibre crops

Molecular markers closely associated with genes for adaptation and tolerance to water-deficit, cytoplasmic male sterility, fertility, restoration and semigamy were identified in cotton using differential display and were cloned.

A gene coding for a membrane active peptide (magainin) inhibiting the growth of pathogens was constructed and tobacco, as a model system, was genetically engineered with this gene. A study is underway to develop a new transformation system for cotton *in planta* by *Agrobacterium* to avoid the need for callus regeneration.

Upland cotton (*Gossypium hirsutum*) was transformed with DNA of other *Gossypium* species carrying virus resistance or high fibre quality using DNA-mediated embryo transformation technique. Gamma-irradiation of donor DNA at low doses increased the introgression rate of desired traits into upland cotton.

2.2. Induced biodiversity for breeding

In all industrial crop species, promising mutant and breeding lines were obtained through the various approaches adopted during the CRP (Table I). Various traits were improved affecting yield, early maturity, oil and protein content of oil seeds, nutritional quality of oil and meal of oilseeds, fatty acid modification of oilseeds for potential use as specialised industrial oils and biodiesel, resistance to biotic stress factors (e.g. fungal and virus diseases, insect pests, nematodes), plant architecture and response to photoperiod.

2.3. Exchange of germplasm, and isolated genes and DNA sequences among participating research groups

Varieties, germplasm collections and breeding lines of various industrial crops developed through mutagenesis, interspecific hybridisation, cross breeding and doubled-haploid technique were shared among participating groups and their national collaborators. In addition to sharing seed material, DNA sequences and isolated genes were exchanged for assessment of genetic diversity, mapping and genetic transformation among research groups working on the molecular level in breeding research (Table II). The transfer of the the FATB gene isolated from Cuphea by Oregon State University, to the Canadian linseed programme, successfully resulted in a linseed oil containing medium chain fatty acids and increased C 16:0 levels, with potential for use in new industrial applications.

3. RECOMMENDATONS

3.1. Oilseed crops

Significant achievements were made during this CRP, in both public and private sectors regarding modification of oil content and composition of oilseeds using induced mutations and gene transfer from related species. As a consequence, a vast array of fatty acid variants is available, offering possibility for the development of specific genotypes and varieties for the production of various kinds of specialty oils, both for food and non-food uses.

At the same time demand for vegetable oil continues to increase, especially for human consumption and particularly in developing countries.

In this field the private sector is very active in the development of novel varieties, with progressive increases in yield potential. However, in order to stabilize production and realize the yield potentials, the disease and pest resistance of relevant oilcrop species must be substantially enhanced. Such an approach contributes to sustainability through Integrated Pest Management (IPM) and hence to reduced environmental degradation.

The available sources of genetic variation for disease and pest resistance are not adequate. Therefore, ways of base-broadening respective genetic variation for resistance to biotic stresses are urgently required, by using:

— Introgression of genes from alien germplasm via interspecific and intergeneric hybridisation either through biotechnology assisted sexual hybridisation (with or without radiation-assistance) or by protoplast fusion (asexual hybridisation, somatic hybridisation);
— Radiation or chemically induced mutations yielding lines and varieties resistant to major diseases and pests;
— Identification and cloning of respective genes controlling disease reaction(s) of the host plant(s);
— Directed mutagenesis of respective disease-related genes on a molecular level;
— Transfer of wild type resistance genes or newly created alleles into the respective, susceptible crops.

The enhancement of variability of oilseed crops is in line with the base-broadening concept of the Global Plan of Action for Plant Genetic Resources for Food and Agriculture.

Future research networks in the area of industrial crops should focus on enhanced production stability (improved tolerance and/or resistance to biotic and abiotic stresses) and concentrate on related plant species or genera, respectively, in order to be able to make use of similar genes and biosynthetic pathways within respective taxonomic groups (e.g., *Brassicaceae* or *Compositae* families).

3.2. Fibre crops

When objectives for improvement of specific growth characteristics, quality parameters, agronomic traits, or yield are established, researchers should examine all options for attaining the goals. This should include a survey of the available literature for approaches that may have been used by others with similar needs. Execution of a plan of action should firstly optimise production and management practices including time of planting, fertilization, plant population (spacing within and between rows), and disease and pest management. When additional genetic diversity for the desired trait(s) must be identified, induced mutations can be an option, as well as a wide survey of germplasm (with potential for possessing the desired diversity). Additionally, when mutants or introgressed lines are developed, production and management protocols should be reconfirmed and modified as necessary to optimise expression of the desired traits.

In cotton, fibre yield is genetically complex due to quantitative traits associated with the development of bolls and fibre, which cannot be easily incorporated into viable breeding programmes. The identification of mutational changes of quantitative traits can be improved

by dissecting them into component traits. When component or specific traits (qualitative) are desired that cannot be found among available germplasm or in well-adapted germplasm, then induced mutations can be considered a viable approach. Bast fibre crops may be even more amenable to the creation of useful diversity by induced mutation because vegetative growth is the important yield component and modifications in the yield can be more easily identified.

Future cotton improvement for quantitative traits, such as yield, will also depend upon utilization of a broader range of germplasm. Marker-assisted selection can improve the efficiency of incorporating novel traits from exotic germplasm and mutants into elite lines. This requires a substantial set of publicly available genome markers (preferably PCR-based, such as SSRs). Agencies interested in technology transfer should promote the development, availability and utilization of such markers in conjunction with broadened germplasm utilization.

Genetic engineering for specific traits will remain a viable approach, especially for traits that improve broad tolerance to biotic and abiotic stresses versus "magic bullet" genes targeted to specific pests. An exception to this might be when a single pest is the major limiting factor to economic production in a region, e.g. Cotton Leaf Curl Virus (CLCuV).

Economically, yield enhancement and stability (while maintaining fibre quality) must be the over-riding objective of breeding. Specific areas that need to be addressed and improved which contribute to lower production costs or which help in reaching the genetic yield potential of a genotype include one or more of the following characters (depending on the production region):

- Drought tolerance
- Heat tolerance
- Cool temperature tolerance
- Improved carbon partitioning (harvest index).

More attention should be given to the development of cotton-based niche markets and speciality crops. This could include, among others, “tailored” fibre colours, novel engineered fibre traits, antibody production, pharmaceuticals, etc.

3.3. Information systems

The FAO established an interactive information system on ecology knowledge for natural resources managers (ECOPORT), which includes information on the industrial crops worked on during this CRP. Participants are invited to get actively involved to contribute information on their breeding programmes (e.g. induced mutations) and successes to the existing ECOPORT information system (http://ecoport.org).

3.4. General

In view of the many beneficial developments emanating from CRPs, as clearly demonstrated in this one, it is recommended to sustain and enhance the CRP approach. The present CRP exemplifies so well the tangible benefits of such an approach in terms of collaborative research, exchange of breeding lines and genetic stocks and sharing of information and techniques. Other benefits, especially of longterm research contacts and training opportunities, should also be recognized.

Use of induced mutations and biotechnology to tailor industrial crops for new crop rotations and quality improvement

D.C. Joshua, S.J. Jambhulkar

Nuclear Agriculture and Biotechnology Division,
Bhabha Atomic Research Centre,
Trombay, Mumbai, India

Abstract. The induced mutation approach was employed to develop photoperiod insensitive mutants in jute (*Corchorus olitorius*), black hull in sunflower (*Helianthus annuus*) and double zero lines in *Brassica napus.* A late flowering mutant isolated in the M_2 generation of jute variety TJ-40 was insensitive to the inductive short photoperiod for a significantly longer time than the parent. This trait of the mutant was confirmed by protracted sowing and exposure to short-days. In sunflower, black hulls are preferred by consumers. Black-hulled mutants with high seed yield were isolated in the variety 'Surya' having black and white zebra patches. Three of these mutants had significantly higher yield than the parent at two different locations. In *Brassica napus*, early flowering, high yielding mutants with double zero traits were developed. The fatty acid profile showed significant increase in the oleic acid content in these mutants.

1. JUTE

1.1. Introduction

Jute (*Corchorus capsularis* and *C. olitorius*) is a bast fibre crop. The fibre is obtained from bark and hence fibre yield is positively correlated with plant height. Plant height is determined by floral initiation. Plant growth in height stops with the onset of flowering. Hence, early flowering is an undesirable trait. Jute, being a short-day plant, flowers early when exposed to short-days. Jute can be planted without the risk of early flowering and the consequent low yield only during long-day periods. Developing varieties insensitive to photoperiod is thus a major objective in jute breeding. A day neutral mutant was earlier developed in *C. capsularis* using the induced mutation approach [1]. In *C. olitorius* such types are not available [2] and this species is sensitive to the inductive photoperiod as early as cotyledonary leaf stage. The experiments under this project are aimed at developing photoperiod insensitive mutant in *C. olitorius*.

1.2. Materials and methods

1.2.1. Radiation treatment and M_1 generation

A high fibre yielding *C. olitorius* variety TJ-40 was used in this study. Five hundred seeds (7% moisture) per dose were exposed to gamma ray doses of 700, 900 and 1100 Gy from ^{60}Co source at a dose rate of 20 Gy/min. The M_1 generation was grown during the normal season (June sowing). At maturity, five pods from different regions of each plant were harvested from all the M_1 plants and the seeds were bulked dose wise.

1.2.2. M_2 and M_3 generations

The M_2 generation was grown during the month of short-days (Dec. sowing) to isolate late flowering mutants. The M_2 population had 71,000 plants. Control plants were grown at

regular intervals after every 25 lines. Plants which flowered 10 days later than the control or more were labelled and seeds were collected at harvest. Seeds of the 19 M_2 plants, which flowered later than the parent, were sown during early March 1998 along with the control to study the flowering response of the putative mutants in M_3.

1.2.3. Experiments to assess the flowering response of the late flowering mutant

The late mutant (LFM) which flowered later than all the putative mutants and the parent variety, TJ-40, were sown on different dates viz. Feb. 10 and March 16 (short-day months) and June 2 (long-day month) to study their flowering response to photoperiod. Data on the time of flowering and plant height at flowering were recorded. The parent variety and the late flowering mutant were exposed 15 and 30 days after sowing to short days (8 h) by keeping them in the dark for 16 h. The time of flowering and plant height at flowering were recorded.

1.2.4. Mutant × parent crosses

Reciprocal crosses were made to select recombinants having the desirable morphological traits of the parent and the short-day insensitivity character of the mutant.

1.3. Results and discussion

The field survival of the plants in the highest dose of 1100 Gy was 75% indicating that jute is relatively resistant to radiation. The control plants in M_2 generation grown during the months of short-days flowered 30 days after sowing. From the data on the time of flowering, 19 M_2 plants, which flowered later than the control, were isolated. These mutants had a vegetative growth period of 47-60 days. In the M_3 generation, when they were sown in early March, one of the mutants flowered later than all the others. This late flowering mutant (LFM) was used in further studies.

In the staggered sowing tests, sowing in February and March induced early flowering in the parent TJ-40. The days to flower were 28 and 31 days respectively (Table I). The corresponding numbers of days for the LFM were 231 and 208 days (Table I). Also when sown in June, the mutant had a significantly longer vegetative phase than the parent (Table I).

The plants of the parent variety exposed artificially to 8 h day flowered earlier than the LFM (Table II). In spite of exposure to the inductive short-days the LFM flowered only after 133-138 days (Table II).

Highly productive multiple cropping systems are essential to improve agricultural production. This demands changes in the traditional sowing time. Such changes in sowing time without affecting the yields are possible only with crop varieties insensitive to photoperiod and temperature. In crops like jute, which are typical short-day plants, even minor changes in sowing time lead to early flowering resulting in economically low biomass yield. In the present study, a mutant was induced which remains insensitive to the inductive short-photoperiod for a very long time compared to the parent. The photoperiod insensitivity and the long basic vegetative phase of the mutant were confirmed by staggered sowing and exposure to short-days. However, the mutant has the tendency to produce axillary branches, which is an undesirable trait. The mutant is being crossed to the parent to isolate recombinants with the non-branching trait of the parent and photoperiod insensitivity of the mutant.

TABLE I. DATE OF SOWING, DAYS TO FLOWER AND PLANT HEIGHT OF TJ-40 AND LATE MUTANT (LFM)

Genotype	Date of sowing	Date of flowering	Days to 50% flowering	Mean plant height, cm
TJ-40	Feb.10, 98	Mar.10, 98	28	29 ± 2
LFM	Feb.10, 98	Sept.29, 98	231	262 ± 18
TJ-40	Mar.16, 98	Apr.16, 98	31	67 ± 2
LFM	Mar.16, 98	Oct.10, 98	208	283 ± 13
TJ-40	Jun. 2, 98	Aug. 28, 98	87	316 ± 14
LFM	Jun. 2, 98	Oct. 11, 98	131	288 ± 10

TABLE II. FLOWERING DATA AND PLANT HEIGHT OF TJ-40 AND LATE MUTANT EXPOSED TO SHORT DAYS 15 AND 30 DAYS AFTER SOWING (DAS)

Genotype	Date of sowing	Time of exposure, DAS	Date of flowering	Days to flower, No.	Mean plant height, cm
TJ-40	Jun. 2, 98	15	Jul. 29, 98	57	115 ± 14
LFM	Jun. 2, 98	15	Oct.18, 98	138	266 ± 10
TJ-40	Jun. 2, 98	30	Aug. 5, 98	64	142 ± 9
LFM	Jun. 2, 98	30	Oct.13, 98	133	256 ± 5

2. SUNFLOWER

2.1. Introduction

Sunflower was introduced to Indian agriculture in the late 60's. From a mere 500 ha in 1972-73 its area increased to 2.1 million ha with the production of 1,500,000 ton in 1997-98. Being an introduction, the variability is limited. It is very important to enhance the range of variability for morphological characters [3], yield and its contributing characters [4]. The existing efforts in India to generate variability are mainly through hybridisation, whereas studies on mutation breeding are meagre [5,6].

'Surya' is the first high yielding open-pollinated variety released for cultivation in India. The yield potentiality (1,300-1,600 kg/ha) of this variety is comparable with hybrids. However, this variety is not preferred in market because of the black and white zebra patches on the hulls. The preference of the farmers and traders is for black hulled varieties. Therefore isolation of a high yielding black hull mutant has been identified as an important objective in the sunflower mutation breeding programme.

2.2. Materials and methods

The high yielding variety Surya was selected for mutation studies. Approximately 400 seeds per dose of an inbred line (S_8) containing less than 5% moisture were irradiated with 50,

100, 150 and 200 Gy doses of gamma rays at 20 Gy/min. The M_1 generation was grown along with the control and observations on germination, chlorophyll sectors, survival, leaf deformities and chimera were recorded during the crop growth. All plants were selfed at the time of flowering, using muslin cloth bags. Their plant to row progenies were raised in M_2 generation where chlorophyll and morphological mutations were isolated. At maturity, individual plants were screened for hull colour mutations. Isolated mutants were studied in subsequent generations for their breeding behaviour and yield potential. The hull mutants were evaluated in unreplicated 2×2 m net plots for yield and its components.

2.3. Results and discussion

2.3.1. Plant injury

Increasing doses of radiation reduced the germination rate and increased the frequency of chlorophyll sectors and leaf deformities in the M_1 generation. Maximum chlorophyll sectors and leaf deformities like flecking, bifurcation and wrinkling were found in the 200 Gy dose of gamma rays (Table III). The frequencies of chlorophyll mutations like chimera, xantha and virescent in M_2 generation were also higher in the plants from the 200 Gy dose. It provided a good index of the radiation effect. Reduction of germination up to 20-30% in 200 Gy dose of gamma rays has been reported [4,7].

TABLE III. OBSERVATIONS IN M_1 GENERATION ON SUNFLOWER VARIETY 'SURYA'

Dose Gy	No. seeds treated	Germination %	Survival %	Frequency of chimera %	Leaf deformities %	Sectorial male sterility %
0	100	97.3	96.5	-	-	-
50	382	77.0	76.2	0.33	1.74	-
100	349	83.4	82.2	1.71	1.03	-
150	388	68.6	66.2	1.87	2.25	-
200	406	62.6	59.9	3.18	5.90	0.78

2.3.2. Morphological mutations

A large spectrum of variability for morphological characters was isolated and characterised [8]. Among them 3 were for chlorophyll, 9 for leaf, 3 for stem, and 8 for capitulum (Table IV). All of them bred true in subsequent generations. Distinct mutants were yellow leaf vein, fasciation and zigzag stem. Three characters were mutated in the wrinkled leaf mutant: the lamina was dark green and highly wrinkled, the petioles were thick and shortened and the ray florets were dissected. These novel mutations have not been reported so far, among the large number of mutations for morphological characters that were isolated and characterised for their inheritance pattern earlier [3]. Single recessive gene and two genes with complementary effect controlled most of them. Similar gene action for various morphological mutants has also been reported [9]. Genetic analysis of the fasciated mutant in our studies showed that it is governed by a single recessive gene.

TABLE IV. TRUE BREEDING CHLOROPHYLL AND MORPHOLOGICAL MUTATIONS IN M_4 GENERATION

Characters	No. mutants
Chlorophyll	3
Leaf morphology	9
Stem	3
Flower morphology	8
Hull colour	4

2.3.3. Hull colour mutations

The variety used in the present studies possessed black and white zebra patches on the hulls. Four hull colour mutations, viz. black, brown, white and horizontal brown patch were isolated. In the black hull mutations, seven mutants having relative differences for black hull colour were isolated. Among them, 3 were from 100 Gy, 2 from 150 Gy and 2 from 200 Gy dose. Their individual plant to row progenies were grown in M_3 and M_4 generations and were selfed at flowering to isolate true breeding mutants. All of them bred true for black hull colour. However, relative differences for blackness were found within the progenies. Therefore individual plant selections based on black hull colour were made up to M_5 generation. About 85 lines were selected. Of these, 40 stable lines were advanced to M_6 generation. Based on uniformity, only 25 lines were retained. At maturity, data on yield and its contributing characters were recorded on 25-30 plants each for preliminary yield assessment. Some selections were also tested in another location (Gauribidnur). The performance of these selections is presented in Table V. Almost all selections were shorter than the control.

TABLE V. MEAN YIELD AND YIELD CONTRIBUTING CHARACTERS OF SELECTED BLACK HULL MUTANT LINES IN M_6 GENERATION AT TROMBAY, AND THEIR YIELDS AT GAURIBIDNUR

Line	Plant height cm	Head diameter cm	100 seed wt. g	Oil content %	Yield /plant Trombay, g	Yield/plant Gauribidnur g
TS 16-3	144.6 ± 2.8	17.1 ± 1.9	8.1 ± 1.0	36.4 ±0.8	28.5 ± 1.2	44.9 ± 2.9
TS 19-2	138.0 ± 3.0	13.5 ± 0.8	6.8 ± 0.4	37.0 ± 0.6	26.7 ± 1.5	50.1 ± 1.9
TS 32-2	182.2 ± 2.8	15.6 ± 0.8	8.9 ± 0.5	37.0 ± 1.0	15.9 ± 1.1	48.5 ± 1.9
TS 37-3	180.3 ± 3.2	15.6 ± 1.5	8.5 ± 2.3	35.3 ± 0.8	18.3 ± 1.1	56.0 ± 2.2
TS 42-2	186.0 ± 2.2	18.0 ± 2.5	8.5 ± 0.3	36.2 ± 0.7	25.7 ± 1.2	60.3 ± 2.5
TS 54-2	171.4 ± 3.1	16.1 ± 0.6	10.2 ± 0.5	30.5 ± 0.7	19.4 ± 0.8	56.0 ± 3.0
TS 86-11	165.8 ± 2.8	17.3 ± 0.6	9.0 ± 0.3	35.2 ± 0.5	35.5 ± 1.6	65.2 ± 3.2
TS 87-7	177.6 ± 2.8	16.3 ± 0.7	13.2 ± 4.4	30.7 ± 0.3	31.9 ± 1.0	83.1 ± 3.1
TS 88-6	173.3 ± 3.4	16.0 ± 0.5	8.2 ± 0.4	36.5 ± 0.4	32.0 ± 1.7	63.5 ± 3.5
Surya	193.0 ± 2.9	15.4 ± 0.4	7.3 ± 0.3	32.5 ± 1.5	25.0 ± 1.2	49.1 ± 2.1

In some cases the reduction was up to 50-60 cm. No significant differences were found for head diameter and 100 seed weight except TS 42-2 for height and TS 54-2 and TS 87-7 for 100 seed weight. The large seed size mutant had less oil. It may be due to higher fibre content. Such lines could be used for confectionery purposes. Other lines had 2-5% oil above the control. It may be due to the thin hulls and lower fibre content. This could be the main reason for the preference of black hull varieties by farmers and traders. Single plant yields at Trombay were comparable with control. However, yields at Gauribidnur location were either comparable or superior to control. Three lines namely TS 86-11, TS 87-7 and TS 88-6 showed higher yields at Trombay as well as outyielding the parent at Gauribidnur. This has provided a very good index to exploit their yield potentiality at various locations. It has been proved that induced mutations have improved the yield and its contributing characters [5,7,10].

2.3.4. In vitro rooting of excised leaves

Rooting of excised leaves was standardised in the varieties 'Morden' and Surya to screen large populations for resistance to the leaf spot disease caused by *Alternaria helianthi* under controlled conditions. Leaf samples from field grown plants were taken at regular intervals of 5 days starting from 20 days after germination up to 40 days. The excised leaves were kept in a tray with the petioles immersed in tap water at 23 ± 2^0C and 12 h light (7000 lux). Healthy rooting was observed in the 3rd, 4th, and 5th leaves of 20 days old plants. The rooting started within 7 days and the roots grew profusely keeping the leaves healthy for 40 days. Samples taken after 20 days did not show symptoms of rooting in older leaves, which wilted within 2-3 days. However, younger leaves showed root growth but it was not uniform in all leaves. Using this technique, screening of black hull mutants for resistance to *Alternaria* leaf spot disease will be carried out.

3. RAPESEED (*Brassica napus*)

3.1. Introduction

Rapeseed-mustard comprising of *B. juncea, B. napus, B. campestris* and *B. carinata* is the second most important group of oilseed crops in India after groundnut. These crops account for 27.5% of the total oilseed production and 13% of the gross cropped area. Antinutritional factors in the oil and the oil cake have limited its use and export. None of the released varieties have the desired quality characters, i.e. zero erucic acid and zero glucosinolates. Therefore, reducing erucic acid and glucosinolates to zero is the prime breeding objective in the rapeseed-mustard improvement programme in India [11].

3.2. Materials and methods

A late flowering introduction, Culture 2, a *B. napus* line having low erucic acid (20%) and low glucosinolate (ca. 40μM/g defatted meal) was selected for mutation studies. Approximately 1000 seeds per dose were irradiated with 500, 750, 1000, and 1500 Gy dose of gamma rays. In M_2 generation, emphasis was placed on isolating early plants. These plants were studied in subsequent generations in five to ten row plots 3 m long to characterise for erucic acid, glucosinolates and yield. In M_7 generation data on yield and its contributing characters were recorded on 25 plants each. Screening for erucic acid and glucosinolates was carried out using paper chromatography and Tes-tape respectively [12]. Quantitative analysis of fatty acids was done by gas liquid chromatography, using single seed analysis [13]. Oil content was estimated using MQA6005 NMR (Nuclear Magnetic Resonance).

3.3. Results and discussion

A total of 61 early flowering plants from the four doses of gamma rays were isolated from the large M_2 population of 65,000 plants. Out of these only 2 mutants found in 750 and 1000 Gy dose had zero erucic acid and zero glucosinolates. They were grown in plant to row progeny and found to be true breeding for early flowering in the M_3 and M_4 generations. In the M_5 generation 988 plants of the double zero mutants were screened for erucic acid and glucosinolates, where 117 plants were found to be double zero. Eighty-three plants were advanced to the M_6 generation as plant to row progenies. Based on their breeding behaviour for morphological characters, 40 were selected for further analysis. Among them only 13 progenies were found to be true breeding for zero erucic acid and low glucosinolates. These lines were advanced to the M_7 generation. Among them 6 were rejected because of lodging. At maturity, data on yield and its contributing characters were recorded on 25 plants each of the remaining 7 lines (Table VI). The first three lines were derived from the mutant obtained in the 750 Gy dose and the remaining ones are from the second mutant obtained in the 1000 Gy dose. It indicated that plant height, oil content and yield per plant have either increased or decreased. However, significant decrease in height and increase in oil and seed yield has been observed in line TN 1-2. All lines were found to be true breeding for zero erucic and glucosinolates content was either low or zero. Zero erucic acid has been reflected in the doubling of the oleic acid content. As expected this is the result of alteration in the fatty acid biosynthetic pathway. Desirable alterations in fatty acids and glucosinolates by induced mutations have been reported [14,15] in *B. napus*.

These results show that the induced mutation approach can be effectively employed to produce genetic variations in physiological, morphological and biochemical traits.

TABLE VI. MEAN PLANT HEIGHT, OIL CONTENT AND YIELD OF SELECTED M_7 DOUBLE ZERO MUTANT LINES AND THEIR ERUCIC ACID, OLEIC ACID AND GLUCOSINOLATE CONTENT

Lines	Plant height cm	Oil content %	Yield/plant g	Fatty acid content of oil (%)		Glucosin-olates
				Erucic acid	Oleic acid	
TN- 1-2	128.6±2.5	40.7±0.2	7.8±0.6	0.0	63.0	Trace
TN-222-10	155.3±2.1	35.1±0.6	4.6±0.4	0.0	62.0	"
TN-320-4	134.7±1.5	38.0±0.2	6.6±0.7	0.0	66.0	0
TN-437-6	143.5±2.1	37.5±0.2	7.1±0.4	0.0	65.0	0
TN-545-5	130.2±3.3	37.9±0.3	6.3±0.5	0.0	64.0	Trace
TN-584-7	151.2±4.0	36.0±0.5	5.7±0.3	0.0	63.0	0
TN-788-7	158.4±2.8	35.5±0.3	4.7±0.3	0.0	64.0	0
Cul.-2	143.0±3.4	38.4±0.3	5.7±0.4	20.1	33.8	>40 µM

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Genetic modification of oil crops for optimisation of oil quality regarding industrial uses

W. Friedt

Institute of Crop Science and Plant Breeding,
Justus-Liebig-University Giessen,
Giessen, Germany

Abstract. Oil crops – like linseed, sunflower and oilseed rape - are important sources of energy, both for human consumption and for feeding livestock. They are also sources of many non-edible uses, providing raw materials for a wide range of industrial products. Oilseed rape (*Brassica napus* L.) is one of the leading crops, which benefit from the application of genetic engineering through recombinant DNA technology. Due to substantial progress in breeding and cultivation practices rapeseed has become the world's third most important source of vegetable oil. Modification of the fatty acid composition to make rapeseed oil more competitive in various segments of the food and industrial oil markets has been an important objective of plant breeding and molecular genetics in recent years. While the primary demand by the food and animal feed industry is met by 'double-low' quality rapeseed ('canola'), interest has grown in 'Biodiesel' feedstocks or specialty materials being directed to several industrial niche markets, because of their higher value than commodity oils. Rapeseed oil is unique in having a large spectrum of usability and good properties for non-food applications, such as relatively homogeneous composition, high degree of refinement, freedom from contaminants and biodegradability, giving it advantages over petrochemicals. Consequently, one of the most important objectives of rapeseed breeding is the genetic modification of the seed oil by maximizing the proportion of specific fatty acids, like laurate, erucate or functionalised acids, in order to obtain tailor-made raw materials suited for industrial purposes.

Present rapeseed oil (canola) is characterized by a high content of unsaturated C18 fatty acids. In order to improve its industrial usefulness a current project aims at the genetic modification of saturated fatty acids content and the development of transgenic *B. napus* accumulating medium-chain triacylglycerols in its seed oil. For this purpose relevant genes from unrelated plant species forming unusual storage oils are isolated and transferred to oilseed rape. Particularly in *B. napus* the efficiency of *Agrobacterium tumefaciens* mediated transformation mainly depends on the susceptibility of the starting material to agrobacteria, the ability to select for newly grown tissue derived from the transformed cells, and the potential to regenerate plants from the selected tissue. In the course of a preliminary study we have investigated intraspecific differences towards shoot regeneration by genetically transforming the resynthesized high-erucic acid rapeseed line 'RS 306' and the spring canola cultivar 'Drakkar' with the gene construct pASBnDES1. The latter harbours a chimeric gene based on a *Cuphea lanceolata* seed-specific promoter (ClFatB4) and the coding sequence from rapeseed Δ9-desaturase in antisense orientation, in order to modify the content of oleic acid, which is the major precursor for subsequent fatty acid pathways (desaturation, elongation) in both rapeseed genotypes.

Stable transformants amongst the progeny of primary transformants can be identified by different means. An attractive modern technique would be the application of molecular cytology tools, i.e. fluorescence *in situ* hybridisation (FISH) techniques, which enable the direct chromosomal localisation of labeled DNA probes. Such approaches have been increasingly applied to plant genome mapping in recent years. We have developed FISH methods for the accurate localisation of repetitive DNA sequences at chromosomal sub-arm level in *Brassica* species. In addition, we apply genomic *in situ* hybridisation (GISH) for identification and characterisation of parental genome components in rapeseed hybrids. The detection of short, low-copy molecular markers is not possible by FISH, however this shortcoming could be overcome by physical localisation of megabase DNA clones containing markers of interest. High-resolution FISH can provide information about ordering and

physical distances between molecular markers, both important considerations for physical mapping and positional cloning. Practical applications of FISH and GISH in rapeseed breeding are discussed.

In sunflower, hybrid varieties are used almost exclusively for oilseed production. Since the genetic basis is considered narrow, broadening of the variability seems to be particularly necessary in this crop. With this aim, numerous interspecific hybrids have been made in *Helianthus*. Interspecific hybrid progenies with superior ability to regenerate shoots from apical meristems were successfully selected, which now facilitate the development of lines for improved biotechnological applications. Early generations of interspecific hybrids originating from crosses between two *H. annuus* CMS lines 'HA89' and 'Baso', and 10 wild species were screened for their ability to regenerate *in vitro*. Evaluation of 36 progenies led to identification of four progenies from crosses involving *H. mollis*, *H. giganteus*, *H. strumosus*, and *H. decapetalus*, which showed a superior regeneration potential. For these progenies, means of 2.3-3.5 shoots per cultured explant at a frequency of 51.3-62.4% of explants producing shoots were observed. Regeneration *in vitro* was clearly determined by the genotype. Fifty percent of all investigated interspecific hybrids performed better than the inbred line 'HA89' demonstrating that the high regeneration potential available in the wild species can be efficiently transferred to cultivated sunflower. In addition, two of the interspecific hybrids derived from *H. strumosus* and *H. decapetalus* not only showed a superior regeneration potential but also proved to be competitive to commercial hybrids with regard to important agronomic traits, e.g. oil content and seed weight.

1. RAPESEED BREEDING: SYNTHESIS OF CLASSICAL METHODS AND BIOTECHNOLOGY APPROACHES

1.1. Introduction

At present, the four major globally traded oil crops are soybean (*Glycine max*), oil palm (*Elaeis guineenis*), rapeseed - originally derived from several locally adapted *Brassica* species - and sunflower (*Helianthus annuus*), respectively. Together, these four crops account for approximately 77% of the worldwide vegetable oil production, comprising a total of some 78 million mt [1]. In particular the cultivation of oilseed rape (*Brassica napus*) has increased tremendously during the last decades due to rapid progress in rapeseed breeding [2]. Further adjustments of rapeseed oil composition will not be realized satisfactorily by conventional breeding methods. For these purposes genetic engineering using recombinant DNA technology is required for efficiently transferring specific foreign genes between distant species [3]. In a time frame of only one decade more than 60 different plant species, including most of the economically important crops, have been successfully engineered genetically since 1983, and the list is rapidly growing each year [4–6]. In particular, oilseed rape has been among the leading agricultural crops to benefit from the application of genetic engineering. The variety of novel traits introduced into rapeseed plants and being evaluated in field trials since the early 1990s includes commercial hybrid seed production based on genetically engineered pollen control systems (e.g., Seedlink® developed by PGS, Gent, Belgium), tolerance to broad-spectrum herbicides (e.g., glyphosate or glufosinate ammonium), modified oil and protein composition, and many others [7–11].

This chapter reviews the progress which has been achieved regarding the genetic alteration of fatty acid and storage lipid metabolism leading to oil compositions other than the C16 to C18 range normally found in canola oil, as well as other vegetable oils and fats. Furthermore, tasks of managing specialty oil production, which can be anticipated for the very near future, are also discussed.

1.2. Rapeseed breeding: combination of classical methods and biotechnology

1.2.1. Conventional breeding assisted by modern techniques

Until recently, commercial rapeseed breeding has been entirely focused on the development of open pollinated varieties, either populations or (inbred) lines. With the advent of suitable systems for the control of male sterility/fertility, hybrid oilseed rape breeding programmes have been initiated in most if not all of the commercial companies and many public institutions, e.g. in Canada and France. One system is based on cytoplasmic male sterility (CMS) which has earlier been developed, e.g. via intergeneric hybridization between rapeseed and radish (*Raphanus sativus*). The principle scheme for the development of a hybrid variety by such a procedure is presented in Fig. 1: a) Basic breeding pools with sufficient agronomic performance have to be established, both on the female and the male side; b) Lines in both pools – i.e. inbreds, doubled haploids, etc. will be tested for combining ability with materials of the respective other pool – eventually aided by marker assisted approaches; c) Experimental hybrids will be produced and tested at multiple locations.

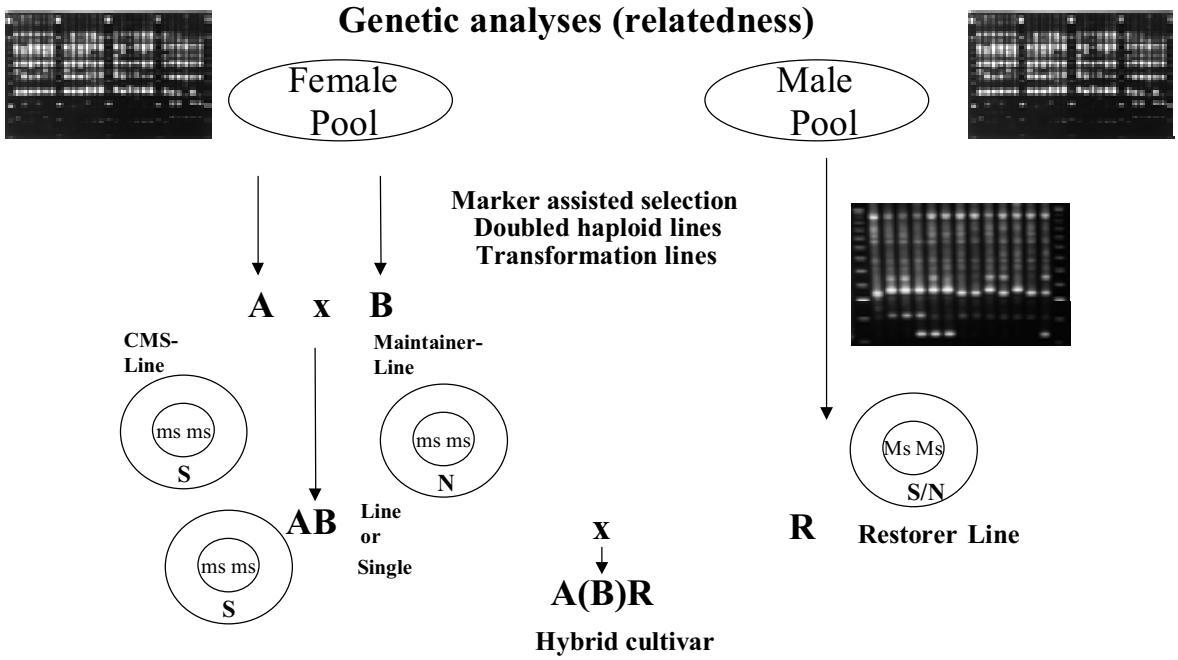

FIG. 1. Hybrid seed production on the basis of a CMS system, e.g. in rapeseed.

It is obvious from this scheme that a sufficient genetic distance (non-relatedness) between potential female and male parents is a prerequisite for the appearance of heterotic effects (hybrid vigour) in the cross progeny and, therefore, for an outstanding agronomic performance (hybrid yield) of the experimental hybrid(s). Since the actual genetic rapeseed material seems to be comparatively closely related, the creation of novel genetic variability is urgently needed. For this purpose, interspecific and intergeneric hybridization has been shown to be a very useful tool. Such wide crosses within the *Brassicaceae* family under natural conditions have led to the existing allotetraploid *Brassica* species, i.e. *B. carinata, B. juncea*, and *B. napus* (Fig. 2).

By means of artificial sexual hybridization followed by application of "embryo rescue" technique(s) novel allotetraploid genotypes can be obtained. For example, by crossing specific types of *B. oleracea*, like cauliflower, with particular *B. rapa* (*B. campestris*)

accessions, e.g. Indian Turnip (Yellow Sarson) (Fig. 2), novel types of oilseed rape have been obtained which now prove to be highly valuable in broadening the genetic variation of the existing oilseed rape gene pool. However, in such cases, complete genomes of different species are combined which may or may not lead to a useful product, i.e. novel plant with satisfactory agronomic performance. For example, with regard to the improvement of seed oil composition the change of only one or a few genes – or their respective function(s) - is often supposed to be critical or sufficient to achieve the respective goal, i.e. a certain fat quality (see below 1.4,1.5).

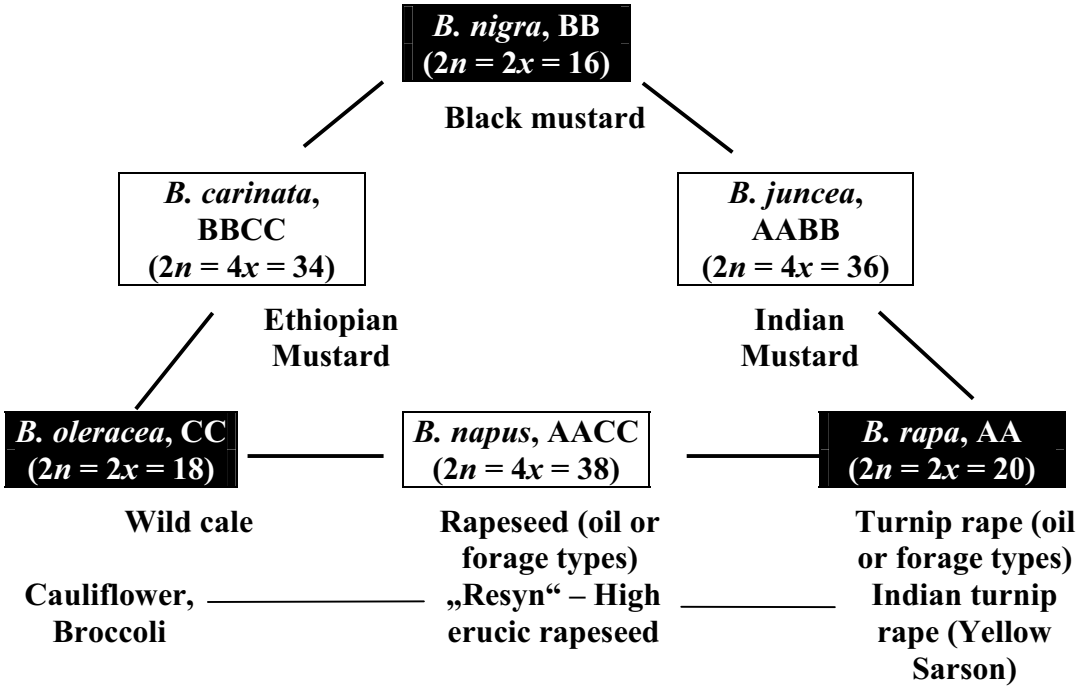

FIG. 2. Relationships in the genus Brassica: Natural allotetraploid species (light boxes) derived from interspecific hybridization between diploids (dark boxes); artificial alloploids are obtained by biotechnology-aided interspecific hybridizations via embryo rescue.

Regarding the genetic alteration of the oil composition of industrial rapeseed in Germany, joint research activities between private breeding companies and several research institutes were started at the beginning of the 1990s. They cover a broad range of fatty acid modifications, as follows:

- Super-high erucic acid content (above 70%) via biosynthesis of trierucin and promotion of elongation capacity.
- Very high oleic acid content above a value of 80%.
- Medium-chain fatty acids (MCFA), such as myrisitic and capric acid.

First transgenic candidates from this research project have been grown in ”transgenic nurseries” under controlled field release conditions since spring 1997 in three locations in Germany.

1.2.2. Specialty rapeseed – on the way to the market

In recent years an increasing number of releases of genetically engineered rapeseed involved traits for seed quality improvement, such as fatty acid chain length distribution of

the oil or seed storage protein and amino acid composition of the meal [12–16). This development was promoted by the findings that major quality properties are genetically controlled by a limited number of genes. Therefore, this would make it technically and commercially attainable to effect desirable alterations in such traits by transferring just a few genes to create a value-added transgenic rapeseed variety in an acceptable time frame. In the future, the number of compounds developed in genetically engineered plants following a ”molecular farming” approach will increase rapidly, since it has already proved feasible to produce modified carbohydrates, high-value pharmaceutical polypeptides, industrial enzymes and even biodegradable plastics [17–20].

Projects dealing with alterations of oil and protein quality are especially at a relatively advanced stage of development. That means, there are transgenic plants produced, field trials underway or even commercialization is realized [15]. With regard to the latter, the most notable progress in oil modification achieved to date is the development of specialty rapeseed varieties containing up to 40 wt% lauric acid (C12:0) in their seed oil; they have reached already the highest level of genetically improved organism release – the successful implementation into the market. The first commercial acreage of high-laurate rapeseed was harvested during May 1995 in southern Georgia, USA [21].

1.3. Genetically modified rapeseed as a source of medium-chain fatty acids

1.3.1. General aims and achievements

Vegetable oils with a high content of lauric acid (C12:0) are of economic interest. These so-called laurics are widely used as raw materials for both edible purposes and non-food applications, such as detergents, soaps, personal care products and oleochemicals. The demand for lauric oils is met almost entirely by coconut and palm kernel oil [2]. In the past few years, several projects have been initiated in order to increase the availability of laurate and other medium-chain fatty acids (C8 to C14) by genetic engineering of rapeseed. Scientists of Calgene Inc., Davis, California [22, 23], have successfully demonstrated for the first time that a chain termination mechanism, a C12:0 specific acyl-acyl carrier protein (ACP) thioesterase derived from California bay laurel (*Umbellularia californica*), is responsible for the accumulation of about 45-50 mol% (35-40 wt%) lauric acid in the storage triacylglycerols of genetically engineered rapeseed (see Table I). High-laurate rapeseed was approved for unrestricted release (deregulation) in the USA in October 1994 by the United States Department of Agriculture (USDA) and has been commercialized by Calgene under the brand name Laurical®. Although this specialty fat was custom-tailored to resemble the fatty acid profile of other lauric oils, it has functional effects of its own. The triacylglycerol composition of coconut (*Cocus nucifera*) and the genetically engineered high-laurate rapeseed, bearing the thioesterase gene from California bay laurel (*UcFatB1*) were compared by HPLC to show the drastically different nature of these two oils containing appreciable amounts of lauric acid. The triacylglycerols of coconut oil are dominated by those species consisting of two moieties lauric acid and as third a medium-chain fatty acid, such as caprylic, capric, lauric or myristic acid. In contrast, the high-laurate rapeseed oil contains only minor portions of trilaurin and is particularly characterized by having a high degree of dilauroyl species of the type C12:0-C18:x-C12:0, where only a minor proportion (2.2%) of the novel lauric acid is esterified in the central position. On the other hand, the desaturated C18 fatty acids, namely oleic (61.7%), linoleic (25.4%) and linolenic acid (10.7%), are esterified in a rapeseed-typical fashion in the *sn*-2 position of the triacylglycerol fraction as analysed by pancreatic lipase hydrolysis by using a method described by Christie [24] and W. Lühs [unpublished].

This unique kind of structured triacylglycerols has a beneficial effect leading to a specialty product that has many applications - especially in the food industry. Resembling cocoa butter in a certain way the first products derived from Laurical® are predominantly targeted at edible markets for use in confectionery fats, coffee whiteners and milk formulas [25]. With regard to non-edible applications products derived from high-laurate rapeseed have shown definite advantages over conventional coconut oil-based soap formulations in terms of mildness and foaming [26].

TABLE I. FATTY ACID COMPOSITION OF HIGH-LAURATE RAPESEED

Fatty acid[a]	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	Rest
mol%	45.8	4.8	3.4	1.0	27.1	11.3	6.1	0.5
wt%	38.0	4.6	3.6	1.1	31.7	13.2	7.1	0.7
Laurical® (wt%)[b]	36.7	3.9	3.1	1.3	33.2	11.9	7.4	2.5

[a]Line '11-DH 128', means of 3 replications: C12:0 = laurate, C14:0 = myristate, C16:0 = palmitate, C18:0 = stearate, C18:1 = oleate, C18:2 = linoleate, C18:3 = linolenate [W. Lühs, unpublished].
[b]As specified by Calgene Inc.

For oleochemistry however, a higher purity of lauric acid is required. Calgene scientists expect that the introduction of a coconut-derived lysophosphatidic acid acyltransferase (LPA-AT) gene into their proprietary high-laurate rapeseed lines will increase the lauric acid content beyond the 40 wt% level achieved until now. Progenies from crosses between plants expressing both the laurel bay thioesterase (Uc FatB1) and the coconut LPA-AT gene indicate the possibility of developing very high-laurate rapeseed [27, 28].

In the past years further attention has focused on species in the genus *Cuphea* belonging to the family *Lythraceae*. The seeds of these herbaceous species most commonly have 30-33% oil, which is a rich source of medium-chain fatty acids ranging from caprylic (C8:0) to myristic acid (C14:0). In particular, one important advantage of cuphea seed oils as compared to traditional sources of lauric oils, is that high proportions (up to 95%) of specific single fatty acids, such as C8:0 or C10:0, are feasible [29]. Recently, several acyl-ACP thioesterase cDNAs and genes belonging to the FatB gene family were isolated from *C. lanceolata*, an annual plant which contains predominantly capric acid (C10:0, up to 83%) in its seed oil. The expression in transgenic *Brassica napus* has shown that the C8 to C18 fatty acid composition (calculated on a mol% basis) of mature T_2 seeds is drastically changeable [30]. For example, the transfer of one FatB gene conferred the production of 6 mol% C10:0, while the transfer of another FatB gene yielded 23 mol% myristic (C14:0) and 29 mol% palmitic acid (C16:0).

Following similar approaches genetic engineering of the thioesterase from *Cuphea hookeriana* (ChFatB1) or nutmeg (*Myristica fragrans*) into rapeseed has resulted in oils with high amounts of palmitate or myristate, respectively [31,32]. Introduction of a seed-specific thioesterase gene (ChFatB2) from *C. hookeriana* resulted in rapeseed that contains significant concentrations of medium-chain fatty acids (MCFA) such as 11 mol% caprylate and 27 mol% caprate [33]. It is expected that the better availability of a stable source of oil containing C8:0 and C10:0 fatty acids from MCFA rapeseed rather than the traditional coconut oil could lead to an increased use of medium-chain lipids in food markets specialized in medicinal and nutritional products [25].

1.3.2. Specific transgenic approach to the biosynthesis of medium-chain triacylglycerols in oilseed rape (Brassica napus)

1.3.2.1. Introduction

In order to improve the industrial usefulness of rapeseed oil in oleochemistry the long-term aim of this study is to develop *B. napus* which is able to store a large amount of C10-C14 fatty acids in its seed oil [34]. These medium-chain fatty acids (MCFA) are very common for members of the *Lauraceae* family, but they are completely absent from rapeseed oil. Obviously, the adjustment of rapeseed oil composition leading to an accumulation of medium-chain triacylglycerols cannot be achieved by conventional breeding methods. As shown in the cases of laurate canola or rapeseed high in myristic and palmitic acid [23,35] genetic engineering is the most promising breeding route for transferring relevant genes between rapeseed and distant species. The efficiency of *Agrobacterium tumefaciens*-mediated transformation protocols, as indicated by the percentage of transgenic plants, depends on the following main factors:

- Susceptibility of the *B. napus* starting material to *Agrobacterium*.
- The ability to select for newly grown tissue derived from the transformed cells.
- The potential to regenerate plants from the selected tissue [5,9].

In a first step, we established the transformation protocol developed by De Block et al. [36] under our specific laboratory conditions. Regarding the alteration of fatty acid composition we used the spring canola cultivar 'Drakkar' and the resynthesized high erucic acid rapeseed (HEAR) line 'RS 306' as donor plant material. Due to differences in regeneration response after co-cultivation with *Agrobacterium*, the optimum growth regulator concentration and combination in the selectable medium must be found for each genotype, allowing sufficient regeneration of potentially transformed plants [37].

1.3.2.2. Materials and methods

1.3.2.2.1. Plant material used for transformation

The genetic stock 'RS 306' is a resynthesized high erucic (HEAR), high glucosinolate line which was developed via the interspecific cross *B. rapa* ssp. *trilocularis* ('Yellow Sarson') x *B. oleracea* conv. *botrytis* var. *botrytis* (cv. 'Super Regama'); it requires moderate vernalisation but is without winter hardiness [37,38]. Seeds of cv. 'Drakkar' were obtained from the breeding company Norddeutsche Pflanzenzucht Hans-Georg Lembke KG (Hohenlieth, Germany).

1.3.2.2.2. Bacterial strain and vector

The *A. tumefaciens* strain GV3101/pMP90RK was transformed with the construct pAS*Bn*DES1 carrying the *B. napus* stearoyl-acyl carrier protein (18:0-ACP) desaturase (Δ9-desaturase) gene fused in antisense orientation to the *Cl*FatB4 promoter (N. Martini, unpublished). The binary vector used is pLH9000 [L. Hausmann, unpublished] with the neomycin phosphotransferase (NPTII) gene as selectable marker.

1.3.2.2.3. Transformation procedure

For genetic transformation and the subsequent selection steps the method of De Block and co-workers [36] was applied with minor modifications, including the use of 1) etiolated rapeseed hypocotyls, 2) ticarcillin/potassium clavulanate (Betabactyl^{TM}; SmithKline Beecham Pharma, Germany) instead of carbenicillin for elimination of the agrobacteria after stopping co-cultivation, and 3) Gelrite^{TM} (Serva, Heidelberg/Germany) as gelling agent in the tissue culture media. Seeds were surface-sterilized in 70% ethanol for 5 min, then for a further 10 min in a 3% NaOCl solution. The rape seedlings were germinated on A_1 medium [36] in darkness. After 7 days, the etiolated hypocotyls were cut in 1 cm segments and co-cultivated with *A. tumefacien*s for 3 days in a liquid A_3 medium [36] using 9 cm petri dishes. Following co-cultivation, the hypocotyl explants were placed on petri dishes (2 cm high and 14.5 cm in diameter; 25 explants per dish) containing A_5 medium [36] with Kanamycin (50 mg/l) as a selective agent. Further sub-culturing of the explants was conducted at intervals of two weeks. After 6-8 weeks of selection the first shoots were formed and removed from the hypocotyl explants and transferred to A_6 medium [36] (with 15 mg/l Kanamycin) on which they continued growing until normal phenotypic characteristics appeared. They were transferred to A_8 rooting medium [36] and then, following the formation of roots, to the greenhouse.

1.3.2.2.4. Testing the hormone composition of the selectable media

Several variants of the A_5 selectable media were used after the co-cultivation step, differing in the concentration of the supplemented growth regulators as follows: naphthalene acetic acid (NAA) in the range of 0.1-0.5 mg/l and benzyl amino purine (BAP) ranging from 1.0 to 5.0 mg/l. The hormone concentration 0.1 NAA mg/l and 1.0 mg/l BAP corresponds to the one formerly used in the transformation protocol of De Block et al. [36].

1.3.3. Results and discussion

Following a modified protocol of De Block et al. [36] we have co-cultivated etiolated hypocotyl segments of the *B. napus* genotypes cv. Drakkar and the resynthesized rapeseed line RS 306 by using the *Agrobacterium* strain GV3101/pMP90RK harbouring the gene construct pAS*Bn*DES1 with the NPTII gene as selectable marker (Table II). The results of the experiment revealed a significant difference between the two genotypes regarding their regeneration response after co-cultivation with *A. tumefaciens*. A large variation in regeneration efficiency was found within the genotype RS 306 ranging from 1 to 14% (Table II). The shoot regeneration of Drakkar was very insufficient and did not exceed 3%, although this spring canola cultivar is usually appreciated for its superior transformation and regeneration response [cf. 39]. As shown in a previous study, RS 306 is clearly more suitable for our laboratory conditions and the modified transformation protocol [37].

Antibiotics used after the co-culture step to eliminate agrobacteria may influence the regeneration response of the transformed explants in that they decrease the shoot differentiation, especially if kanamycin is used as a selectable agent. On the one hand, it is reported that Betabactyl^{TM} (ticarcillin/potassium clavunate), unlike the widely used carbenicillin and cefotaxime, is light-stable and resistant to inactivation by β-lactamase [40]. On the other hand, it is recommended to use carbenicillin, which prevents the medium from turning brown and eliminates the toxic effects of prolonged use of silver nitrate on plant tissue [36]. Possible differences between the above mentioned antibiotics (carbenicillin and Betabactyl^{TM}) were investigated but based on the number of regenerants the differences found were not significant (Table III).

In order to ameliorate the regeneration of potentially transformed plants from the spring cultivar Drakkar, hypocotyl segments were placed on selectable A_5 medium differing in the concentration of the supplemented growth regulators. A regeneration rate of 12% was achieved through the increase of the NAA concentration from 0.1 mg/l to 0.5 mg/l (Fig. 3). This effect was observed in all combinations with BAP where the NAA concentration amounted to 0.5 mg/l. These results thus demonstrate a clear NAA effect. However, for the Drakkar genotype which can reach a maximum regeneration rate of 28% potentially transformed plants [39], further optimisation of the transformation protocol will be necessary.

TABLE II. KANAMYCIN-RESISTANT SHOOT REGENERANTS OF *B. napus* 'RS 306' AND 'DRAKKAR' AFTER CO-CULTIVATION WITH THE *Agrobacterium* STRAIN GV3101 BEARING THE BINARY VECTOR PASBNDES1

Genotype	RS 306			Drakkar		
Agrobacterium	Explants in	Regenerated shoots		Explants in co-	Regenerated shoots	
strain/gene construct[a]	co-culture	Number	%	culture	Number	%
GV 3101 (pAS*Bn*DES1)	200	24	12.0	200	0	0
	200	4	2.0	200	1	0.5
	200	13	6.5	200	2	1.0
	200	2	1.0	200	0	0
	200	11	5.5	200	0	0
	200	28	14.0	200	6	3.0
Mean		13.6	6.8*		1.5	0.8

[a] The experiment consisted of 2 combinations of *Agrobacterium* strain x *B. napus* genotypes, each of the six replications comprised 200 hypocotyl explants (8 dishes x 25 explants).

* Significant difference (P=0.05).

TABLE III. INFLUENCE OF ANTIBIOTICS ON SHOOT REGENERATION FROM CO-CULTIVATED *B. napus* HYPOCOTYLS (CV. DRAKKAR)

Antibiotic	No. co-cultivated explants/replication	Number of explants, total[a]	No. regenerated shoots, total	Regeneration rate, %
Betabactyl™	225	1,125	7	0.6
Carbenicillin	225	1,125	13	1.2

[a] Each experiment consisted of five replications.

Further improvement of the modified transformation protocol was tested by using different hormone concentrations and combinations. The above experiment was repeated using the RS 306 genotype instead of the Drakkar genotype (Figure 4). The experiment demonstrates that unlike the spring canola cultivar Drakkar, in which the 0.5 NAA mg/l / 1.0 mg/l BAP hormone concentration gives the best results, in the resynthesized rapeseed line RS 306 the 0.3 NAA mg/l / 3.0 mg/l BAP concentration provided the best regeneration rate. The latter genotype has also shown an excellent regeneration response using the *Agrobacterium* DNA-delivery system in combination with other gene constructs [37,38].

The results of Table III as well as Figures 3 and 4 indicate the importance of the genotype itself, the culture conditions after transformation and the medium supplements [9,41,42,43]. The results also demonstrate once again the pronounced genotype x treatment interactions found *in vitro*. Consequently, for each genotype, favourable conditions after co-cultivation and medium supplements must be found which allow the regeneration of sufficient numbers of plants from the transformed cells [5,9].

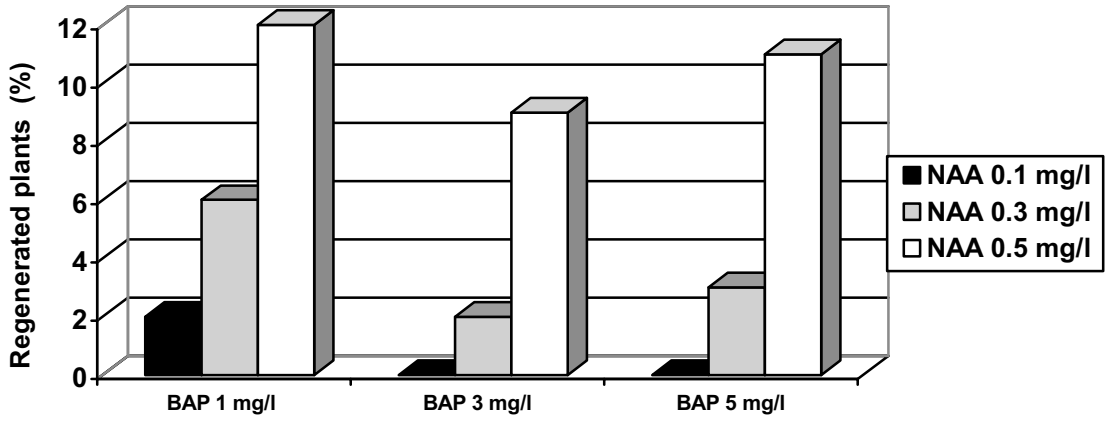

FIG. 3. Influence of the hormone composition on the regeneration of the spring cultivar B. napus cv. Drakkar after co-cultivation with Agrobacterium tumefaciens.

1.3.4. Conclusion

Medium-chain fatty acids derived from plant oils are preferred for industrial applications, including commercial detergents, surfactants, cleaning agents and cosmetics. Since rapeseed oil does not furnish this need, this biosynthetic capacity has to be introduced by genetic engineering. As plant oil composition can be substantially modified by either increasing or reducing the expression of defined enzyme activities [44,45] the strategy

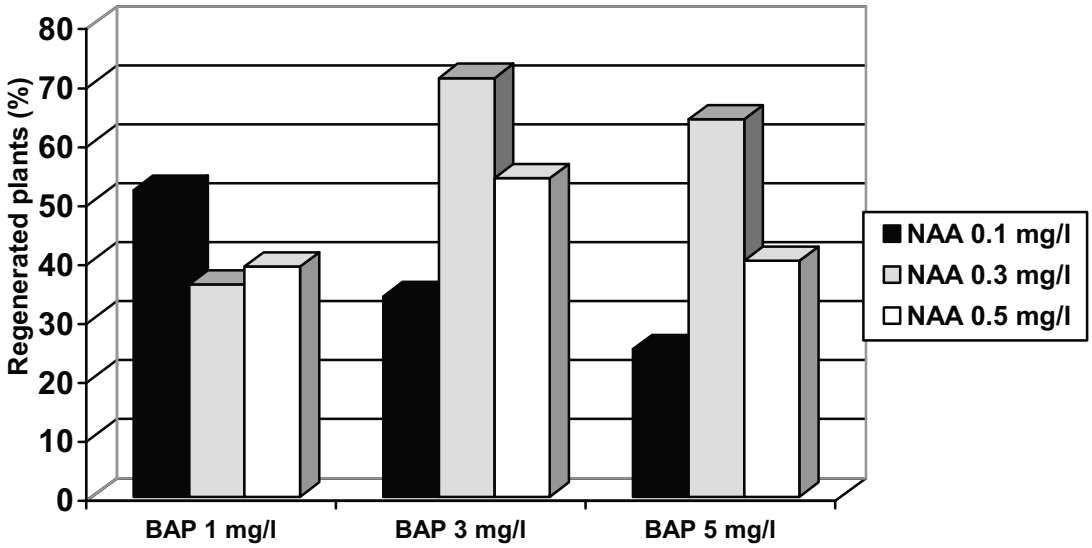

FIG. 4. Influence of the hormone composition on the regeneration of the resynthesized rapeseed line RS 306 after co-cultivation with Agrobacterium tumefaciens.

consists of two-steps: firstly, the content of unsaturated C18 fatty acids will be reduced by antisense inhibition of the endogenous Δ9 or 18:0-ACP desaturase leading to an increase in the proportion of stearic acid (and its precursor fatty acids) at the expense of oleic acid in both Canola and HEAR oil. So far, in the course of the transformation experiments a comparable high number of regenerants has been developed.

Following their characterization by using PCR or NPTII ELISA assays, the transformants have to be analysed for their altered fatty acid composition. Finally, the transformed rapeseed plants showing a significant shift to more saturated fatty acids, are considered ideal for further *Agrobacterium*-mediated transformations, transferring relevant genes encoding the synthesis of medium-chain fatty acids from the *Lauraceae* family.

1.4. Rapeseed oil with increased erucic acid content

1.4.1. Background and situation

Apart from crambe (*Crambe abyssinica*) [46] and yellow mustard (*Sinapis alba*) [47] - relatively low-yielding spring oilseed crops - the seed oil of traditional varieties of *Brassica* species, i.e. high-erucic acid rapeseed (*B. napus*) and several mustards (*B. juncea*, *B. carinata*), are the primary sources of erucic acid (*cis*-13-docosenoic acid, C22:1). This very long chain monounsaturated fatty acid (VLMFA) is detrimental to the food quality of rapeseed oil, but it has a wide array of potential industrial uses. The major market of C22 oleochemicals is to produce erucamides performing as an antiblock, slip-promoting agent in the manufacture of polyolefine sheeting. Other uses include personal care products, surfactants and detergents, paints and coatings, plastics and nylons as well as lubricants, fuel supplements and high-temperature functional fluids [48,49,50,51,52].

Breeding of high-erucic acid rapeseed (HEAR) has revived and a couple of new cultivars have been registered since 1991. Low glucosinolate content as a surplus trait is realized both in spring and winter type varieties, such as ‘Hero‘, ‘Mercury‘, ‘Venus‘ and ‘Neptune‘ (Canada), ‘Industry‘ (Denmark) as well as ‘Erox‘ and ‘Maplus‘ in Germany. Until now, HEAR breeding has been concerned with the improvement of seed and oil yield rather than with the increase of erucic acid content. It is obvious that a seed oil bearing only 45-50% of the desired fatty acid is not a very useful feedstock for most industrial applications. In order to obtain a rapeseed oil which is more preferable as a raw material for industry, concerted research efforts in plant breeding and genetic engineering have been undertaken to maximize the proportion of erucic acid [38,53,54].

In most members of the *Brassicaceae* and especially in natural *B. napus*, erucic acid and other VLMFAs are not incorporated into the central position of the glycerol backbone. This obstacle prevents the synthesis of trierucin (trierucoylglycerol, C69:3) and restricts the content of erucic acid in rapeseed oil to a theoretical maximum of 66.7%. However, in practice even levels of ca. 55% and more have turned out to be very difficult to realize in HEAR oil [54]. Trierucin (trierucoylglycerol) is predicted to provide the main breakthrough in breeding and production of high-erucic acid oils, because it would make the processing of comparatively pure erucic acid much easier and more attractive commercially. The lack of trierucin biosynthesis in rapeseed is attributed to both a reduced capacity of erucic acid synthesis as such, and to the unique properties of the lysophosphatidic acid acyltransferase (LPA-AT) being involved in the acylation reaction at the *sn*-2 position of the triacylglycerols [55].

1.4.2. Development of genotypes with improved elongation capacity

For enhanced erucic acid synthesis as such, the elongase enzyme complex catalysing the production of VLMFA with chain lengths of C20 to C24 is an attractive target for genetic modification and more sophisticated techniques of metabolic engineering. As compared to the amphidiploid oilseed rape (*B. napus*, 2*n*=4*x*=38 chromosomes) the progenitor diploid species, *B. rapa* (2*n*=2*x*=20) and *B. oleracea* (2*n*=2*x*=18), have been shown to possess a larger magnitude of variation for high erucic acid content in the seed oil, ranging from 30 to 61% and from 28 to 63%, respectively. Through a biotechnological approach, resynthesized rapeseed has been generated via wide interspecific hybridization, crossing high erucic acid *B. rapa* X *B. oleracea* genotypes [54,56, cf. Fig. 2]. Subsequent genetic studies involving this spring-type resynthesized rapeseed material (RS lines) with up to 60% C22:1 in the seed oil have revealed that the two genes (four alleles, respectively) capable of erucic acid synthesis in *B. napus* are additive contributing to the total erucic acid content of ca. 16-17% C22:1 per allele [57]. This is considered to be equivalent to a high capacity for fatty acid elongation (FAE) in the RS lines, since for traditional spring rapeseed lower contributions of about 9-10% C22:1 per allele are usually observed, leading to a lower total erucic acid content of only ca. 40% [58].

In order to clarify the molecular basis of VLMFA biosynthesis, the *FAE1* gene of *Arabidopsis thaliana*, probably encoding a condensing enzyme (β-ketoacyl-CoA synthase, KCS), has been isolated by using direct transposon tagging [59]. The activity of the KCS, representing the rate-limiting step of the microsomal fatty acid elongation reaction, determines the acyl chain length of the VLMFA produced by developing seeds [60]. More recently, an immature embryo cDNA coding for a *B. napus* KCS was described to be tightly linked to one (*E1*) of the two loci associated with the variation of C22:1 content in rapeseed oil [61,62]. Due to the high degree of both homology of nucleotide sequences and identity of the deduced amino acids there is strong evidence that C22:1 biosynthesis in rapeseed and other *Brassica* species is controlled through the expression and property of KCS enzymes being encoded by gene(s) homologous to the *FAE1* gene from *A. thaliana* [62,63]. The introduction of a KCS gene cloned from jojoba (*Simmondsia chinensis*) confirmed this assumption by showing the complementation of the canola fatty acid elongation mutation (*fae*) leading to the restoration of erucic acid synthesis in transgenic rapeseed [64]. The isolation and expression of cDNAs encoding KCS from the crucifer *Lunaria annua* (honesty) in transgenic rapeseed led to alterations in the fatty acid composition of the transgenic seed oil, but did not dramatically increase the VLMFA content of the oil [10].

1.4.3. Development of transgenic rapeseed with altered acyltransferase properties

Different routes have been followed in order to modify genetically the properties of the *sn*-2 acyltransferase (LPA-AT) in *B. napus* and to increase the overall proportions of erucic acid in rapeseed oil. Following molecular breeding procedures, the erucoyl-compatible LPA-AT of meadowfoam (*Limnanthes douglasii*, L. *alba*) [38,65,66] and non-plant sources, such as yeast [67] or *Escherichia coli* [68] were functionally expressed in HEAR genotypes revealing significant alterations in both the stereochemical composition and the trierucin content of the transgenic seed oil. Especially, in the transformants with the LPA-AT of *L. douglasii*, which were developed from the above mentioned RS lines as recipient the highest trierucin content (up to 8%) has been detected [38], which can mainly be attributed to the capacity to produce erucoyl-CoA being higher for the RS lines than for the traditional HEAR cultivars used before in similar experiments. The present state in the development of high-

erucic acid *Brassica* genotypes accumulating erucic acid in the *sn*-2 position and trierucin in the seed oil is summarized in Table IV.

TABLE IV. ERUCIC ACID CONTENT OF INTACT TRIACYLGLYCEROLS (TAG) AND OF THE *SN*-2 POSITION DETECTED IN DIFFERENT *Brassica* SEED OILS CONTAINING TRIERUCIN [SOURCE: 34]

Species	Genotype[a]	Source LPA-AT[a]	C22:1 TAG	C22:1 *sn*-2	Trierucin[b]	Ref.
B. oleracea	'Sesam'	inherent	61.6	23.7	trace	69
B. oleracea	n.s.	inherent	60.0	55.6	n.s.	70
B. rapa	n.s.	inherent	49.6	51.7	n.s.	70
B. napus (transgenic)	'SLC1-1 Hero 8-6'	*Saccharomyces cerevisiae*	50.5	3.5	up to 0.4	67
"	'TN-E6'	*E. coli*	43.2	3.9	0.5	68
"	'7695-1'	*Limnanthes alba*	37.5	15.1	Signif. peak	65
"	'SCV144-2'	*L. douglasii*	32.1	28.3	2.8	66
"	'TR-E14'	*L. douglasii*	43.1	30.1	2.7	38
"	'T02-RS239'	*L. douglasii*	54.6	40.8	6.4	38

[a] LPA-AT = lysophosphatic acid acyltransferase.
[b] n.s.= not specified.

Apart from these molecular approaches, which have confirmed the feasibility of using chimeric LPA-AT genes to alter the stereochemical composition of rapeseed oil, more recently natural genotypes of both *B. oleracea* and *B. rapa* have been reported having the capability to insert up to more than 50% erucic acid into the *sn*-2 position of the glycerol backbone [69,70]. These materials possessing the property of interest in both parental genomes would be prime candidates for *B. napus* resynthesis experiments in order to genetically modify the LPA-AT enzyme in rapeseed by a non-transgenic procedure [71].

Molecular studies have demonstrated that the development of rapeseed plants, which can accumulate trierucin, is an elementary step in order to increase the erucic acid content of transgenic rapeseed oil. However, the genetic transformation of the responsible *sn*-2 acyltransferase revealed also that this approach was not a wholly sufficient route towards rapeseed oil with a super high erucic acid content, i.e. more than 70% C22:1. Even corresponding transgenic rapeseed needs improvement by stimulating the biosynthetic capacity for fatty acid elongation. In this context, further work aims to identify, characterize molecularly and introduce more effective alleles and/or additional genes for erucic acid synthesis in *B. napus*, involving transgenic HEAR lines with significant trierucin in their seed oil [10,38,62].

1.5. Rapeseed oil with uncommon fatty acid functionalities - future perspectives

Numerous seed oils containing unusual fatty acids have been described so far [72,73,74] indicating a broad flexibility with regard to storage lipid biosynthesis in the plant kingdom. Thus chain length is actually just one fatty acid property that can be altered by metabolic engineering. Functionalities such as the degree of desaturation or the positions and stereochemistry of double bonds also have the potential to be altered by recombinant DNA

technologies including sense or antisense suppression and overexpression of heterologous enzymes [75,76]. Furthermore, increased flexibility and new raw materials for industrial purposes have to come from genetically engineered plant oils providing fatty acids with unusual chain lengths and/or functionalities, such as unique double bond positions or functional groups, e.g. hydroxy, epoxy, acetylenic, or keto groups, respectively [53,54,77]. A prominent example in this context is the aim to develop rapeseed with unsaturated hydroxy fatty acids in its seed oil, which are usually found in castor beans (*Ricinus communis*) or cruciferous desert plants belonging to the genus *Lesquerella* [78,79,80,81,82,83,84]. More recently, the expression of a cDNA encoding the oleate 12-hydroxylase from castor bean in transgenic *Arabidopsis thaliana* resulted in an accumulation of up to 17% hydroxy fatty acids including ricinoleic (C18:1-OH), lesquerolic (C20:1-OH) and densipolic acid (C18:2-OH) [85]. Similar efforts in the area of epoxy fatty acids currently focus on cloning the responsible linoleate epoxidase gene from *Euphorbia lagascae*, a wild spurge native to Spain, with the goal of producing oil rich in vernolic acid, C18:1 epoxy [77].

Most of the genetically engineered rapeseed varieties currently under development are substitutes for raw materials established at existing markets. For example, high-laurate rapeseed is an annually growing domestic source of lauric acid leading to certain replacement of tropical oils derived from perennial crops like coconut or oil palm. The market relevance of such substitutes for traditional vegetable oils or even petrochemical feedstocks is uncertain and may rely on improved yields of the novel fatty acid desired [2,15]. It may also be influenced by special market situations due to periodic shortages in raw materials supply. For instance, in the Philippines it is estimated that ca. 100 million coconut trees were damaged by heavy typhoons in 1996. Thus, tight tropical lauric oil supply may enhance high-laurate rapeseed production, which could lead to a stable supply reducing price volatility [1]. Furthermore, efforts to domesticate and develop so-called new oilseed crops, such as crambe, lesquerella or *Euphorbia lagascae*, for specific oil types having unusual properties would obviously be superseded by the production of similar oils in a genetically engineered adapted present-day crop, like rapeseed or sunflower. The latter can be grown in a wider range of climates or are compatible with existing farm practices and trade infrastructure as well as processing methods.

Although many of the specialty rapeseed varieties mentioned in this paper may not reach large-scale cultivation, it is obvious that trade and industry will be faced with numerous rapeseed varieties which will be essentially identical in seed properties, growth habit and yield potential, yet contain seed oils completely matching various industrial purposes. Even though a specialty rapeseed may look just like standard commodity rapeseed, its identity must be preserved from planting until consumption – as already demonstrated for laurate canola production in the USA [21]. Consequently, a reliable system of identity preservation is indispensable, including all the necessary precautions to assure that the composition of the specialty rapeseed oil will not be affected by admixtures and cross-contamination with commodity rapeseed or with any other oilseed crop, either during operations of growing, harvesting and handling, or subsequently during storage, transport, crushing and refining of the final product to produce the desired oil. Thus, in some instances the quantities of these specialty oils will create demands for small or medium-size processing mills. Contract growing, fields' isolation and geographical separation as well as quality assurance and product integrity will have to be monitored regularly. This will require supervision of the crop through all stages of production and processing in order to ensure that genetically modified plant varieties become a commercial reality. At the same time, an important determinant to the success of specialty oilseeds will be whether the end users will be willing to pay a higher price to cover the higher costs associated with such a system of identity preservation [11].

1.6. Molecular cytogenetic techniques as a supplementary tool in *Brassica* breeding

1.6.1. Introduction

Knowledge of the physical organisation of DNA sequences within the genome is critical for the understanding of genome structure and function. The technique of fluorescence *in situ* hybridisation (FISH) allows the precise physical localisation of genes or DNA sequences on cytological preparations. In recent years FISH has facilitated enormous progress in studies of genome organisation in humans and other mammals. Coupled with rapid technological advances, it is being increasingly adapted for the localisation and characterisation of various classes of DNA sequences in plant genomes. Applications of FISH methods in plant genome mapping have been reviewed in detail by Jiang and Gill [86,87].

The use of total genomic DNA as a FISH probe (genomic *in situ* hybridisation, or GISH) is especially useful for diagnostic studies of the amount and integration of foreign chromatin in interspecific and intergeneric plant hybrids [see 88]. Hybrids between high-yielding rapeseed cultivars and related species are relatively easy to produce. They are used often to develop new lines containing desired traits like pest or disease resistance. Great progress in rapeseed breeding has resulted from the application of *in vitro* techniques for the generation of viable offspring from interspecific and intergeneric hybrids [34]. Here we describe the application of methods for GISH analysis of intergeneric *Brassica* hybrids [89] for the characterisation of backcross progeny from *B. napus* hybrids exhibiting nematode resistance introduced from *Raphanus sativus* [90], and *Phoma* resistance from *Sinapis arvensis* and *Coincya monensis* respectively.

The use of more specific DNA sequences as FISH probes enables their localisation to discrete chromosomes or chromosomal regions. Localisation of repetitive DNA probes gives important information on the distribution of repetitive sequence motifs throughout the genome [91,92], an important aspect in the genetic mapping of molecular markers. Physical information about the extent and localisation of both repetitive and low-copy sequences can be compared with genetic maps of molecular markers, allowing associations to be made between distinct chromosomal regions and molecular marker linkage groups. Additionally, hybridisation patterns of repetitive DNA sequences can be used as chromosome markers in plants like rapeseed which have numerous small chromosomes with few cytological features to allow their identification using traditional cytogenetic methods.

The heavily condensed chromatin characteristic of plant chromosomes prevents the reliable localisation of single- or low-copy sequences shorter than 10kb on plant metaphase chromosomes [86,93]. The development of megabase-DNA (BAC, YAC) libraries, however, provides a way to overcome this limitation: low-copy BAC or YAC clones can now be localised directly onto metaphase chromosomes or in interphase nuclei. This development has important implications for the integration of molecular marker linkage maps with physical information, since simultaneous hybridisation of genetically linked clone sequences can give important information about the spatial distribution of molecular markers along chromosome arms. Using multicolour FISH to extended chromatin fibres it is now also possible to estimate physical distances among genetically linked megabase-DNA fragments containing genes or markers of interest [94]. Such physical data is extremely useful for positional cloning strategies.

We have developed methods for the reliable chromosomal localisation of FISH signals in *Brassica* species and a GISH technique that allows identification of alien chromatin in

intergenomic rapeseed hybrids. Use of FISH with repetitive probes for the identification of chromosomes is discussed, along with the practical application of GISH techniques for the characterisation of various rapeseed hybrids containing genes of interest. Future aims include the application of fibre-FISH to obtain physical information about spatial relationships among molecular markers.

1.6.2. Materials and methods

Mitotic metaphases were generated from seedlings of diverse *Brassica* cultivars for FISH and from hybrid plants (see Table V) for GISH. Cytological preparations were made from young root tips using a modification of the droplet method of Schwarzacher et al. [95]. Briefly, whole seedlings or excised roots were incubated in 2 mM 8-hydroxyquinoline for 90 min at room temperature and a further 90 min at 4°C before fixation in ethanol-acetic acid (3:1). After digestion in cellulase and pectinase, root tips were subjected to 30 min hypotonic treatment in 75 mM KCl, then washed for 20 min in 60% acetic acid to clear cytoplasm before being suspended in ethanol-acetic acid (3:1) and spread on clean slides at -20°C. Fibre-FISH preparations were generated from *B. oleracea* L. (white cabbage cv. ′Braunschweiger′) following the method of Jackson et al. [94].

TABLE V. INTERGENERIC HYBRIDS ANALYSED USING GISH

Target genome	Donor	Resistance character	Backcrossed with
B. napus cv. ′Drakkar′	*Raphanus sativus*	Beet cyst nematode resistance	*B. napus* cv. ′Lisandra′
B. napus cv. ′Madora′	*Sinapis arvensis*[a]	*Phoma* resistance	*B. napus* cv. ′Ceres′
B. napus cv. ′Loras′	*Coincya monensis*[a]	*Phoma* resistance	*B. napus* cv. ′Loras′

[a] *Sinapis* and *Coincya* crosses were kindly provided by Prof. Marisol Sacristan, FU-Berlin, Germany

In situ hybridisation followed methods described previously [89], with slight modifications. For multicolour FISH, a 25S rDNA clone from *Arabidopsis thaliana* and the 5S rDNA subunit from *Beta vulgaris* were labelled by nick translation with the fluorochromes Cy3 and fluorescein, respectively. For GISH probes, genomic DNA extracted from *Raphanus sativus*, *Sinapis arvensis* and *Coincya monensis* was directly labelled with Cy3 and mixed with a 50-fold volume of unlabelled, sheared *B. napus* genomic DNA. GISH probes were pre-annealed for 20 min at 37°C before hybridisation. All slides were washed at 42°C for 5 min in 2x SSC and 10 min in 0.2x SSC before being counterstained with DAPI. Composite fluorescence images were obtained using a Leica DMR fluorescence microscope fitted with specific single-band filters for DAPI, FITC and Cy3 and an integrating black and white CCD camera driven by Leica QFISH software.

1.6.3. Results and discussion

Multicolour FISH was used for the co-localisation of 25S and 5S rDNA loci on mitotic metaphase and prometaphase chromosomes from various *Brassica* species. In *B. napus*, co-localisation of the six 25S rDNA loci [96,97] with the seven 5S rDNA loci enables the reliable identification of 10 chromosome pairs. Using conventional cytogenetic methods the identification of rapeseed chromosomes is often difficult or impossible. FISH with rDNA and other repetitive DNA probes provides molecular cytogenetic markers for accurate chromosome identification, opening the possibility for a correlation of molecular marker linkage groups with individual chromosomes.

Using GISH, backcross offspring from three different intergeneric *B. napus* crosses could be effectively characterized. Addition chromosomes were identified in BC_3 individuals from *B. napus* crosses with *Raphanus sativus*, *Sinapis arvensis* and *Coincya monensis*, respectively. In both the *R. sativus* and *S. arvensis* crosses, fertile BC_3 individuals exhibiting the desired resistance characters were found which contained monosomic addition chromosomes from the respective donor genome. GISH is now being used for the characterisation of resistant BC_4 offspring.

The ultimate aim for each of the hybrids described above is a high-quality rapeseed cultivar containing the desired resistance genes on a stable chromosome introgression. Although intergenomic recombination appears to be low in the hybrids described here, it is hoped that useful introgressions will be present in resistant BC_4 plants. Donor-genome introgressions can potentially be observed by GISH as was confirmed by the discovery of a small translocation (along with two complete addition chromosomes) in a non-resistant BC_3 *B. napus* x *R. sativus* individual. However, the visualisation of small translocations in rapeseed hybrids by GISH can be problematic, because the chromosome arms of *Brassica* and closely related species contain few of the dispersed repetitive sequences that contribute to GISH signals [88]. This is not necessarily a major problem, however, because in eventual late-backcross introgression lines it will be the plant performance - rather than the quantity of donor chromatin - which will be the deciding factor in the selection of breeding material.

A fibre-FISH technique for the high-resolution localisation of FISH signals on extended chromatin fibres is presently being adapted for use in *Brassica* species. Until now only repetitive probes have been hybridised. With further improvements in the technique, however, it is hoped that reliable detection of low-copy markers will also be possible. As described by Jackson et al. [94], this would open the possibility to compare physical distances between linked molecular markers with the corresponding genetic distances. Such information will be extremely useful for future physical mapping and positional cloning efforts.

2. SUNFLOWER BREEDING: CONVENTIONAL METHODS AND APPLICATION OF BIOTECHNOLOGY

2.1. Conventional and commercial sunflower breeding

Commercial sunflower varieties are essentially single cross hybrids derived from the hybridisation of a male-sterile female line (A-line) – based on cytoplasmic male sterility (CMS) - with a fertile restorer line (R-line). The principal scheme for the development of a hybrid variety by such a procedure is presented in Fig. 1. The essential steps of such a breeding programme are identical in different crops species, i.e. a) Development of basic breeding pools with satisfactory agronomic performance (e.g. disease resistance) on the female and the male side; b) Testing of lines in both pools – i.e. inbreds - for combining ability with materials of the respective other pool – eventually aided by marker assisted selection; c) Production of experimental hybrids and testing at multiple locations.

Extensive interspecific hybridisations have been carried out for broadening genetic variation, e.g. with regard to sources of resistance to diseases. The materials tested in field experiments and the results are summarized in Tables VI and VII.

TABLE VI. SUNFLOWER INTERSPECIFIC HYBRID PROGENIES USED FOR *Sclerotinia* s*clerotiorum* TESTING

Entry[a]	Interspecific hybrid progeny (cross combination)	Generation[d]
2	'Baso' (cms)[b] x *H. mollis* Pop. Rh[c]	S_2
3	'Baso' (cms) x *H. mollis* Pop. Rh	S_2
4	'Baso' (cms) x *H. mollis* Pop. Rh	S_2
5	'Baso' (cms) x *H. mollis* Pop. Rh	S_2
6	'Baso' (cms) x *H. mollis* Pop. Rh	BC_1S_1
8	'Baso' (cms) x *H. mollis* Pop. Rh	S_2
9	'HA89' (cms) x *H. decapetalus* Pop. Dij	BC_2S_1
10	'HA89' (cms) x *H. mollis* Pop. 187*3*	S_3
11	'HA89' (cms) x *H. mollis* Pop. 187*3*	S_3
12	'HA89' (cms) x *H. mollis* Pop. 187*3*	S_3
13	'HA89' (cms) x *H. mollis* Pop. 187*3*	S_3
15	'HA89' (cms) x *H. giganteus* Pop. 1897	S_3
16	'HA89' (cms) x *H. giganteus* Pop. 1897	S_3
17	'HA89' (cms) x *H. maximiliani* Pop. 40	S_3
18	'HA89' (cms) x *H. mollis* Pop. 1873	S_3
19	'HA89' (cms) x *H. pauciflorus*	S_3
21	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
22	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
23	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
24	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
25	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
26	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
27	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
29	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
30	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
31	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
32	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
34	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3
36	'HA89' (cms) x *H. tuberosus* Pop. 1705	BC_2S_3

[a] Entries No.1 (Sunking 256), 7 (Alphasol), 14 (Frankasol) are commercial hybrids used as checks [98].
[b] Cytoplasmic male sterile inbred line.
[c] Populations of *Helianthus* wild species maintained at the Inst. Crop Sci. & Plant Breeding, Giessen/Germany.
[d] S = self-pollination generation, BC = backcross generation (backcrosses were performed with the line 'HA89'; the F_1 hybrids were used as male parents).

Corresponding inbred materials are now used for further breeding, i.e. production of experimental hybrids. Such interspecific progeny are also used for basic studies in order to improve the *in vitro* culture ability of sunflower.

TABLE VII. ANALYSIS OF VARIANCE FOR THE SIZE OF LESIONS CAUSED BY ARTIFICIAL MID-STEM INFECTION OF *Sclerotinia sclerotiorum* IN 29 INTERSPECIFIC HYBRID PROGENIES AND 3 COMMERCIAL HYBRIDS (2 REPLICATIONS, 1995 I, II)

Source of variation	df	SS	MS	F value	LSD 0.05
Blocks	1	5.63	5.63	0.00 NS	88.35
Genotype (G)	31	1657.69	53.47	1.95 **	7.79
Time (T)	1	1375.17	1375.17	0.89 NS	88.35
G x T	31	712.29	22.98	0.81 NS	10.86

** Significant at P =0.01; NS, not significant.

2.2. Regeneration *in vitro* of sunflower lines derived by interspecific hybridisation as a basis for the establishment of an efficient transformation protocol

2.2.1. Introduction

Interspecific hybridisation of the cultivated sunflower (*Helianthus annuus* L.) with annual or perennial wild species serves to increase the genetic variation regarding resistance against pathogens, abiotic stress tolerance [99], and the development of new CMS sources [100,101,102]. However, for other traits such as seed oil quality, the natural variation in the genus *Helianthus* is quite limited [103]. Genetic engineering offers here the possibility to introduce novel fatty acid patterns into the cultivated sunflower making this crop suitable also for non-food purposes. However, this requires an efficient transformation system. Regeneration of intact plants of sunflower represents the major bottle neck regarding transformation [104]. Until now apical meristems represented the only explants which gave rise to transgenic plants from inbred lines of sunflower [105]. This tissue was first used as the target explant for *Agrobacterium*-mediated gene transfer by Schrammeijer et al. [106]. However, the yield of transgenic plants was very low in this approach. Subsequent studies [107] led to an improvement of the system by combining *Agrobacterium* co-culture with particle bombardment of the explants prior to inoculation with Agrobacteria. Knittel et al. [108], using a similar transformation protocol, concluded that the improvement of the transformation efficiency requires higher rates of adventitious shoot formation which are formed *de novo*. However, Burrus et al. [109] excluded a unicellular origin of shoots from a single transformed cell by analysis of transformation patterns of transgenic shoots emerging from apical meristems. He showed that such shoots originated from transformation events that occurred in the meristem. Therefore, transformation efficiency of apical meristems might be enhanced by using genotypes which regenerate both types, meristem-derived and *de novo*-formed shoots, from shoot apices, at a high frequency.

Wild *Helianthus* species, e.g. *H. resinosus*, *H. laetiflorus* and *H. tuberosus*, seem to bear a higher regeneration potential *in vitro* than the cultivated sunflower [110]. Therefore, the use of interspecific hybrids may help elevate transformation efficiency in sunflower by developing genotypes with higher frequencies of shoot formation.

In the genus *Helianthus*, postzygotic incompatibility observed in wide crosses can be successfully overcome by embryo rescue [111]. Considering the high proportion of the wild type genome in the initial generations of interspecific hybrids [112] and the hypothesis that selection during the domestication process may have led in cultivated sunflower to a loss of genes necessary for regeneration *in vitro* [113], progenies of interspecific crosses in the early generations represent the most promising material to examine for regeneration potential *in vitro*. Simultaneous characterisation of such progenies by agronomic traits may provide seedstocks that combine both: satisfactory agronomic performance and a high regeneration potential *in vitro* for the use in breeding oriented research.

2.2.2. Materials and methods

2.2.2.1. Plant material

Interspecific hybrid progenies originating from crosses between cytoplasmic male sterile *H. annuus* lines 'HA89' or 'Baso' (CMS lines) and the wild species *H. argophyllus* (ARG-Gat), *H. decapetalus* (DEC-Dij), *H. giganteus* (GIG-1897), *H. laetiflorus* (LAET), *H. maximiliani* (MAX-40), *H. mollis* (accessions MOL-1873 and MOL-RH), *H. rigidus* (RIG-1848), *H. strumosus* (STR-1974) and *H. tuberosus* (accessions TUB-5 and TUB-1705), respectively, were recovered via embryo rescue [111]. The experimental design consisted of two trials with three progenies in early inbred or backcross generations for each of the 12 interspecific hybrid cross combinations. The parental CMS lines 'HA89' and 'Baso' and the commercial hybrid 'Albena' served as checks, resulting in a total of 39 investigated genotypes.

2.2.2.2. *In vitro* culture

Preparation and culture of apical meristems were carried out as described by Knittel et al. [108]. The pericarp was removed and the seeds were surface sterilized in 5.6% NaOCl. After soaking in sterile distilled water for 4 h, the seed coat was removed prior to germination on MS medium [114] with 3% sucrose for 2 days. The culture conditions were 16/8 h day/night photoperiod at 25°C.

Shoot apices were excised by removing the cotyledons and the radicles. Subsequently, the shoot tips were divided in half by cutting between the two leaf primordia which were discarded afterwards. The resulting explants were cultured on MS medium with 3% sucrose supplemented with 0.1 mg/l BAP and solidified with 4 g/l Gelrite® in 100 mm Petri dishes. The dishes were sealed with Nesco film to prevent dessication and incubated as described above. In the first experiment 55 seeds of each genotype were processed in order to obtain an average number of 100 explants per genotype. In a second trial an average number of 50 explants was cultured per genotype.

The frequency of shoot formation was examined 20-24 days after explant preparation. Genotypic performance *in vitro* was scored (1) by the mean number of shoots produced per cultured explant, (2) by the frequency of responding explants and (3) by the frequency of explants producing more than three shoots. In addition, the origin of the shoots was recorded in order to obtain information about the potential for the induction of adventitious shoots.

2.2.2.3. Shoot regeneration

Responding explants from genotypes with superior regeneration frequency were subcultured after determination of shoot formation on regeneration medium (MS with 3% sucrose, 4 g/l Gelrite®) for two weeks. Elongated shoots were cut and subcultured on the same medium for root induction. Plantlets that exhibited adventitious root formation were transferred to soil and adapted to greenhouse conditions.

2.2.2.4. Evaluation of agronomic traits

The most responsive interspecific hybrid progenies were characterised for their major agronomic traits such as plant height, days to flowering and physiological maturity in the field. Seeds of selfed plants were analysed for oil content (%) and for thousand grain weight (TGW). The evaluation was carried out at our field station in Gross Gerau south of Frankfurt/Main, Germany (light sandy soil) for two years. Each interspecific hybrid progeny was grown in three rows, consisting of 14 plants each. The commercial hybrid 'Albena' and the two CMS lines 'HA89' and 'Baso' were used as checks for comparison.

2.2.3. Results

2.2.3.1. *In vitro* culture

Cultivation of apical meristems was used to evaluate the regeneration potential *in vitro* of 36 progenies of interspecific hybrids involving 10 wild species of the genus *Helianthus*. Responding shoot apices showed induction of shoots 3-7 days after dissection. Callus formation in varying degrees - which occurred predominately at the explant base - was observed for all responding explants. The shoots developed either directly from the meristematic region or *de novo* as adventitious shoots, and from differentiated leaves. Shoots which were formed *de novo* appeared later, i.e. 12 to 16 days after explant preparation [cf. 115].

Regeneration of plants was carried out using 10 highly responsive explants for each of the four genotypes that showed the best results concerning shoot formation. Shootlets elongated rapidly upon transfer of such explants to regeneration medium. Shoots of meristematic origin showed adventitious root induction when subcultured on regeneration medium at an average frequency of 46% and could be transferred to soil whereas those derived from the cross with *H. giganteus* (GIG-1897) exhibited rooting at a frequency of 65%. In comparison, shoots formed *de novo* remained small and failed to produce roots.

Rooted plantlets originating from meristem-derived shoots were all fertile and allowed seed production at a frequency of 20% irrespective of the genotype although the plants exhibited a stunted growth. However, seed were not set when the plantlets flowered prematurely, which represents a general problem in sunflower. In total, 40 plantlets were transferred to soil, 8 of which finally showed seed set.

2.2.3.2. Regeneration potential *in vitro*

The number of shoots regenerated per explant plated was determined by the respective genotype. The mean number of shoots per cultured explant for both trials was 1.6. Most of the shoots (80%) originated directly from the meristem. The frequencies of ‘responding explants’ and ‘explants producing more than three shoots’ were also determined by the genotype with

an average of 37.4% and 17.3%, respectively. In addition, the frequency of 'explants producing more than three shoots' (Fig. 5) proved to be a feasible indicator for shoot formation since the parameters 'shoots per explant' and 'explants producing more than three shoots' were closely related ($r^2 = 94.9$ at P = 1%).

In total, nine interspecific hybrid progenies regenerated a significantly higher number of shoots than the commercial hybrid 'Albena', while 50% of the investigated genotypes were superior to the inbreds 'HA89' and 'Baso'. Progenies of the hybrid combinations between *H. annuus* (female) and *H. decapetalus*, *H. giganteus*, *H. mollis* and *H. strumosus* as male parents, respectively, showed superior results for the overall shoot formation with 2.3 - 3.5 shoots per cultivated explant and a superior frequency of 'explants producing more than three shoots' ranging from 51.3 to 62.4%.

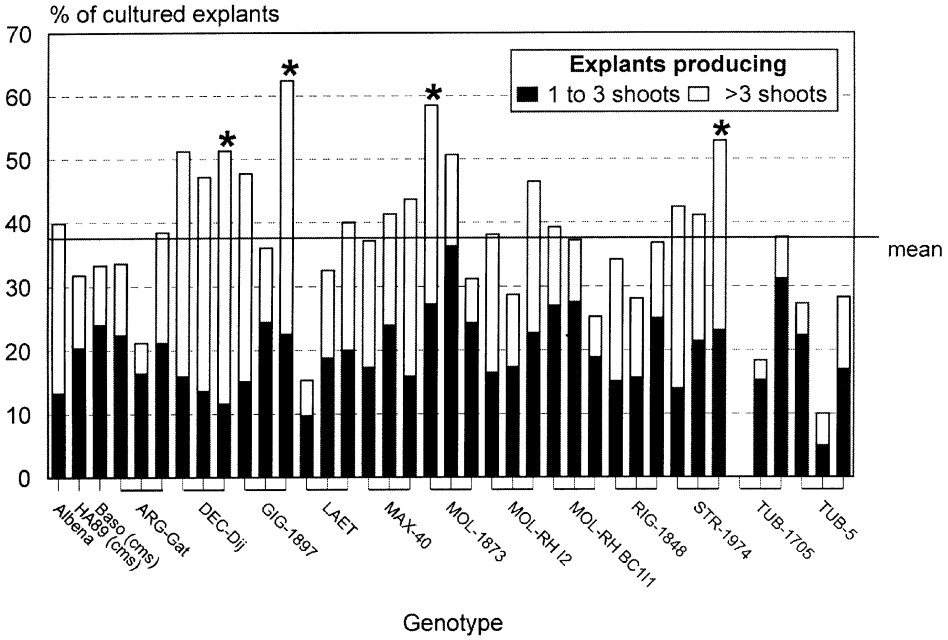

FIG. 5. Shoot formation in vitro from apical meristems: frequencies (%) of explants with shoot regeneration [115].

All three progenies of crosses with *H. decapetalus* (DEC-Dij.) and *H. strumosus* (STR-1974) as male parents exhibited an outstanding potential for regeneration *in vitro* while considerable variation was detected between single progenies of the cross combinations involving *H. mollis* (MOL-1873) and *H. giganteus* (GIG-1897) as wild parents. Maximum shoot formation was observed for the progeny sf 2789/91 (of 'HA89' x *H. decapetalus*) with a mean of 3.5 shoots per cultured explant (6.8 shoots per responding explant). Up to 25 shoots on a single explant were observed for the genotype sf 2787/91 derived from the cross with *H. decapetalus*.

Regarding the origin of shoots, progenies derived from the cross combination with *H. decapetalus* (DEC-Dij.) exhibited a superior ability for adventitious and secondary shoot regeneration, as well as the selected progeny sf 3143/90 from the cross involving *H. mollis* (MOL-1873) with up to 30% of the shoots formed *de novo*. The selected progenies sf 3565/90 and sf 2803/91 originating from crosses with *H. giganteus* (GIG-1897) and *H. strumosus* (STR-1974), respectively, regenerated shoots mainly directly from the meristem while *de novo* shoot formation was only recorded at a frequency of 15% each. From each of these cross combinations the best performing progeny was selected for regeneration potential *in vitro*.

More than 50% of the responding explants of the progenies selected produced more than three shoots (Fig. 5).

Statistical analysis verified that performance *in vitro* estimated by the number of shoots per explant strongly depended on the genotype (significance level $\alpha<0.01$).

2.2.3.3. Genotype characterisation on the basis of agronomic traits

In the field trials, the four interspecific hybrid progenies selected by their superior *in vitro* performance demonstrated a high degree of homogeneity despite their being in comparatively early generations. The phenotype of the interspecific progenies was closer to that of cultivated sunflower than to the respective wild species. All of the selected lines flowered later than the cultivars. Physiological maturity varied from mid/mid-early to mid-late for the selected interspecific hybrids and was comparable to that of the check cultivars. The thousand grain weight (TGW) - a major yield determining parameter – ranged between 39.2 and 59.4 g for these lines. Here, the progenies sf 2789/91 (HA89 x DEC-Dij.) and sf 2803/91 (HA89 x STR-1974) were in the range of the inbred lines ‘HA89’ and ‘Baso’ while sf 3565/90 (HA89 x GIG-1897) and sf 3143/90 (HA89 x MOL-1873) showed lower values. Regarding oil content, a low variation was observed between 37.1 to 41.9% in the progenies of the investigated cross combinations. The oil content was comparable to that of the inbred ‘HA89’. However, none of the investigated genotypes reached the oil content of the commercial hybrid ‘Albena’ [cf. 115].

2.2.4. Discussion

The culture of apical meristems *in vitro* can be considered a reproducible method for examination of regeneration potential in sunflower. Selection for superior performance *in vitro* proved to be possible since the examined parameters 'shoots per explant', 'responding explants' and 'explants producing more than three shoots' were clearly determined by the genotype. For cultivated sunflower Sarrafi et al. [116] demonstrated by estimation of general combining ability (GCA) that the organogenic response, scored by the number of responding explants and the number of shoots per explant, was under additive genetic control. In addition, the authors demonstrated by analysis of specific combining ability (SCA) genetic variation due to dominance effects.

The interspecific hybrids used in our study revealed a fairly large variation of regeneration potential between, and to some extent within, single cross combinations. Nine lines responded better than the commercial hybrid cultivar 'Albena', and as many as 50% of the interspecific progenies were superior to the inbred lines ‘HA89’ and ‘Baso’ regarding this trait. This can be explained by introgression of “wild” alleles into these cultivated sunflower lines. Obviously, high potential for regeneration from *in vitro* culture can be found in various interspecific hybrid combinations but not in each progeny. However, the presented results support the hypothesis that wild species of *Helianthus* possess a higher regeneration potential *in vitro* than common cultivated sunflower materials [113].

The best cross combinations regarding regeneration *in vitro* involved the wild species *H. decapetalus*, *H. giganteus, H. mollis* and *H. strumosus* as parents. Three of these four wild species have already demonstrated their capacity to contribute to the success of various biotechnical approaches, i.e. *H. decapetalus* [117], *H. giganteus* [118,119], and *H. mollis* [120]. *H. tuberosus,* which has earlier been used with success for somatic embryogenesis

[121], anther culture [122], and genetic transformation [123], showed a very low response in apical meristem culture.

Regarding the origin of the shoots regenerated from shoot apices, interspecific hybrids were identified which exhibited a superior ability to regenerate adventitious and secondary shoots. Konov et al. [124] studied epiphyllus bud formation in sunflower using *in vitro* cultured shoot apices and described these buds appearing 9-11 days after excision of the leaves. However, the secondary shoots observed in the present study appeared at nearly the same time on leaves of previously developed shoots and therefore can be considered consistent with the epiphyllus buds described by Konov et al. [124]. Meristems of such buds are formed by a multicellular process from subepidermal cell layers on the adaxial leaf side which is cytokinin-dependent. The obviously high ability to form secondary shoots observed in progenies derived from crosses with the wild species *H. decapetalus* and *H. mollis* additionally suggests a genotypic control of this regeneration pathway. The second type of shoots, those formed *de novo,* corresponds to the description of adventitious shoots observed by Burrus et al. [109]. Stable transformation of such shoots at a very low frequency has been reported. Interspecific progenies selected for their superior regeneration potential represent a promising starting material for transformation experiments employing shoot apical meristems since they deliver shoots of different origin at a high frequency.

Regarding the recovery of shoots, the adaptation to greenhouse conditions and seed production, the regenerated shoots often exhibited premature flowering, that lowered the efficiency to a considerable extent, irrespective of the genotype used. This phenomenon has recently been shown to be influenced by culture conditions [125]. The latter authors could reduce the frequency of *in vitro* grown plantlets flowering precociously by applying shorter day lengths (8/16 h day/night) and a lower culture temperature (20°C). In addition, the genotype and the composition of the culture media affected the occurrence of premature flowering.

Finally, field trials demonstrated that the narrow genetic background of the two parental female inbred lines, ‘HA89’ and ‘Baso’, was broadened to a large extent by introgression of “wild” germplasm into the cultivated sunflower. Therefore, the aim of the work, i.e. to develop lines carrying a high regeneration potential *in vitro* combined with a phenotype adequate for incorporation in breeding programmes, could already be achieved. The investigated crosses showed a comparatively high degree of homogeneity within single cross combinations in the field although they represented early generations. Regarding the results of TGW and oil content as major yield and quality determining parameters, the data indicate that a loss of agronomic performance is not necessarily caused by interspecific hybridisation. The selected progenies which combine both a high regeneration potential *in vitro* and an adequate agronomic performance, such as lines sf 2789/91 of *H. annuus x H. decapetalus* (DEC-Dij.) and sf 2803/91 of *H. annuus x H. strumosus* (STR-1974), represent ideal breeding materials for further crop improvement involving application of biotechnological methods, which have been characterized by a comparatively low efficiency so far [105,113]. The introgression of wild germplasm into cultivated sunflower offers the possibility to increase the efficiency of *in vitro* techniques and may therefore be used not only to increase genetic variability in sunflower *per se* but also to make this crop more accessible for transgenic approaches. The selected interspecific progenies can now be used to increase the efficiency of transformation protocols using apical meristems as explants.

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Biotechnology trends in oilseed crops in the USA

S.J. Knapp

Centre for Gene Research and Biotechnology,
Department of Crop and Soil Sciences,
Oregon State University,
Corvallis, Oregon, United States of America

Private biotechnology companies (primarily DuPont and Monsanto) have isolated hundreds of genes from developing seeds of a wide range of plant species that produce novel fatty acids in addition to more generic fatty acids. Both companies have completed massive expressed sequence tag (EST) sequencing programs. Genes underlying most of the key activities for engineering novel oils have been cloned and many have been engineered. However, despite phenomenal progress in isolating genes and producing novel phenotypes (fatty acid profiles) in engineered plants, the field is still a long way from producing so-called "designer oils" by genetic engineering.

Numerous target phenotypes have been very difficult to reproduce in transgenic plants (e.g. the development of oils with novel unsaturated fatty acids has been particularly difficult). Dow Elanco Co. is funding a significant collaborative research project in several public laboratories in the USA geared towards understanding why certain fatty acids are not incorporated into triacylglycerides in high concentrations and how the glycerolipid biosynthetic pathway is regulated.

Among the oilseeds, DuPont has concentrated on genetically engineering soybean with some work on sunflower, while Monsanto has concentrated on genetically engineering rapeseed. Several genetically modified organisms (GMOs) have been developed and tested and many are being produced commercially. This trend is expected to continue; however, there seems to be a growing resistance to GMOs that could dramatically impact the delivery of transgenic technology to the marketplace. Regardless, the biotechnology industry will become more and more proficient at producing novel oils by genetic engineering. Novel oils produced by induced mutations still play a role and are economically significant. There are still many crops or species where induced mutations are needed or can play a role.

The US is investing millions of dollars in plant genomics. Research is being funded by the US Department of Agriculture (USDA), US Department of Energy (DOE), National Science Foundation (NSF), and the National Institute of Health (NIH), in addition to private companies and non-profit organizations (e.g. the American Soybean Association). The *Arabidopsis* genome is to be fully sequenced by 2001.

The greatest investment in the US in an oilseed has been made in soybean. Several million dollars have been directed towards EST sequencing, physical mapping, genetic mapping, and the development of high throughput genotyping technologies in soybean and cotton. Sizable sums have been directed towards genetic mapping and the development of high throughput genotyping technologies in sunflower and rapeseed. The present focus on genotyping technologies is on the development of molecular markers for simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) and the development of microarray and microchip technologies for SNP analysis. Almost nothing is being invested in flax, safflower, sesame, and many other neglected and under-utilized species.

Cotton biotechnology

J.McD. Stewart

Cotton Biotechnology and Germplasm Development Laboratory,
Crop, Soil & Environmental Sciences Dept.,
University of Arkansas, Fayetteville, Arkansas, United States of America

Abstract. The contribution of the laboratory to the CRP related to the development of a new crop (*Gossypium arboreum*) for the USA, and to biotechnological applications for cotton (*G. hirsutum*) improvement. Within the framework of the CRP, a genotype of *G. arboreum* suitable for USA production and production strategies shown to be compatible with the existing cotton production and processing infrastructures were developed. In the area of biotechnology, molecular markers closely associated with genes which restore fertility to cytoplasmically male sterile lines of cotton were identified. Gene transcripts possibly associated with restoration were isolated and identified. Gene transcripts putatively associated with semigamy expression were also identified. A gene coding for a membrane-active peptide was constructed and tobacco, as a model system, has been genetically engineered with this gene. New methods for transformation of cotton are under investigation, and a number of new projects are being initiated. For the benefit of readers interested in biotechnology resources, a short list of website addresses are annotated in this report.

1. INTRODUCTION

The original collaborator contract for my participation in this CRP stipulated work on the development of Asiatic cotton (*Gossypium arboreum* L.) as a new crop in the USA. While that research was pursued, at the organizational meeting of the CRP a broader interest by the organizers and participants in the application of biotechnology to cotton was apparent. As a consequence related research in the Cotton Biotechnology and Germplasm Development Laboratory, University of Arkansas, was reported at subsequent meetings. This report summarizes recent and current research projects by personnel associated with the laboratory, and indicates a few of the new directions that are underway. In addition, an annotated list of internet websites are included for readers interested in availing themselves of the biotechnology resources relating to cotton.

2. SUMMARY OF RESEARCH PROJECTS

2.1. Asiatic cotton

In the USA the fibres of Asiatic cottons are used in padding and other non-woven materials requiring high absorbency, such as disposable diapers and feminine products. The fibre walls are very thick and, although fibre length is usually less than an inch, the micronaire can exceed 8.0. It is the high cellulose content of the fibres that makes them highly absorbent and resilient to pressure. Traditionally the fibre has been imported, and no Asiatic cotton is produced in the USA. Industrialists are interested in having a domestic source for the fibre, so this project was undertaken to identify germplasm and production methods suitable for the USA. A brief summary of the research has been published [1].

In 1996, 75 lines of Asiatic cotton from the USDA Cotton Germplasm Collection that had high micronaire (7.0+) in previous tests were tested for yield in single 10-meter rows at Rohwer, Arkansas. The 1996 production year was exceptionally good and the top yielding variety had about 1700 kg/ha of lint. Some of the lines had high yield potential but dropped a

high percentage of the locules on the ground after boll opening. Nine lines, based on yield potential and other agronomical traits, particularly ability of the bolls to retain the locules after they open, were grown again in 1997 to determine their suitability for mechanical harvesting and ginning. Replicated yield data were not taken that year because a spindle picker was used at harvest, and this proved inadequate for this cotton. The fibres adhered very poorly to the picker spindles and a high percentage of the seedcotton dropped to the ground. The Asiatic cotton that was harvested was ginned on a USDA research gin, indicating that it could be processed on existing commercial gin stands. Of the nine lines four were selected, primarily on the basis of earliness of crop maturity, and grown the next year in replicated plots for large scale evaluation and for seed increase in Keiser, Arkansas. The plots were mechanically harvested with a stripper, and efficiency of harvest was good. It was confirmed that Asiatic cotton could be ginned on commercial gin stands designed for upland cotton. The best Asiatic cotton selection was grown on 4 ha in the Mississippi Delta region in 1999 under ultra-narrow row (25 cm) production with no irrigation. The crop received no water after June 20 because of an extremely hot/dry weather pattern in that area in 1999. The field yielded 320 kg/ha of lint from the gin, which was comparable to Upland cotton (*G. hirsutum* L.) grown under the same conditions. The selected genotype has been planted to 40 ha in 2000, half at 1 m row spacing and half at 25 cm row spacing.

From among more than 70 genetic lines one has been selected that is suitable for production in the Mississippi Delta region using available equipment and infrastructure in place for upland cotton. The yield potential is comparable with conventionally grown upland cotton cultivars. With continued interest by the manufacturers in terms of advance contracts for the cotton produced, production of Asiatic cotton can be an economical alternative for a limited number of cotton producers in the USA.

2.2. Cytoplasmic male sterility and restoration

A new cytoplasm male sterile/restorer system was developed for cotton based on the cytoplasm (D8) of *G. trilobum* Skov. [2]. Restoration is conditioned by one nuclear gene (Rf_2) transferred into upland cotton from the cytoplasmic donor parent [3]. The D8 cytoplasm affects the viability of upland nuclear male gametes and restoration is gametophytic. Tests determined that Rf_2 is not allelic to a restorer for *G. harknessii* (D2-2) but is closely linked to it (0.81% recombination frequency) [4]. The Rf_1 gene is sporophytic in action but is preferentially transmitted over the non-restoring allele rf_1 through the male gametes.

In efforts to identify the restorer gene, comparisons of anther gene expression were made between normal plants and plants heterozygous for the restorer. Thirty-eight gene transcripts related to CMS restoration were isolated from differential displays [5] of anther cDNAs and subjected to reverse Northern dot-blotting to verify differential expression. Compared with the normal recurrent parent, the heterozygous restored plants had 5 genes up-regulated and 12 genes down-regulated (one half of the pollen grains of the heterozygous plants are non-functional). The down-regulated genes included phosphoribosylanthranilate transferase, calnexin, starch synthase, polyubiquitin, ascorbate oxidase and others of unknown function. These genes are involved in tryptophan synthesis (and IAA), protein and starch synthesis, protein maturation and targeting for degradation, suggesting that the metabolically depauperate condition of non-restored pollen accounts for its non-function [6]. We have observed microscopically that starch does not accumulate in these pollen grains whereas it does accumulate in pollen containing the restoring allele.

As an aid in developing parental lines for hybrid production, an effort was made to identify molecular markers linked to the restorer genes, Rf_1 and Rf_2. Bulk segregate analyses [7] were used on separate populations segregating for Rf_1 and Rf_2. Random amplified polymorphic DNA (RAPD) fragments, putatively linked to one of the genes in the bulked samples, were tested on all the plants in the test population. A RAPD marker was identified that co-segregated with Rf_1, and another was identified that was located approximately 3 cM from Rf_2 [8]. Site-specific polymerase chain reaction (PCR) primers are currently being developed for these molecular markers.

2.3. Semigamy

Semigamy (*Se*) is a type of facultative apomixis in cotton in which male gametes do not fuse with female gametes after entering the embryo sac, leading to production of paternal and maternal haploid progeny [9,10]. It provides a potential system to investigate reproductive biology and a convenient way to generate haploids at will in cotton breeding. It can be observed only in the female, therefore it is necessary to observe the next generation for haploids in order to determine if an existing plant carries the trait. A molecular marker associated with this trait would be very useful.

The plant materials consisted of Pima S-1, a normal *G. barbadense* (*sese*), and the semigametic line (*SeSe*) Pima 57-4. The genetic dissimilarity between 57-4 and S-1 was evaluated at the DNA level with molecular markers (RAPDs). The polymorphic molecular markers between 57-4 and S-1 provide putative molecular markers for tagging the *Se* gene, so that development of new semigametic lines can be facilitated. To isolate genes related to *Se* expression, mRNA differential display [5] was employed to compare cDNA profiles between 57-4 and S-1. The differentially displayed cDNA bands were excised, re-amplified, cloned into pGEM-T vectors, and the nucleotide sequence determined. Based on 171 RAPD markers generated from 20 informative primers, 57-4 was 93% identical to S-1, while each shared only 57% of the markers with upland cotton (*G. hirsutum*). When mRNAs from ovule and anther tissues were compared between 57-4 and S-1, more than 60 differentially expressed cDNAs were detected, cloned and sequenced. Some of the sequences had homology to genes coding for cell division-related proteins. The *Se* gene is likely related with cell division. Gene expression studies associated with *Se* will help understand its molecular mechanism and mode of action and may eventually lead to isolation of the *Se* gene.

2.4. Magainin analog

Insects and microbial pathogens are ever present challenges to crop production. Pesticides and fungicides have been the control methods of choice. However, biotechnology is now offering an alternative through enhancement of host plant resistance by incorporation of genes with antibiotic properties from foreign sources. Fungal and bacterial pathogens pose a major challenge to cotton, and fungal pathogens alone contribute to a significant reduction in yield in the United States and elsewhere.

Several organisms provide us with sources of peptides with potential antibiotic activity against pathogens. One such group, called magainins, are small (approximately 23-30 amino acids) peptides isolated from the skin of the African clawed frog (*Xenopus laevis*) [11]. The effectiveness of magainins against lower organisms is based on disruption of membrane integrity by inserting into the lipid bilayers and formation of ion channels. Membranes of higher plants and animals are relatively insensitive to these peptides because of differences in membrane chemistry. We determined that magainin 2 at 0.05 mg/ml completely inhibited

hyphal growth of several species of pathogenic fungi including *Rhizoctonia solani, Fusarium oxysporum, Verticillium dahliae* and *Thielaviopsis basicola* [12]. Consequently we have undertaken to synthesize a gene coding for this peptide and use it in the transformation of plants to enhance resistance [13]. Gene constructs harboring the magainin coding sequence and a signal peptide sequence were made. The codons used for the construct were those most favored by cotton. The function of the transit peptide is to localize the gene product in the endoplasmic reticulum. Various over-lapping fragments of the magainin gene were synthesized then linked and replicated by PCR. Following synthesis the PCR products were cloned into pGEM-T as vector and sequenced to verify that the sequence was correct and free of PCR errors. The gene was excised from the pGEM-T vector and cloned into a pBIN binary vector under the control of the CaMV 35S constitutive promoter. The gene insert was verified by restriction digestion and PCR. Plasmid DNA was isolated and mobilized into *Agrobacterium* super virulent strain EHA 105. Again the presence of the gene in *Agrobacterium* was confirmed by PCR analysis. As a preliminary model to determine expression potential of the construct, tobacco leaf discs were transformed by co-cultivation with *Agrobacterium* harboring the binary vector. Putatively transformed shoots were selected based on resistance to the antibiotic kanamycin. Shoots were transferred to rooting media. After primary roots were formed the plants were transferred to pots containing potting soil. Gene integration was confirmed by gene-specific priming of PCR with the tobacco genomic DNA as template. Currently the plants are growing and will be tested for enhanced antimicrobial activity. Following a positive result, cotton will be transformed with the construct. In addition to evaluation of effectiveness against pathogens, the effect of the peptide on mycorrhizae will be evaluated to determine potential negative influences.

3. NEW PROJECTS

At the conclusion of the CRP, four new areas of research are in the planning stage or have been recently initiated in the Cotton Biotechnology and Germplasm Development Laboratory.

3.1. Cotton transformation

Cotton regeneration from callus tissue is a major bottle-neck to progress in the production of transgenic plants. Also, the ability to regenerate is very genotype specific. We have initiated a project to determine if cotton meristems can be efficiently transformed *in planta* by *Agrobacterium* following various permutations in mechanical injury and infiltration.

3.2. Tissue specific gene regulator

A morphological gene that is expressed only in the anther walls of cotton has been identified. We are attempting to isolate the gene to obtain the tissue specific promoter. Anthers are a major feeding site for plant bugs, boll worms and boll weevils. An anther wall-specific gene promoter could be used to restrict the production of a transgenic pesticidal protein toxin to the anther wall for maximum effect at minimum cost to the plant or to non-target insects.

3.3. Reniform nematode (*Rotylenchulus reniformis*) resistance

This is a multi-phased project involving: a. Transfer of immunity to the reniform nematode from a wild species of *Gossypium* to upland cotton via wide hybridization

strategies. b. Development of molecular markers associated with the resistance. c. Finally, isolation and cloning of the gene if the immunity is monogenic.

3.4. Novel QTLs

Cotton has a large pool of exotic germplasm from which to draw new genetic combinations, however this resource is rarely used. Part of the effort in my laboratory is directed toward the development of recombinant inbred lines from exotic crosses. These populations will be evaluated with molecular markers in an effort to identify novel QTLs in the wild materials.

4. COTTON WEB SITES

The advent of the Internet has made information and resources much more available to almost any cotton researcher in the world. For readers who may not be fully aware of the resources that are available, a few particularly useful sites that provide information on cotton and/or biotechnology directly or through links are annotated below. The list is not complete by far, but serves as a starting point through which one can make other links of interest.

4.1. http://www.nalusda.gov/

National Agricultural Library. This is an extremely valuable site that provides entrance through links to many resources including on-line literature searches, DNA sequence data bases and searches, plant genome data bases (including cotton), biotechnology resources, and many other types of information. The cotton data base can be searched through the Agricultural Genome Information Service (AGIS) program list on a site menu under Services.

4.2. http://algodon.tamu.edu/htdocs-cotton/cottondb.html

Cotton DB data collection site. Cotton researchers can add data to the base through this site. It links to the data base for category searches. It also has a link to a cotton "genomics workbook" in which information on a cotton BAC library and cotton SSR can be obtained. Some of the links listed at this site are out of date.

4.3. http://www.genome.clemson.edu/projects/cotton/index.html

Clemson University Genomics Institute Cotton Project. A large number of cotton expressed sequence tags (ESTs) are available through this site.

4.4. http://taipan.nmsu.edu/aght/cotton/genetic.html

New Mexico State University Cotton Genetics Laboratory. This site, developed and maintained by Dr. Roy Cantrell, contains information relating to cotton genetics and breeding research at NMSU and useful links to other Home Pages. Sequences to 10-mer primers giving PCR polymorphic fragments between *G. hirsutum* and *G. barbadense* can be found at this site.

4.5. http://cottongenomecenter.ucdavis.edu/

Cotton Genome Center. This is a newly developing entity founded by a small group of researchers who received a large National Science Foundation grant for research on cotton

genomics. The website is a developing resource that should be useful to cotton researchers as participating directors and collaborators place resources and data in the public domain. As of 1 June, 2000 the center has made available by request through its website: (1) a cotton genomic library (*G. hirsutum* cv. Acala SJ-2); and (2) several cDNA libraries including a) 7-10 dpa fibre from *G. arboreum* cv. AKA8400; b) -3 dpa ovule cDNA of *G. hirsutum* cv. Acala SJ-2; c) 0 dpa ovule of Acala SJ-2; d) leaf cDNA of Acala SJ-2; e) anther cDNA expression library of Acala SJ-2; f) 10 dpa fibre cDNA expression library of Acala SJ-2; and (3) BAC libraries of *a) G. barbadense* cv. 'Pima S6'; and b) *G. raimondii* (un-named accession). Other planned resources are discussed at the website.

4.6. http://www.cotton.org/

Home page of the National Cotton Council of America. Various news items and information relating to cotton are regularly posted at this site. Part of this site is for members only, but there is a wealth of information on cotton at this site that is publicly available.

4.7. http://www.jcotsci.org

Journal of Cotton Science. This electronic journal, begun in 1997, publishes refereed articles in all aspects of cotton research. This journal could be a good avenue for international researchers to present their publishable research to a wide audience. The journal maintains a good scientific and editorial standard of acceptance of manuscripts and it is listed by the major abstracting organizations.

4.8. http://www.genome.ad.jp/kegg/metabolism.html

This site provides extensive information on metabolic pathways including intermediates and enzymes. The metabolic maps are organized according to function.

4.9. http://isb@gophisb.biochem.vt.edu/

Information Systems for Biotechnology (ISB). ISB is funded by a grant from the U.S. Department of Agriculture to Virginia Polytechnic Institute and State University in Blacksburg, Virginia. It is an excellent site for new developments and information on environmentally responsible use of agricultural biotechnology products. One can find documents and searchable databases pertaining to the development, testing and regulatory review of genetically modified plants, animals and microorganisms within the U.S. and abroad.

4.10. http://www.ars-grin.gov/npgs/

USDA, ARS National Plant Germplasm System. One can enter the Germplasm Resource Information Network (GRIN) through this home page to do searches for information on the germplasm (any species) available in the system. The number of cotton (*Gossypium*) accessions exceeds 6000. Germplasm within the system, for which there are seeds, is freely available to anyone in the world with a *bona fide* request. Generally, *bona fide* means the seed is requested for growth, increase, research, evaluation, and utilization by someone with the training and knowledge to accomplish the objectives.

4.11. http://www.nal.usda.gov/pgdic/Map_proj/cotton.html

This is primarily a summary catalog of research projects in the USA in which cotton molecular genome mapping is listed as an objective.

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Improved earliness of two locally adapted cotton cultivars by induced mutations

F.P. Xanthopoulos, U.E. Kechagia

National Agricultural Research Foundation,
Cotton and Industrial Plants Institute,
Thessaloniki, Greece

Abstract. Seeds of two locally adapted cotton cultivars, Eva and Zeta-2, were irradiated by 300 Gy γ-rays in order to create useful variability for earliness within each cultivar, and then to select desirable genotypes. Selection for earliness was applied in the M_2 generation and the earliest 2% of the mutants were selfed for further evaluation. After eliminating the undesirable genotypes the remaining material was sown in M_3 progeny rows. Selection for earliness based upon morphological and physiological characteristics resulted in five early mutants from Eva and three early mutants from Zeta-2. In the following year the M_4 lines were further evaluated for earliness, lint yield, fibre quality and seed quality in three locations across the Greek Cotton Belt. Among the five early mutants of cultivar Eva only one was consistently early in the three locations, while the other four mutants showed significant differences in the first growth stages. From the three early mutants of cultivar Zeta-2, one was consistently early in all three locations while another one was early in one location only. Plant height, lint yield and fibre length, fineness-maturity and strength, as well as oil, protein and gossypol content in the seed were not significantly different from the untreated checks.

1. INTRODUCTION

Cotton is one of the most important cash crops over the world. Although it originated in tropical-subtropical regions, it is nevertheless well-adapted far from its center of origin. It is, in fact, a perennial plant that has been adapted to annual production systems.

Commercial cotton genotypes, when cultivated as annuals, have no separate vegetative and reproductive stages and have an indeterminate type of growth. Therefore, flowers and closed bolls as well as open ones are found on the plant at the same time. Some bolls never reach maturity and much of the photosynthetic capacity is wasted. An earlier termination of flowering would permit the plants to use the photosynthetic products more efficiently.

Climatic conditions in many cotton growing areas are marginal, therefore the cultivars must have special adaptability to them. When the spring is rainy or cold, as is usual in the Northern Hemisphere, cotton sowing is delayed and the harvest period shifts towards late November/early December. Usually this period is wet and it leads to reduction in yield and fibre quality. Consequently cotton revenue decreases about 1-3% daily due to harvesting and ginning problems. Also, seed quality declines rapidly with harvest delays.

There are three classical ways to incorporate new genes into cultivated *G. hirsutum* varieties in order to increase variability. The first, the easiest, is by transferring them from other *G. hirsutum* materials, the second from *G. barbadense* accessions, and the third and most difficult from wild diploid species. In all cases crosses between suitable parents and selection for improved earliness in the segregating generations are a common practice [1]. The use of biotechnology will be the new tool for developing early cotton varieties.

Mutants with changes in the maturation time have been successfully induced in numerous experiments and early cultivars have been released in barley and other crops [2].

Mutation breeding has been utilized in cotton; successful results in developing early cotton varieties have been reported in India and Pakistan [3].

Nevertheless, earliness is very difficult to assess in cotton. Cotton breeders disagree on the criteria for the term earliness (of crop or boll maturity) and on the appropriate methods for estimating this character; this is due primarily to the indeterminate flowering and fruiting habit of the cotton plants. Usually earliness is estimated by the percentage of the total harvest obtained in the first picking, but the time of first picking depends on various factors including weather and cultivation conditions. The first picking may start earlier or later, thus enhancing or diminishing the accuracy of the estimate.

The aim of this research was to improve earliness by induced mutations in two locally adapted commercial cotton cultivars. The study was performed in three locations across the Greek Cotton Belt.

2. MATERIALS AND METHODS

Two commercial cotton cultivars, Eva and Zeta-2, locally adapted in North and South Greece, respectively, with noticeable differences in earliness were chosen as experimental material and a sufficient quantity of their seeds was irradiated with 300 Gy γ-rays. The germination rate of the treated and untreated seeds was evaluated, in order to assess the effectiveness of 300 Gy doses on these cultivars.

The treated seeds from each cultivar were carefully sown because of the detrimental effect of the mutagen. All field conditions, such as sowing time, seedbed preparation and soil moisture, irrigation, soil nutrient level and weed control were optimal. Planting spacing was doubled in order to avoid any stress conditions. Data for seedlings' emergence, seedlings' survival, various abnormalities (small leaves, reduced height and long bolls), and sterility were recorded during the M_1 generation growth, but no selections were made.

Of the 6000 seeds originally sown in the 1995 growing season, 2,701 plants from Eva and 2,556 plants from Zeta-2 ultimately made up the M_1 generation. Two flowers from many of these plants were selfed and the seeds produced in the resulting bolls were collected and kept separately.

In the following year (1996) six seeds from each of the selfed flowers were bulked and sown under optimal conditions at the end of April. In each experimental field, untreated seeds were sown every 10th row as a check. In total, the M_2 population consisted of 12,000 plants (75 rows, 10m long) of Eva and 12,500 plants (90 rows, 10m long) of Zeta-2.

The first opened flower served as the criterion for earliness, therefore the earliest 2% of the first blooming plants in each population were marked and their flowers were selfed for a 45 day period. The products of the selected and selfed plants were collected and ginned separately, while technological evaluation of fibre quality helped in eliminating undesired phenotypes. The M_3 seeds obtained from 82 Eva and 56 Zeta-2 M_2 plants, were sown in progeny rows in April 1997.

After evaluation for earliness, taking into account morphological and physiological characteristics as well as yield, the fibre and seed quality components were also evaluated. All comparisons were made to the untreated checks. Self-pollination was applied on each mutant

line in order to ensure the purity of seeds for the M_4 generation. Ultimately, five early mutant lines, resulted from cultivar Eva and three from cultivar Zeta-2.

During the 1998 growing season, the five and the three mutant lines from Eva and Zeta-2, respectively, were further evaluated in three different locations along the Cotton Belt. The experimental design was a randomized complete block with four replications. In all cases the untreated checks were used as comparative standards for all evaluations. Each experimental block consisted of three 10m rows spaced 1m apart. The following characteristics were noted in all locations:

— days after planting (DAP) to 50% seedlings emergence, No.
— DAP to the 3rd true leaf, No.
— DAP to the appearance of first pinhead, No.
— DAP to the appearance of first flower, No.
— DAP to the appearance of first open boll, No.
— ratio (%) of number of open bolls at first picking to the total number of bolls.
— ratio (%) of number of open bolls at the second picking to the total number of bolls.
— ratio (%) of weight of seedcotton harvested at the first picking to total seedcotton weight.
— ratio (%) of seedcotton weight at 1st and 2nd pickings to the total seedcotton weight.
— height of plants at 50% flowering.
— total yield.
— fibre quality (length, fineness-maturity, strength).
— seed quality (oil, protein, gossypol content).

All physiological observations and measurements were tabulated and analysed in comparison to the checks in order to evaluate the irradiation effect on earliness.

3. RESULTS AND DISCUSSION

3.1. Preliminary studies (1994)

The germination rates of the seeds of the two cultivars after irradiation with 300 Gy, along with the controls, are given in Table I. The most effective doses for inducing desirable mutations in cotton were defined in earlier experiments [4]. The dose of 300 Gy drastically reduced seed germination in Eva and Zeta-2 compared to the control and may be regarded as approaching the LD_{50} for these cultivars, as well as providing a good indication of the radiation effect. Compared to the laboratory results the germination in the field was slightly lower, as expected.

TABLE I. SEED GERMINATION UNDER CONTROLLED CONDITIONS AFTER 300 GY IRRADIATION

Treatments	Germination %	
	Eva	Zeta-2
300 Gy	58.6	60.9
Check	94.8	92.6

3.2. M_1 generation (1995)

The rates (%) of seedlings' emergence and survival for each treatment and cultivar are given in Table II. Plant growth in the M_1 generation was depressed; a few plants were sterile while others had smaller leaves and were shorter than the checks. The frequencies of some morphologically different plants are also given in Table II. Zeta-2 had a higher percentage of plants with small leaves and low stature, while a higher percentage of plants with long bolls was observed in Eva. The frequencies of sterile and semisterile plants in the two cultivars were similar.

TABLE II. EFFECT OF 300 GY SEED IRRADIATION ON M_1 SEEDLINGS EMERGENCE AND SURVIVAL AND ON PLANT MORPHOLOGY

Treatments	Seedlings, %		Frequency (%) of plants with			
	Emergence	Survival	Small leaves	Short plants	Long bolls	Sterility
Eva (300 Gy)	52.8	43.8	12.8	12.4	7.9	2.6
Eva (Check)	89.6	82.7	0	0	0	0
Zeta-2 (300 Gy)	54.5	45.9	18.9	14.6	3.8	2.7
Zeta-2 (Check)	90.6	84.3	0	0	0	0

3.3. M_2 generation (1996)

Table III shows some of the morphological and physiological characteristics of the selected earlier mutants (M_2) and the untreated checks. The mean number of days from sowing to 50% seedlings emergence was more or less similar in all cases. Small decreases in the number of days after planting (DAP) to 3rd true leaf, were found in the selections from both cultivars. The differences between treated and untreated representatives in both cultivars were increased in the more advanced growth stages. Additional 0.9 days for Eva and 2.3 days for Zeta-2 were needed for the untreated checks to reach pinhead squares compared to the mutagenized selections. The appearance of first flower was 3 days earlier in irradiated Eva and 5.2 days in Zeta-2 compared to their respective controls. The first boll opened 2.8 days earlier in the irradiated Eva and 3.2 days earlier in the irradiated Zeta-2 compared also to the untreated controls. Thus, the differences were greater in the case of Zeta-2. From Table III it is obvious that earlier selections were obtained from the γ-irradiated populations.

TABLE III. MEANS OF SOME MORPHOLOGICAL AND PHYSIOLOGICAL MEASUREMENTS OF SELECTED EARLIER M_2 MUTANT PLANTS

Treatments	Plants, No.	No. DAP to					Ratio to total produced bolls of open bolls at		Ratio to total wt. of seed cotton of		Plant ht., cm
		Emergence	3rd true leaf	1st pinhead	1st flower	1st open boll	1st picking	1st & 2nd picking	1st picking	1st & 2nd picking	
Eva 300 Gy	240	8.8	28.1	49.9	68.5	140.8	0.692	0.951	0.786	0.946	73.1
Eva (check)	30	8.8	28.4	50.8	71.5	143.6	0.667	0.922	0.748	0.919	77.9
Zeta-2 300 Gy	240	8.7	30.9	54.3	76.6	152.7	0.651	0.885	0.706	0.875	84.1
Zeta-2 (check)	30	8.8	31.9	56.6	81.8	155.9	0.604	0.843	0.664	0.840	92.7

The γ-irradiation reduced the mean height of plants in the cultivars under study. The selected earlier mutants were shorter than the controls in all cases. The mean differences in height at blooming between the treated and untreated plants were greater in Zeta-2 (8.6 cm), than in Eva (4.8 cm). Early maturing cultivars generally have shorter plants than late ones, thus our data are in accordance with this general observation [5].

Estimates of earliness based upon boll and yield measurements are also given in Table III. At first picking selections from irradiated Eva had 69.2% of the bolls open compared to 66.7% for the check. For Zeta-2 the percentages were 65.1% and 60.4%, respectively. All pickings were synchronous, therefore the later maturing Zeta-2 had a lower percentage of open bolls compared to the earlier Eva. In the selections from irradiated Eva 95.1% of the bolls were harvested in the two pickings compared to 92.2% in the check. In Zeta-2 the percentages were 88.5% and 84.3%, respectively. The same results concerning earliness were obtained for seedcotton weight (yield). In the Eva selections 78.6% of the seedcotton was harvested at first picking compared to 74.8% in the check, while in Zeta-2 the percentages were 70.6% and 66.4% respectively. The percentage of seedcotton harvested in the 1st and 2nd harvests combined, also were greater for the selected plants as compared to controls.

3.4. M_3 generation (1997)

Table IV presents the morphological and physiological characteristics of the selected early maturing M_3 mutants and the checks. The M_3 generation included 82 selections of Eva and 56 selections of Zeta-2. The mean number of DAP to 50% seedlings emergence was more or less the same in all treatments. Also, there were no differences in earliness up to the 3rd true leaf stage. In subsequent stages, the differences between the treated and untreated lines increased progressively. Again, generally in the early selections the plants were shorter compared to the standards. The ratios of open bolls at first picking to total bolls, and of first and second picking to the total were higher in the early selections. The same trend was also observed for seedcotton weight. The early mutants had the higher ratio of first picking to total and also the higher ratio of first and second picking to total seedcotton weight.

TABLE IV. MEANS OF SOME MORPHOLOGICAL AND PHYSIOLOGICAL MEASUREMENTS OF M_3 MUTANT LINES SELECTED FOR EARLINESS

Treatments	Plants, No.	No. DAP to					Ratio to total produced bolls of open bolls at		Ratio to total wt. of seed cotton of		Plant ht., cm
		Emergence	3rd true leaf	1st pinhead	1st flower	1st open boll	1st picking	1st & 2nd picking	1st picking	1st & 2nd picking	
EVA 300 Gy	82	8.2	29.1	50.6	73.3	142.9	0.701	0.976	0.795	0.940	83.1
EVA (check)	10	8.2	29.9	52.3	74.2	143.6	0.686	0.939	0.752	0.910	88.9
ZETA-2 300 Gy	56	8.5	31.7	56.9	78.1	154.5	0.648	0.888	0.714	0.862	94.1
ZETA-2 (check)	7	8.5	32.5	58.6	81.8	156.8	0.614	0.843	0.670	0.854	102.7

3.5. M_4 generation (1998)

In the M_4 five selected lines derived from Eva and three selected lines derived from Zeta-2 were retained. Tables V and VI present the results of replicated trials across the Cotton Belt. In Table V the means of the earliness criteria, across the three locations, of the selected

mutant lines from Eva, showed no significant differences from the check in the number of DAP to emergence, DAP to 3rd true leaf and DAP to first pinhead. In the next growing stage, all the selected early mutants showed significant differences compared to the check. They had open flowers three days earlier then the check line. For the next measurement, DAP to 1st open boll, lines 9 and 66 had the first open boll about four days earlier than the check.

Concerning the other earliness measurements, only line 9 showed significant differences in comparison to the check with LSD_{05}. In the selected mutant line 9, 74.7% of the total produced bolls were open at the first picking, while the check line had 70.8%. The percentage of open bolls in the mutants at the 1st and 2nd picking was 94.5% vs. 90.5% for the check. The weight of seedcotton at the 1st picking stage was 75.3% of the total, while for the check it was 71.2%. The seedcotton weight at 1st and 2nd picking was 95.2% of the total weight, significantly higher than that of the check.

The differences in plant height were not significant, although a general trend for the earlier mutants to be shorter was apparent.

TABLE V. MEAN VALUES OF EARLINESS CHARACTERISTICS OF PROMISING M_4 EVA MUTANT LINES ACROSS LOCATIONS

EVA lines	No. DAP to					Ratio to total produced bolls of open bolls at		Ratio to total wt. of seed cotton of		Plant ht., cm
	Emergence	3rd true leaf	1st pinhead	1st flower	1st open boll	1st picking	1st & 2nd picking	1st picking	1st & 2nd picking	
9	7.9	27.8	50.1	67.5*	140.7*	0.747*	0.945*	0.753*	0.952*	80.1
24	7.9	27.6	50.3	67.4*	141.2	0.727	0.933	0.734	0.944	81.2
65	7.9	27.7	50.3	67.4*	141.2	0.728	0.928	0.732	0.938	82.2
66	7.9	27.8	50.1	67.5*	140.8*	0.728	0.921	0.727	0.934	82.7
81	7.9	27.7	50.1	67.7*	141.1	0.725	0.921	0.725	0.928	80.9
Check	7.9	28.5	51.2	70.6	144.6	0.708	0.905	0.712	0.913	82.5
LSD_{05}	**0.15**	**1.16**	**1.95**	**2.53**	**3.79**	**0.0385**	**0.0385**	**0.038**	**0.0369**	**3.89**

* Significant at the 5% level

Table VI shows the means of earliness measurements and comparisons of the early selected mutants from the cultivar Zeta-2, and the check. For the three first growing stages, DAP to emergence, DAP to 3rd true leaf and DAP to 1st pinhead, no significant differences were found between the early mutants and the Zeta-2 check. In the next growing stage DAP to 1st flower, the selected mutant lines 2, 28 and 33 had first open flower at least 3.31 days earlier than the check. Significant differences regarding DAP to 1st open boll were shown only by line 33. This line had the first open boll 4.4 days earlier than the untreated check.

The other four indirect earliness measurements showed that only line 33 was earlier across the three locations. At the first picking stage, 68.1% of total produced bolls were open in line 33, while in the check this percentage was 62.2%. At the second picking stage, the open bolls were 89.1% of the total in line 33 and 83.9% in the check. The ratio of seedcotton weight at 1st picking to the total of line 33, showed significant difference compared to the check: about 71.3% of total seedcotton had been collected at the 1st picking of the early

mutant line 33 and 66.6% of the check. The seedcotton weights at the second picking were 88.9% and 84.0% of the total weight for the line 33 and the check, respectively.

The height of the plants followed the same pattern as in Eva. All selected early mutants from Zeta-2 showed a general trend to be shorter but the differences were not significant.

TABLE VI. MEAN VALUES OF EARLINESS CHARACTERISTICS OF PROMISING M_4 ZETA-2 MUTANT LINES ACROSS LOCATIONS

ZETA-2 lines	No. DAP to Emergence	3rd true leaf	1st pinhead	1st flower	1st open boll	Ratio to total produced bolls of open bolls at 1st picking	Ratio to total produced bolls of open bolls at 1st & 2nd picking	Ratio to total wt. of seed cotton of 1st picking	Ratio to total wt. of seed cotton of 1st & 2nd picking	Plant ht., cm
2	8.0	29.2	53.1	76.3*	152.8	0.660	0.869	0.690	0.871	94.0
28	8.0	29.2	53.3	76.0*	152.0	0.973	0/877	0.696	0.880	94.7
33	8.0	29.3	53.3	76.2*	151.8*	0.681*	0.891*	0.713*	0.889*	96.0
Check	8.0	29.6	55.0	79.7	156.2	0.622	0.839	0.666	0.840	99.3
LSD_{05}	**0.13**	**1.07**	**2.22**	**3.31**	**4.21**	**0.0512**	**0.0449**	**0.0350**	**0.0413**	**4.84**

* Significant at the 5% level.

TABLE VII. MEAN LINT YIELD AND FIBRE QUALITY OF PROMISING EARLY M_4 COTTON MUTANT LINES ACROSS LOCATIONS

Mutants	Lint yield kg/ha	Fibre Length mm	Fibre Maturity micronaire	Fibre Strength pressley	Oil %	Protein %	Gossypol %
EVA lines							
9	1045.3	28.4	4.3	8.3	24.0	26.6	0.918
24	1058.7	28.8	4.1	8.4	23.5	26.5	0.921
65	1048.7	28.7	4.1	8.2	23.8	26.4	0.924
66	1039.3	28.6	4.1	8.3	23.9	26.3	0.935
81	1038.3	28.6	4.1	8.5	23.5	26.4	0.938
Check	1045.3	28.8	4.2	8.4	23.5	26.6	0.929
LSD_{05}	**52.38**	**0.95**	**1.34**	**0.57**	**1.33**	**1.89**	**0.959**
ZETA-2 lines							
2	1080.7	28.4	4.2	8.4	23.7	26.0	0.915
28	1083.0	28.5	4.1	8.5	23.3	26.3	0.925
33	1079.3	28.6	4.2	8.6	23.5	26.2	0.938
Check	1081.7	28.7	4.2	8.5	23.5	26.2	0.928
LSD_{05}	**44.35**	**0.89**	**1.09**	**0.65**	**1.01**	**1.04**	**0.928**

The means of yield and quality of the eight lines retained for their earlier maturity are given in Table VII. Comparisons with LSD_{05}, showed that lint yield of the selected early mutant lines in both cultivars did not differ significantly from the source cultivars. Also, the

quality measurements of fibre length, fineness-maturity and strength of the early mutants and checks, were not significantly different.

These results indicate that irradiation did not affect the commonly measured fibre characteristics. There were no significant differences in the seed quality measurements including oil, protein and gossypol contents (%) among the test entries. It should be pointed out that no selection for these traits was applied in the earlier generations.

4. CONCLUSIONS

The most effective dose for inducing desirable mutations in cotton was the treatment with 300 Gy γ-irradiation. The γ-irradiation improved the earliness of tested cultivars marginally, under the conditions of this experimentation. Yield and important fibre and seed quality parameters did not change in the selected mutant lines.

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Development of improved germplasm of cotton through radiation and DNA-mediated embryo transformation technique — Evaluation and confirmation of novel genotypes

M. Aslam, M.T. Elahi, N. Iqbal

Nuclear Institute for Agriculture & Biology,
Faisalabad, Pakistan

Abstract. Studies were carried out to incorporate *Gossypium arboreum* and *G. barbadense* genes for disease resistance and quality traits, respectively, into *G. hirsutum* through DNA-mediated embryo transformation technique. The self-fertilized flowers/ovaries of the recipient were injected with donors' DNA solutions irradiated at low doses, i.e. 2.5 Gy of gamma rays. Incorporation and preservation of traits from the donor species into the recipient were observed in the D_1 and D_2 generations. A higher percentage of CLCuV (cotton leaf curl virus) resistant plants and plants with better quality traits were obtained from the irradiated DNA treatments than un-irradiated DNA. The transformed genotypes had higher yield and improvement in other economic traits compared to the untreated recipient genotype. Moreover, expression of qualitative traits, i.e. petal spot, pollen colour and flower colour, of the donor species were observed in the D_2 generation. In D_3 and D_4 generations, the transformed progenies were better in yield and all other economic traits compared to untreated recipient and standard cotton varieties.

Progeny plants with phenotypes suggestive of transformation along with all parental genotypes were analyzed with 20 OPM and 12 OPB random decamer primers, using polymerase chain reaction (PCR). Amongst the OPM primers revealing DNA polymorphism, OPM–4, OPM-11 and OPM-19 confirmed the introgression of DNA fragments of 750 bp, 1 kb and 750 bp, respectively, of *G. arboreum* into *G. hirsutum*. None of the 20 OPM primers revealed polymorphism between *G. hirsutum* and *G. barbadense* parents. However, two of the 12 OPB primers, OPB-3 and OPB-4, detected polymorphic areas between the *G. hirsutum* and *G. barbadense* parents while 6 OPB primers (OPB-05, -06, -07, -08, -11 and -12) showed polymorphism between *G. hirsutum* and *G. arboreum* parents. These OPB primers did not detect polymorphism between parents and their transformed progenies. On the basis of the three polymorphisms detected by OPM primers, the introgression of *G. arboreum* DNA fragments into *G. hirsutum* genome was confirmed.

1. INTRODUCTION

Four cotton (*Gossypium)* species (*G. hirsutum* L., *G. barbadense* L., *G. arboreum* L. and *G. herbaceum* L.) are cultivated. Cotton, *G. hirsutum*, is the world's leading fibre crop and the fifth most valuable oilseed crop. *G. barbadense* L. is the most popular species for its superior quality fibre, while *G. arboreum* L. is best reported for high fibre strength, resistance to insects and diseases [1,2].

Being economically important, the cotton plant has long been subjected to extensive research in the world and in Pakistan. Breeders are striving to change the genetic constitution of the plant, to improve both its yield and fibre quality under diverse climatic conditions of the country. Since the variability for some desirable traits in the existing cultivars is scanty, various methods are being used, e.g., hybridization, backcrossing and ovulo-embryo culture to generate variability by incorporating the desirable genes from exotic sources into *G. hirsutum*. These approaches require extensive time to incorporate desirable gene(s) from the donor/s and are confronted with problems due to differences in ploidy level, interspecific barriers, and undesirable gene linkages present in the genotypes desired to be combined [3]. However, with modern techniques, interspecific introgression of A genome traits into AD genome cotton

may be facilitated by constructing synthetic AD allotetraploids or by crossing with ADD hexaploids [4]. Induced mutations have also been used to create genetic variability in many crop plants, and in recent years a large number of early, high yielding, disease and insect resistant varieties of various crop plants, e.g. wheat, barley, rice and cotton have been developed and released in different countries of the world [5]. However, various transformation approaches are being used to incorporate desirable genes into crop plants more quickly. *Agrobacterium*-mediated transformation is the most commonly used method for gene transfer in plants [6]. Although *Agrobacterium* has been used successfully for the transformation of a number of different plant species, difficulties exist due to limited host range, low efficiency of transformation, and problems with the removal of extraneous *Agrobacterium* genes, following transformation and manipulation of DNA in wide host range plasmids. Electroporation of DNA for gene transfer using protoplasts has also been used successfully for the production of transgenic plants [7,8].

Some *in vitro* transformation methods, e.g. DNA microinjections in tobacco [9] and rye [10], and particle bombardment in cotton [11], have been used. All the *in vitro* transformation methods require callus culture to regenerate whole plants from transformed protoplasts or cells. Moreover, the ability of cotton to regenerate from a cell into a whole plant, through callus tissue is genotype-specific [12,13]. Since cotton has genotype-specificity for *in vitro* regeneration, it is difficult to obtain a whole fertile plant that expresses inserted genes that can be passed on to its progeny. In contrast, DNA-mediated embryo transformation is a straight forward approach involving injection of exogenous DNA solution into the plants' reproductive structures under *in vivo* conditions. The injected DNA solution transforms the developing embryos during zygotic cell division and it is heritable. Positive *in vivo* transformations have been reported in cotton by injecting the flower ovaries with exogenous DNA [14-17], but transformation has not been confirmed through molecular analysis.

Random amplified polymorphic DNA (RAPD) is an easy, simple and reliable DNA fingerprinting technique [18]. It does not require any radioactivity or a specific probe as is necessary in detecting restriction fragment length polymorphism (RFLP). RAPD has been used extensively for genetic diversity evaluation and cultivar analysis of different plants like rice [19,20], celery [21] and cotton [22]. A detailed RFLP map of cotton (*G. hirsutum* x *G. barbadense*) to investigate chromosome organization and evolution has been reported [23]. An extensive study on the molecular analysis of introgression of *G. hirsutum* chromatin into *G. barbadense* has also been described [24].

Since useful genetic variability can be generated through the use of DNA-mediated embryo transformation, desirable genotypes having a few traits incorporated from the donor can be selected in less time than by other methods. Therefore, the research reported here was planned and initiated with the following objectives: i) incorporate into *G. hirsutum* resistance to cotton leaf curl virus (CLCuV) disease and to whitefly (*Bemisia tabaci*) from *G. arboreum,* ii) incorporate good fibre quality trait(s) from *G. barbadense* into *G. hirsutum,* and iii) confirm the incorporation of foreign DNA through molecular analysis.

2. MATERIALS AND METHODS

The locally cultivated tetraploid cotton, *G. hirsutum* (2n=4x=52) var. NIAB-78, was used as recipient in all cases. The donors were a cultivated diploid species of cotton, *G. arboreum* (2n=2x=26) var. Ravi, and a tetraploid long staple species, *G. barbadense* (2n = 4x = 52). Both donors were clearly distinguishable morphologically from the recipient. Selfed seeds of both donors and the recipient were produced and used in these studies.

2.1. DNA extraction

Both donors were grown under controlled conditions to obtain leaf tissue for DNA extraction. Approximately 5 g of leaf tissue of each donor was pulverized in a mortar partially filled with liquid nitrogen. The DNA was extracted, and the DNA solutions were prepared, as previously reported [25].

2.2. DNA injections

More than 200 plants of the recipient were raised from selfed seed. At flowering 50 healthy plants were selected for injection. The self-fertilized flower/ovaries of the recipient were injected via a microsyringe with irradiated or non-irradiated donor DNA solutions through the axial placenta 24 hours post self-pollination. For each treatment 10 microliter DNA solution was used and the injected flower/ovaries were protected from foreign contamination. Matured bolls from the treated flower/ovaries were collected, ginned and the D_0 generation seed was recovered. The D_1 generation was raised from D_0 seed at a spacing of 60x75 cm in the field. The D_1 population was evaluated for phenotypic as well as economic trait changes in comparison to non-irradiated treatments and control plants. The seed cotton from the D_1 population was harvested and the seeds were obtained. The D_2 population was grown from D_1 seed as plant progeny rows for evaluation and confirmation. Moreover, D_1 and D_2 populations from *G. hirsutum* x *G. arboreum* DNA treatments were exposed to CLCuV disease conditions for evaluation [17,25].

The progenies of the D_2 generation plants expressing donor characteristics were grown as the D_3 generation. The D_3 population was evaluated for yield potential and for other agronomic traits. The methodology for CLCuV disease screening was the same as that adopted for the D_2 generation. The D_3 seed cotton was harvested and seed was obtained. The D_4 generation was grown from D_3 seed and evaluated for various phenotypic and economic traits. Materials generated from later DNA injections were evaluated as described earlier [25].

2.3. RAPD analysis

Young leaves obtained from different D_2-derived D_3 transformed plant progenies along with their parents were used for RAPD analysis. The young leaves were collected in liquid nitrogen, ground to a very fine powder and transferred into a 50 ml centrifuge tube. Then 15 ml of hot (65^{o}C) 2 x CTAB [2% cetyldimethyl triethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA (pH 8.0), 0.1 M Tris-HCl (pH 8.0), 1% polyvinyl pyrrolidone (PVP), 1% 2-mercapto-ethanol] was added and incubated in water bath for 30 min at 65^{o}C with occasional shaking. The mixture was emulsified with an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged for 10 minutes at 9000 rpm. The upper phase was again treated with chloroform: isoamyl alcohol and centrifuged for 10 min at 9000 rpm. The aqueous phase was removed and mixed with 0.6 volume of 2-propanol to precipitate the DNA. Then the DNA pellet was transferred with a sterilized loop into another 50 ml tube containing 70% ethanol. The pellet was washed thrice with 70% ethanol, which was discarded each time. The DNA pellet was air dried and redissolved in 0.5 ml of 0.1xTE buffer. After RNAse treatment, the DNA concentration was measured by DyNA Quant 200 Fluorometer. The DNA was diluted in sterilized distilled water to a concentration of 12.5 ng/µl for use in PCR reactions for RAPD analysis.

Random decamer primers (Operon Technologies Inc., Alameda, Calif., USA) were dissolved in sterilized distilled water at a concentration of 15 ng/µl. Thirty two primers

belonging to Operon kits; OPM (20 primers) and OPB (12 primers) were used for PCR amplifications. Amplifications were carried out in a 25 µl reaction volume containing 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 3 mM $MgCl_2$, 0.1 mM each of dATP, dGTP, dTTP and dCTP, one unit of *Taq* DNA polymerase (Perkin Elmer, Norwalk, Conn.), 0.001% gelatin (Sigma, St-Louis, Mo.), 25 ng of template DNA and 30 ng of primer. The reaction mixture was overlaid with two drops of mineral oil in order to avoid evaporation. The amplifications were carried out in a Perkin Elmer Thermal Cycler 480, programmed for a first denaturation step of 5 minutes at 94°C followed by 40 cycles of 94°C for 1 minute, 36°C for 1 minute and 72°C for 2 minutes. After the completion of 40 cycles, the reactions were kept at 72°C for 7 minutes and then held at 4°C until the tubes were removed. PCR products were separated on a 1.2% agarose gel with ethidium bromide in the gel, using 0.5 x Tris Borate EDTA (TBE) buffer.

3. RESULTS AND DISCUSSION

3.1. Interploid interspecific studies

The D_1 population obtained from the irradiated (2.5 Gy) donor DNA treatments, exhibited higher percentage (15-20%) of plants resistant to CLCuV than the D_1 population obtained from the treatments with non-irradiated donor DNA (Table I). The plants exhibiting resistance to CLCuV disease had a higher yield per plant and showed changes in boll size/weight, boll shape, plant type, hairiness, gossypol pigments and plant maturity. The D_1 generation plants were more hairy and had better boll size, more monopodial branches and good boll formation; different individuals had different acquired traits. No changes were observed for flower colour and petal spot in the D_1 generation. The D_2 generation results indicated the persistency of the changes observed during the D_1 generation. Moreover, some of the D_2 progenies manifested qualitative traits, e.g. petal spot, pollen and flower colour, of the donor *G. arboreum.* The CLCuV resistant plant progenies showed a segregation ratio of 3:1 for resistant and susceptible genotypes, indicating that CLCuV resistance behaved as a monogenic and dominant (RR) trait [2,17]. The D_2 generation progenies showing resistance to CLCuV gave higher yields compared to the standard and recipient genotypes (Table II). The yield of the promising genotypes ranged from 2027 to 2602 kg/ha compared to recipient and standard cultivar yields of 1020 kg/ha and 620 kg/ha, respectively. Some of the transformed genotypes also manifested better tolerance to whitefly.

TABLE I. PERFORMANCE OF D_1 PLANTS FOLLOWING THE INJECTIONS OF UNIRRADIATED AND IRRADIATED *G. arboreum* DNA INTO FLOWERS/OVARIES OF *G. hirsutum* COMPARED WITH THE RECIPIENT (R), DONOR (D) AND STANDARD (S) CULTIVARS, 1995-96

Materials	Plants studied No.	Mean			Reaction to CLCuV[a]
		Plant height cm	Boll weight g	Yield/plant g	
G. hirsutum x G. arboreum DNA (0 Gy)	9	91	3.2	190	0-1(10% plants)
G. hirsutum x G. arboreum DNA (2.5 Gy)	32	101	3.9	247	0-1(15-20% plants)
G. hirsutum (R)	20	93	3.0	154	7-8 (100% plants)
G. arboreum (D)	20	120	2.0	82	0 (100% plants)
S-12 (S)	20	102	4.0	55	9 (100 % plants)

[a] Disease rating scale: 0-9 with 0 = immune, 9 = highly susceptible.

TABLE II. MEAN PERFORMANCE OF THE PROMISING D_2 PLANT PROGENIES OF *G. hirsutum* TREATED WITH *G. arboreum* DNA INJECTIONS COMPARED WITH THE RECIPIENT (R) AND THE STANDARD (S), 1996-97

Progenies, D_1 parents and cultivars		Plants studied, No.			Response to CLCuV grade[a]	Yield kg/ha
		Total	Resistant	Susceptible		
G. hirsutum x G. arboreum						
DNA (2.5 Gy)	-1	50	37	13	0-1	2195
"	-3	51	39	12	0-1	1796
"	-4	58	43	15	0-1	2533
"	-5	44	32	12	0-1	2027
"	-6	51	38	13	0-1	2602
"	-8	46	34	12	0-1	2058
"	-9	44	34	11	0-1	1650
" (0 Gy)	-8	45	33	12	0-1	1810
G. hirsutum (R), check		47		47	7-8	1020
S-12 (S), check		49		49	9	620

[a] Disease rating scale: see Table I

TABLE III. MEAN PERFORMANCE OF THE PROMISING D_3 PLANT PROGENIES OF *G. hirsutum* TREATED WITH *G. arboreum* DNA, 1997-98

Progenies, D_1 parents and cultivars[a]		Plant height cm	Boll weight g	Yield kg /ha	Fibre		
					Length mm	Fineness µg/in	Maturity %
G. hirsutum x G. arboreum							
DNA, (2.5 Gy)	-1	145	4.4	3025	28.0	4.9	91
"	-3	136	4.1	2840	27.0	5.7	88
"	-4	135	4.5	2735	28.2	4.8	90
"	-5	128	4.6	3250	27.8	4.8	92
"	-6	136	4.4	2905	27.6	4.7	94
"	-8	128	4.5	3052	28.0	4.5	90
" (0 Gy)	-8	148	3.9	2530	27.5	4.8	88
Recipient		132	3.0	1050	27.3	4.7	90
CIM-448		140	3.2	2165	27.0	4.6	91
S-12		90	4.0	822	27.0	5.0	91

[a] Donor = highly resistant to CLCuV. Recipient = susceptible to CLCuV. S-12 = highly susceptible to CLCuV; CIM-448 = latest standard variety, resistant to CLCuV.

The D_3 progenies of the putative transformed D_2 genotypes were evaluated for important agronomic traits of economic importance in comparison to NIAB-78 and the latest local standard variety of cotton (CIM-448). The transformed progenies gave better boll weight and yield and other characters than the reference cultivars (Table III). One progeny line had an unusually high micronaire value of 5.7, a characteristic of the *G. arboreum* donor. Moreover, plant progenies grown from D_2 plants expressing qualitative traits incorporated from the donor, such as petal spot and yellow pollen colour, showed segregation of these traits. The segregation patterns reflected Mendelian inheritance giving 1:2:1: ratio for incompletely dominant traits (petal spot), and 3:1 for dominant traits like pollen colour. The results from the D_4 generation confirmed the superiority of the transformed genotypes over the recipient and standard in yield and other agronomic traits (Table IV).

TABLE IV. MEAN PERFORMANCE OF THE D_4 PROGENIES OF *G. hirsutum* TREATED WITH *G. arboreum* DNA, 1998-99

Progenies, D_1 parents and cultivars[a]		Plant height cm	Boll weight g	Yield kg/ha	Fibre		
					Length mm	Fineness µg/in	Maturity %
G. hirsutum x G. arboreum							
DNA (2.5 Gy)	-1	147	4.3	3025	28.0	4.9	91
"	-3	138	4.0	3435	27.3	5.7	89
"	-4	135	4.4	3410	28.0	4.8	91
"	-5	128	4.5	2999	28.0	4.8	90
"	-6	132	4.5	3482	27.7	4.7	92
"	-8	130	4.4	3050	27.6	4.5	90
" (0 Gy)	-8	150	3.4	2630	27.0	4.8	89
Recipient		122	3.0	950	27.2	4.7	89
CIM-448		125	3.0	2965	27.5	4.7	91
S-12		110	4.0	722	27.0	5.0	91

[a] As in Table III.

3.2. Intraploid interspecific studies

When *G. barbadense* DNA was injected into *G. hirsutum* NIAB-78, various morphological traits of some D_1 plants indicated changes of plant type, boll type/size, leaf shape, plant vigour, etc. The plants expressing phenotypic changes had greater boll weight, higher yield, and better fibre quality compared to the untreated recipient (Table V). The spectrum of such changes for donor parent traits was higher with the irradiated DNA treatments. The D_2 progeny rows showed consistency in the changes noted during the D_1 generation. Moreover, some of the D_2 generation progenies expressed changes for qualitative traits typical of the donor parent *G. barbadense,* such as red petal spot, pollen colour and flower colour (Table VI). The selected D_2 progenies had better boll weight and their yields ranged from 1487 to 2569 kg/ha compared to 1087 kg/ha for the recipient. The transformed progenies had fibre fineness of 3.9 to 3.6 µg/in compared to 4.7 µg/in for the recipient. Fibre strength of the selected D_2 lines ranged from 97-101 TPPSI. These progenies also had better staple length than the recipient. Various progenies of the D_2 transformed plants were

evaluated in D_3 (Table VII). The D_3 progenies maintained the improved, donor-like agronomic and fibre quality characteristics of their selected D_2 parents. Moreover, D_2 plants having changes in qualitative traits, such as red flower, petal spot and yellow pollen colour, gave Mendelian segregation for these characters in the D_3 generation. The results of the D_4 generation confirmed the superiority of the transformed genotypes over the recipient and standard with respect to yield and other agronomic traits (Table VIII).

TABLE V. RANGE OF PHENOTYPIC AND AGRONOMIC TRAITS OF D_1 GENERATION FROM INJECTIONS OF IRRADIATED AND UNIRRADIATED *G. barbadense* DNA INTO FLOWERS/OVARIES OF *G. hirsutum* COMPARED WITH THE RECIPIENT AND DONOR, 1995–96

D_1 plants and parents	Plants, No.		Plant height cm	Boll weight g	Yield/ plant g	Fibre		
	Studied	Changed[a]				Length mm	Fineness µg/in	Strength TPPSI
G. hirsutum x G. barbadense								
DNA (0 Gy)	31	4	120-154	4.0-4.8	160-320	28-29	3.7-4.0	91-99
" (2.5 Gy)	40	12	125-160	4.2-4.8	180-360	29-30	3.5-3.8	92-101
Recipient	22	-	118	3.0	120	27.0	4.7	91
Donor	19	-	180	4.8	60	34.0	3.4	105

[a] Note: The changed plants: i) grew faster; ii) had larger leaves, conical bolls and long petioles; iii) had larger to medium flower with bigger calyx; iv) had no evidence of marker genes.

TABLE VI. PERFORMANCE OF THE PROMISING TRANSFORMED D_2 PROGENIES OF *G. hirsutum* TREATED WITH INJECTIONS OF UNIRRADIATED AND IRRADIATED DNA OF *G. barbadense*, 1996–97

Progenies, D_1 plants and recipient		Plant height cm	Boll weight g	Yield kg/ha	Fibre		
					Length mm	Fineness µg/in	Strength TPPSI
G. hirsutum x *G. barbadense*							
DNA (0 Gy)	-1	135	4.0	1487	28.5	3.8	99
"	-5	139	4.2	1813	28.5	3.8	98
"	-9	141	4.5	1876	29.0	3.9	99
" (2.5 Gy)	-7	134	4.8	2569	30.0	3.6	99
"	-9	140	4.3	1879	29.7	3.7	100
"	-20	145	4.5	2155	29.3	3.8	101
"	-29	137	4.4	1937	29.2	3.5	98
"	-30	155	4.4	1948	29.1	3.7	97
*G. hirsutum (*recipient)		120	3.0	1087	27.3	4.5	90

The results of the D_1 and D_2 generations originating from *G . hirsutum* x *G. arboreum* and *G. hirsutum* x *G. barbadense* DNA treatments confirmed the enhanced introgression of donors' gene(s) into the recipient, where the DNA was irradiated at low doses (2.5 Gy) of

gamma rays before injections [26]. The transformed genotypes maintained higher yield, better fibre quality and other economic traits superior to the untreated recipient. Some of the transformants originating from *G. arboreum* DNA injections into *G. hirsutum* were resistant to CLCuV disease. Moreover, introgression of some of the qualitative marker traits, such as red petal spot, yellow pollen colour, etc., from the *G. barbadense* and *G. arboreum* donors into *G. hirsutum* was noticed [27]. Material generated from later DNA injections agreed with the results reported herein.

TABLE VII. MEAN PERFORMANCE OF D_3 PROGENIES OF *G. hirsutum* TREATED WITH UNIRRADIATED AND IRRADIATED DNA OF *G. barbadense,* 1997-98

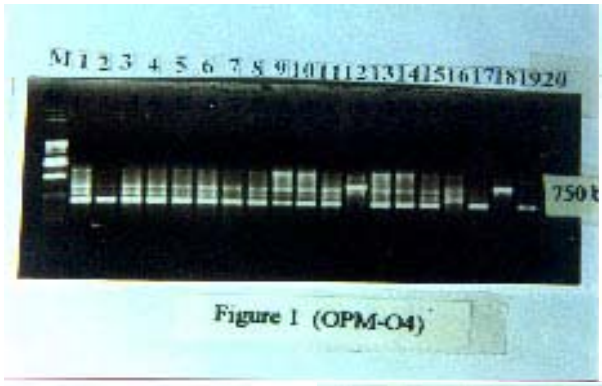
Progenies, D_1 parents and cultivars		Plant height cm	Boll weight g	Yield kg/ha	Fibre		
					Length mm	Fineness µg/in	Maturity %
G. hirsutum x G. barbadense							
DNA (0 Gy)	-1	132	4.0	2095	28.5	4.1	92
"	-5	142	4.1	2320	28.8	3.9	91
"	-9	146	4.4	2075	28.0	4.0	90
" (2.5 Gy)	-7	133	4.8	2485	29.0	3.7	92
"	-9	143	4.4	2645	29.0	3.8	91
"	-20	139	4.4	2476	28.7	4.0	91
"	-29	133	4.5	2109	28.0	4.1	94
"	-30	158	4.3	2515	30.0	3.9	93
*G. hirsutum (*recipient)		125	3.0	955	27.3	4.8	90
CIM-448 (standard)		135	3.2	2450	27.0	4.7	91

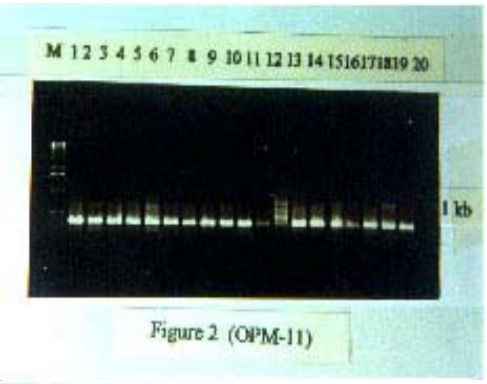
TABLE VIII: MEAN PERFORMANCE OF D_4 PROGENIES OF *G. hirsutum* TREATED WITH UNIRRADIATED AND IRRADIATED DNA OF *G. barbadense,* 1998-99

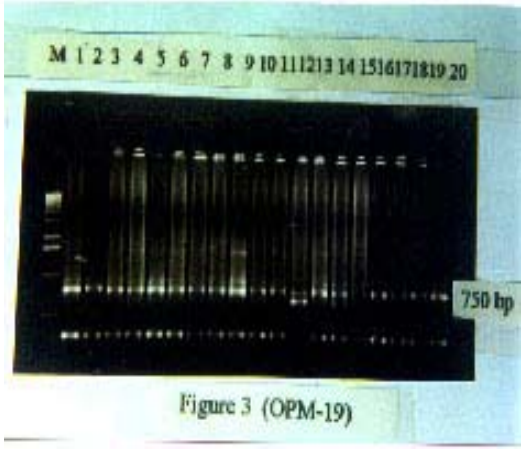
Progenies, D_1 parents and cultivars		Plant height cm	Boll weight g	Yield kg/ha	Fibre		
					Length mm	Fineness µg/in	Maturity %
G. hirsutum x G. barbadense							
DNA (0 Gy)	-1	130	4.0	2318	28.0	3.7	92
"	-5	145	4.2	2298	29.0	3.8	91
"	-9	149	4.5	2060	28.9	4.0	90
" (2.5 Gy)	-7	133	4.7	2850	29.0	3.7	92
"	-9	143	4.3	2649	30.0	3.6	91
"	-20	139	4.4	3146	29.0	4.0	91
"	-29	133	4.5	3010	29.0	4.1	94
"	-30	158	4.4	2815	30.0	3.9	93
G. hirsutum (recipient)		125	3.0	755	27.0	4.8	90
CIM-448 (standard)		130	3.0	2875	27.5	4.7	91

3.3. RAPD analysis

Thirty-two primers were used to amplify the genomic DNA of different D_2-derived D_3 transformed progenies of cotton and their respective parents for PCR analysis [29]. PCR with the primer OPM-04, resulted in polymorphic products among the samples (Fig. 1). A band of 750 bp, present in *G. arboreum* (lane 12) was also present in a transformed progeny, (lane 18) but was absent in all other samples. Another primer, OPM-11, also resulted in a *G. arboreum*-derived polymorphic band of 1 kb present in the same progeny plant (Fig. 2) that was not detected in any other sample. A third primer, OPM-19, revealed a 750 bp band, in *G. arboreum* (lane 12) and a different progeny (lane 14) that was absent in all other samples (Fig. 3). These three fragments contributed by *G. arboreum* provide molecular confirmation that *G. arboreum* DNA fragments were introgressed into *G. hirsutum* following injection of DNA into young ovaries.







FIGs. 1-3. Polymorphic agarose gel of PCR-amplified DNA using various primers:
FIG. 1. Primer OPM-4.
FIG. 2. Primer OPM-11.
FIG. 3. Primer OPM-19.
Lane genotypes: 1. G. hirsutum - R; 2. G. barbadense - D; 3. G. hirsutum (FMC);4-11. G. barbadense DNA - injected into G. hirsutum; 12. G. arboreum – D; 13-19 G. arboreum DNA injected into G. hirsutum.

None of the twenty OPM primers detected DNA polymorphism between *G. barbadense* and *G. hirsutum* that also appeared in the progeny. Among 12 OPB primers, OPB-03 and 04, resulted in polymorphic bands between *G. hirsutum and G. barbadense,* while six primers,

OPB-05, 06, 07, 08, 11 and 12 gave polymorphic bands between *G. hirsutum* and *G. arboreum.* PCR of DNA from all the individuals/ progenies these primers failed to produce polymorphism, and all the progenies were like *G. hirsutum.*

Observations on phenotypic changes in progeny following injection of *G. hirsutum* with foreign DNA indicated that at least some of the DNA was incorporated into the genome. Similar results for gene(s) incorporation in barley [28], tobacco [29] and cotton [11] using different biotechnological approaches have been reported. With a limited number of primers in PCR reactions, we confirmed at the molecular level that some *G. arboreum* DNA was incorporated. However, with this same primer set we could not confirm incorporation of *G. barbadense* DNA. This latter result was probably a consequence of the low DNA polymorphism detected between the two species.

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Varietal improvement of cotton (*Gossypium hirsutum*) through mutation breeding

**K.M. Shamsuzzaman, M.A. Hamid,
M.A.K. Azad, M. Hussain, M.A. Majid**

Plant Breeding Division,
Bangladesh Institute of Nuclear Agriculture,
Mymensingh, Bangladesh

Abstract. Cotton (*Gossypium hirsutum*) mutants induced by gamma-rays were selected in M_2–M_4 generations and evaluated in preliminary, advanced and zonal yield trials. Mutants CTM-10 and CTM-110 consistently revealed early maturity differences and their yields were not significantly different from the control cultivars in the final zonal yield trials. Fresh irradiation and subsequent selections resulted in 12 desirable mutants. The mutant CTM-116 had the highest yield of all and shared equal rank with 6 other mutants in a preliminary yield trial. Moreover, mutant CTM-122 showed earliness and CTM-115 highest fibre length and strength. Introduced and locally collected germplasm were evaluated during the 3 different years; none of them was earlier and higher yielding than the control cultivar, DPL-50. However, a few of the germplasm lines showed better fibre length, strength and fineness together with boll worm and jassid tolerances under field conditions.

1. INTRODUCTION

In Bangladesh, cotton is the second most important fibre crop next to jute. Cotton had very high importance during the 18th century, when the country used to export superfine cotton textiles, "Muslin", to worldwide markets. Due to the negligence of the then British Government, together with the increasing demands for more food crops after the 1843 Bengal Famine, cotton cultivation was steadily forgotten. After the emergence of Bangladesh, a Cotton Development Board (CDB) was established and with its efforts the cotton area and production rose to 35,000 ha and 4,800 tons, during this decade [1]. The total produce is used for blending up to 10–20% with imported cotton [2]. The present cotton cultivars require longer time to mature (180 days) than the current cropping system allows. The yield (1.5 t/ha) is extremely poor compared to developed countries, mostly due to the inherent low productivity and higher jassid (*Ambrasca bigutulla* Ishida) and boll worm (*Helicoverpa armigera* Hubner) incidences. Moreover, with the current rate of 175% cropping intensity [3] there is no possibility of area expansion. Thus, vertical yield improvement is the best alternative option. Mutation breeding has been shown to be an efficient tool for developing early and high yielding cultivars [4,5]. Keeping these ideas in mind, a varietal improvement programme involving mutation breeding was recently initiated for developing early maturing cultivars with acceptable levels of yield, tolerance to jassid and boll worm, and certain quality characters; this paper reports research results in some of these areas.

2. MATERIALS AND METHODS

2.1. Yield trials and evaluation of mutants

2.1.1. Preliminary yield trial (PYT) with 9 mutant families

Dry seeds of three advanced lines: S-71803958, S-71803785 and 398185 (obtained from Bangladesh Agricultural Research Institute, BARI) were irradiated with gamma-rays from a ^{60}Co source of the Bangladesh Atomic Energy Commission with 200, 300, 400 and 500 Gy doses during 1992. Selection based on earliness, number of bolls and seed-cotton

yields on M_2-M_4 generations gave finally 9 mutant lines. Of these, 4 mutant lines: CTM-10, CTM-51, CTM-6 and CTM-107 were obtained from the 200 Gy dose treatment of line S-71803958; the same line yielded another two mutants, CTM-42 and CTM-37, from 400 and 500 Gy doses, respectively. Mutant CTM-30 was isolated from the 300 Gy treatment of line S-71803785 and mutants CTM-110 and CTM-86 were isolated from the 200 and 300 Gy doses, respectively, treatment of line 398185. A preliminary yield trial (PYT) was carried out with the above 9 selected mutant lines at two locations, Ishurdi and Magura farms, during 1995-96. For this, land was prepared with 8-10 ploughings and 2 final ladderings. Fertilizers were applied urea 200 kg, TSP 150 kg, MP 100 kg along with 4 tons of cow manure/ha. A unit plot size was 7.5 m × 4.0 m. Seeds were sown during August 1995 at spacing of 75 cm within rows of 100 cm between the rows. The design followed was randomized complete blocks (RCB) with 3 replications. Crops were raised in rainfed conditions following needed intercultural operations. Data recorded were: days to first flowering and boll opening, maturity, plant height, number of bolls/plant, and seed cotton yield per plot. First flowering and boll opening were recorded as the number of days required from sowing while maturity as the number of days required for 90% boll opening. Plant height was measured from the base of the plant to the first unfolded leaf at the apex; the number of bolls/plant was counted on 10 competitive plants. Seed cotton yields were recorded from plots and converted to kg/ha. Data on jassid infestation was recorded when the fields were heavily infested by jassids and similar scaling for boll worm 10 days before harvesting. The percentages of infestation for both jassid and boll worm were gathered by relating the number of infested plants to totals in each plot.

2.1.2. Advanced yield trial (AYT) with 4 mutant lines

An advance yield trial was conducted during 1996-97 with 4 advanced mutant lines, CTM-10, CTM-110, CTM-6 and CTM-51 together with their 3 parents serving as controls. The locations were Ishurdi, Rangpur and Magura. The design, replications, plant spacing, management practices and data records were exactly as in the PYT, above. In this advanced yield trial, fibre properties such as 2.5% fibre length, strength and fineness were also recorded. At the end, proper statistical analyses were performed with all these recorded data.

2. 1.3. Zonal yield trial (ZYT) with 3 mutant lines

A zonal yield trial was followed during 1998-99 with 3 advanced mutant families, CTM-10, CTM-51 and CTM-110. Once again, two parents, S-71803958 and S-71803785, and a check cultivar DPL-50 were included. Due to heavy showers during the planting time, the seeds were sown in polyethylene bags and after one month the seedlings were transplanted to the main field. Locations were Magura, Rangpur and Ishurdi. Here once again, design, replications, cultural practices and data records were as in the PYT and AYT. Finally, the data were subjected to statistical analyses as per the design used.

2.2. Irradiation of introduced cultivars, DPL-50 and DPL-90, selection and preliminary yield trial

Dry seeds of 'DPL-50' and 'DPL-90' cultivars were irradiated with 200, 250, 300, 350 and 400 Gy from a ^{60}Co source of the Bangladesh Atomic Energy Commission to generate variability for traits pertinent to the objectives above. Selection pressures were put on the M_2-M_4 generations, which were based on earliness, increased number of bolls and seed cotton yields led to 12 M_5 mutant lines. Of these 6 were selected from the 200 Gy dose populations resulting from the treatment of the cultivar DPL-50; they were CTM-111, CTM112, CTM-113, CTM-114, CTM-

121 and CTM-122. Another 3 mutants: CTM-115, CTM-116 and CTM-117 arose from the 250 Gy dose given to the same source. Moreover, 300 and 350 Gy doses irradiated populations from the same source yielded CTM-118 and CTM-119, respectively. Only mutant CTM-120 was obtained from the other parent DPL-90 treated with 200 Gy dose. All these 12 selected lines were finally put into preliminary yield trials at Magura and Ishurdi farms during 1998-99 along with their two parents, DPL-50 and DPL-90. Due to heavy showers during the planting time, seeds were sown in polyethylene bags, after one month the seedlings were transplanted into the main field. Here too, fertilization, plant spacing, designs, and data records were as described before. The data so recorded were then subjected to statistical analyses.

2.3. Germplasm collection and performance evaluation

To satisfy the set objectives, viz. earliness, jassid and boll worm tolerances and high yields, a total of fourteen elite mutant lines, exotic and locally collected germplasm were evaluated along with the two cultivated checks, DPL-50 and DPL-90, during 1996-97 at Ishurdi and Magura Farms. Of the collected germplasm, KARISMA, NIAB-92, NIAB-78, F.H.-87, F.H.-682, F.H.-685, F.H.-679, F.H.-672, F.H.-673, L.S.-1 and R.H.-1 were obtained from the Nuclear Institute of Agriculture and Biology (NIAB), Pakistan. Moreover, advanced lines, S-71803958, S-71803785 and 398185 were supplied by the Bangladesh Agricultural Research Institute (BARI). The country of origin of MACAOS is not known, it was obtained from the Cotton Development Board (CDB), Bangladesh.

Additionally, during the 1997-98 and 1998-99 growing seasons these materials, excluding the elite mutants, were further assessed at Magura, and Magura and Ishurdi farms, respectively. The latter two evaluations included SINDOS-80 and SAMOS, and AFRODITI, B-432 and BAR, respectively, additional genotypes from Greece. All the three consecutive years' assessments used RCB design with 3 replications. Despite differential plot sizes, a uniform plant spacing of 50 cm within the rows and 100 cm between them, was always maintained. In 1998-99 due to heavy showers during the planting time, the seeds were sown in polyethylene bags; after one month the seedlings were transplanted to the main field. Furthermore, fertilization, intercultural operations and data records were as above. At the end of each year's evaluation runs, recorded data were subjected to proper statistical analyses as per design used.

3. RESULTS AND DISCUSSION

3.1. Preliminary, advanced and zonal yield trials with the selected mutants

Generally, the mutants, which reached the AYT, PYT and ZYT arose from the 200 and 300 Gy doses. This means that in future plant breeding applications and production of economic mutations in cotton, 200-300 Gy doses could be the most proper ones. Two lines, CTM-110 and CTM-10, took fewest days of all the mutants/parents to reach flowering, boll opening and maturity (Table I).

The remaining mutants and controls fell into an intermediate group despite showing significant differences within themselves. The two early maturing mutants, CTM-110 and CTM-10, had produced the shortest plants whilst the late and intermediate types produced significantly taller plants. The plant height finding is in agreement with the report of negative correlation between plant height and earliness in cotton [6].

TABLE I. EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF M_5 MUTANTS AND CONTROLS (P) GROWN AT ISHURDI AND MAGURA DURING 1995-96[a]

Mutants	Earliness traits, mean No. days to			Plant height cm	Bolls/ plant No.	Seed cotton yield kg/ha
	First flowering	First boll opening	Maturity			
CTM-110	49e	97f	148d	89b	22b	2120b
CTM-10	48e	106e	148d	88b	22b	2024c
CTM-6	56c	112d	158c	92b	28a	2001c
CTM-42	55c	128b	159bc	100a	23b	1592f
CTM-30	53d	109cd	159bc	92b	20b	1470g
S-71803785 (P)	56bc	130b	159bc	100a	22b	1402h
S-71803958 (P)	58a	121c	160c	98a	18b	1991c
398185 (P)	58a	137a	159bc	100a	21b	1991c
CTM-37	57b	121c	161abc	101a	21b	1728e
CTM-86	58a	137a	163a	102a	27a	1893d
CTM-107	56bc	137a	162ab	90b	21b	1169i
CTM-51	56c	135a	161abc	99a	22b	2271a

[a] Values followed by the same letter in a column do not differ significantly at the 5% level of probability.

TABLE II. REACTIONS TO JASSID AND BOLL WORM OF M_5 MUTANTS AND CONTROLS (P) GROWN AT ISHURDI AND MAGURA DURING 1995-96

Mutants	Jassid infestation, %		Boll worm infestation, %	
	Magura	Ishurdi	Magura	Ishurdi
CTM-37	44.25	100	21.89	5.70
CTM-42	44.05	100	32.19	7.16
CTM-6	41.83	100	27.45	5.65
CTM-107	41.33	100	21.80	5.72
CTM-110	39.96	100	26.29	6.62
CTM-51	34.85	100	25.40	6.61
CTM-86	34.36	100	28.72	10.59
CTM-30	32.80	100	21.62	6.23
CTM-10	22.07	100	26.78	10.35
S-71803958 (P)	37.08	100	26.23	9.77
S-71803785 (P)	32.33	100	24.01	6.20
398185 (P)	40.50	100	23.50	5.96
LSD (0.01)[a]	**NS**	**NS**	**3.54**	**NS**

[a] NS = not significant.

Number of bolls, the most important yield attribute, was highest in CTM-6 and CTM-86. The mutant CTM-51 produced the highest seed cotton yield of all the mutants/parents while CTM-110 produced the second highest yield followed by CTM-10. However CTM-10 was not significantly different from CTM-6 and the two parents, S-71803785 and S-71803958. The mutants/parents were not significantly different in jassid incidence at both locations, which was almost 100% at Ishurdi (Table II). Boll worm infestation was overall lower than jassids, and showed significant differences amongst the mutants/parents only at Magura. The top four mutants, in terms of earliness and yields, were reassessed further for their potentials in an advanced yield trial (Tables III, IV).

TABLE III. MEAN EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF M_6 LINES AND CONTROLS (P,C) GROWN AT ISHURDI, MAGURA AND RANGPUR, 1996-97[a]

Lines	Earliness traits, mean No. days to			Plant height cm	Bolls/ plant No.	Seed cotton yield kg/ha
	First flowering	First boll opening	Maturity			
CTM-110	53d	113c	160c	112a	38a	2048a
CTM-10	54cd	114c	160c	112a	35a	1829a
DPL-50 (C)	59abc	120b	163c	98b	36a	1242b
CTM-51	57bcd	120b	165bc	122a	36a	1834a
CTM-6	58abcd	120b	164c	114a	32a	1757ab
S-71803958 (P)	62ab	124a	171ab	117a	31a	1643ab
398185 (P)	63a	124a	172a	118a	38a	1645ab

[a] Values followed by the same letter in a column do not differ significantly at the 5% level of probability.

TABLE IV. REACTIONS OF M_6 LINES AND CONTROL (P, C) TO JASSID AND BOLL WORM

Lines	Jassid infestation, %		Boll worm infestation, %	
	Magura	Ishurdi	Magura	Ishurdi
CTM-51	2.06	34.69	10.68	58.51
CTM-110	17.41	59.36	20.57	56.34
CTM-10	4.21	76.62	11.48	72.53
CTM-6	1.87	26.67	16.47	55.82
S-71803958 (P)	7.99	45.44	14.71	57.99
398185 (P)	6.58	43.47	10.57	71.29
DPL-50 (C)	68.14	100.00	25.55	75.50
LSD (0.01)	**26.59**	**27.29**	**6.22**	**NS**[a]

[a] NS = not significant.

The two earliest lines at the PYT, CTM-110 and CTM-10 held the same ranking in the AYT. The higher yielding lines of PYT still showed significantly higher yields. Additionally, both jassid and boll worm infestations appeared significantly the highest in the check variety, DPL-50. CTM-6 showed the least infestation of all lines, at both Magura and Ishurdi

locations. Based on the performance in the advanced yield trial, only 3 mutant lines were advanced to zonal yield trials for a confirmatory test of the previous PYT and AYT results.

A detailed zonal yield trial was performed over three locations, viz. Mymensingh, Ishurdi and Magura, during 1998-99. The performance of the mutants/checks is presented in Tables V and VI.

TABLE V. MEANS OF EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF M_7 LINES AND CONTROLS (P, C) GROWN AT ISHURDI, MAGURA AND MYMENSINGH DURING 1998-99[a]

Lines	Earliness traits, mean No. days to			Plant height cm	Bolls/ plant No.	Seed cotton yield kg/ha
	First flowering	First boll opening	Maturity			
CTM-10	76b	141bc	182c	94d	16a	1087a
CTM-110	75b	139c	182c	96d	17a	1079a
CTM-51	76b	151a	187bc	111a	14a	1019a
S-71803958 (P)	76b	146ab	197a	107ab	14a	1012a
S-71803785 (P)	79a	141bc	193ab	103bc	14a	1122a
DPL-50 (C)	77b	145abc	193ab	99cd	16a	1122a

[a] Values followed by the same letter in a column do not differ significantly at the 5% level of probability

TABLE VI. FIBRE PROPERTIES AND REACTIONS TO JASSID AND BOLL WORM OF M_7 LINES AND CONTROLS (P,C) GROWN AT ISHURDI, MAGURA AND MYMENSINGH DURING 1998-99[a]

Lines	Fibre properties			Infestation, %	
	2.5% length cm	Strength PSI	Fineness µ	Jassid	Boll worm
CTM-10	2.72	85.34	4.8	28.32	30.80
CTM-110	2.62	84.06	4.7	27.08	25.68
CTM-51	3.00	84.33	4.8	24.34	31.22
S-71803958 (P)	2.69	85.67	4.7	23.05	30.84
S-71803785 (P)	3.00	85.46	4.9	16.19	34.11
DPL-50 (C)	2.87	86.66	4.6	31.38	26.81
LSD (0.05)	**0.17**	**0.99**	**0.11**	**14.27**	**NS**

[a] NS = not significant.

The results once again revealed earlier maturity in CTM-110 and CTM-10; they took fewer days to first boll opening and maturity. However, the mutant lines/checks were not different in flowering except S-71803785, which took maximal days. The two mutant lines, CTM-10 and CTM-110 along with DPL-50 were short and were not statistically different. All the others fell into a taller group. The mutant lines/checks were not significantly different in boll numbers and seed cotton yields. The above results of PYT, AYT, and finally ZYT consistently revealed the earliness of mutants CTM-10 and CTM-110, which is extremely

important for the existing cropping pattern in Bangladesh. Although non-significant yield differences appeared in the ZYT trials, the mutant lines showed significant differences in the earlier PYT and AYT. This could be due to the fact that the ZYT seedlings were raised in the polythylene bags before being transplanted to the main field, to avoid damage from heavy rainfalls during planting time. Additionally, the approximately 20 days longer period to flowering and maturity compared to PYT and AYT could also contribute to the same effect.

3.2. Irradiation, selection and evaluation of putative mutants from DPL-50 and DPL-90

The performance of 12 M_5 mutant families along with two controls is shown in Tables VII and VIII.

TABLE VII. MEAN EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF M_5 MUTANT LINES AND CONTROLS (P) GROWN AT MAGURA AND ISHURDI, 1998-99[a]

	Earliness, mean No. days to		Plant height	Bolls/plant	Seed cotton yield
Lines	First flowering	First boll opening	cm	No.	kg/ha
CTM-111	83	152	95	14	1596
CTM-112	81	149	97	15	1483
CTM-113	82	155	105	13	1676
CTM-114	83	148	112	16	2107
CTM-115	82	152	105	16	2077
CTM-116	81	150	111	15	2393
CTM-117	80	148	114	14	2213
CTM-118	83	156	108	16	1558
CTM-119	82	152	103	16	2316
CTM-120	83	154	105	11	2153
CTM-121	83	156	102	12	1833
CTM-122	78	148	106	12	1556
DPL-50 (P)	85	154	95	11	1783
DPL-90 (P)	87	164	107	10	921
LSD (0.05)	**3**	**NS**	**NS**	**4**	**697**

[a] NS = not significant.

Mutant CTM-122 had the earliest flowering of all the mutant lines/parents and shared a similar rank with 4 other mutants; it also had shown earliest first boll opening. However, mutants/parent varieties were not significantly different for plant height and days to boll opening. The control parents, DPL-50 and DPL-90 had the lowest boll numbers and shared equal rank with many others. In contrast, 4 mutant lines, CTM-114, CTM-115, CTM-118 and CTM-119 had higher boll numbers (16 bolls/plant). Mutant CTM-116 had the highest yield but did not differ from 6 other mutant lines. In contrast, DPL-90 parent produced the lowest yield of all the entries. The highest yielders had also high boll numbers, agreeing with Bhatnagar [7], that yield in cotton was closely associated with number of bolls/plant.

A wide range of variability existed amongst the mutants/parent lines in quality characters (Table VIII). Mutants, CTM-111, CTM-112 and CTM-115 had longer fibre, with CTM-115 being the longest of all. CTM-115 also had the strongest fibre.

TABLE VIII. FIBRE PROPERTIES AND REACTIONS TO JASSID AND BOLL WORM OF M_5 MUTANT LINES AND CONTROLS GROWN AT MAGURA AND ISHURDI DURING 1998-99

Lines	Fibre properties			Infestation, %	
	2.5% length	Strength	Fineness	Jassid	Boll worm
	cm	PSI	μ		
CTM-111	3.12	84.84	4.6	15.56	30.09
CTM-112	3.12	85.95	4.7	43.16	31.53
CTM-113	2.90	84.53	4.4	9.42	36.65
CTM-114	2.74	85.32	4.8	55.38	33.58
CTM-115	3.18	86.71	4.8	19.08	31.52
CTM-116	2.97	86.30	4.6	60.16	30.31
CTM-117	2.79	86.11	4.9	18.50	17.17
CTM-118	2.72	83.22	4.9	32.63	27.68
CTM-119	2.82	85.40	4.8	39.32	21.39
CTM-120	2.84	85.44	4.7	6.24	35.24
CTM-121	2.67	83.82	4.7	5.89	34.62
CTM-122	2.77	83.66	4.5	17.92	44.43
DPL-50 (P)	2.87	86.66	4.6	8.40	33.61
DPL-90 (P)	2.97	86.24	4.8	9.28	28.24
LSD (0.05)	**0.58**	**0.66**	**0.08**	**25.90**	**8.43**

3.3. Germplasm evaluation

Means over locations of earliness, yield and yield attributes, and jassid infestation of 23 elite mutants, exotic and indigenous genotypes are shown in Table IX**.**

The number of days to first flowering and boll opening ranged from 53 to 59 and from 109 to 127 days, respectively. The entries that showed early flowering did not necessarily show early first boll opening. However, F.H.-673 and L.S.-1 (data not shown) appeared earlier than the rest as per shortest day's requirements. The entry F.H.-87 was tallest but did not differ significantly from F.H.-682, F.H.-679 and CTM-15. In contrast, the check, DPL-50, was the shortest.

The entries, S-71803958, S-71803785 and 398185, DPL-50, NIAB-78 and all the mutants except CTM-14 and CTM-15 produced higher yields and were not significantly different. These entries mostly had also higher boll numbers, which suggests that high boll numbers lead to high yields. Higher jassid infestations were observed in 398185, CTM-13 and DPL-50, although they were not significantly different. The least jassid incidence occurred in NIAB-78, F.H.-679, F.H.-87, L.S.-1, R.H.-1 and CTM-15. Thus, it is possible that these lines have field tolerance.

TABLE IX. ORIGIN, EARLINESS, PLANT HEIGHT, YIELD, YIELD ATTRIBUTES AND REACTION TO JASSID OF MUTANTS, CONTROLS (C) AND INTRODUCED GERMPLASM GROWN AT MAGURA AND ISHURDI DURING 1996-97

Accession	Origin /source	Earliness traits, mean No. days to		Plant height	Bolls/ plant	Seed cotton yield	% leaves infested by
		1st flowering	1st boll opening	cm	No.	kg/plot	jassid
KARISMA	NIAB, Pakistan	56	119	113	24.25	1.644	2.17
NIAB-92	NIAB, Pakistan	56	114	110	27.25	1.908	7.14
NIAB-78	NIAB, Pakistan	57	111	110	29.25	2.741	0.00
F.H.-87	NIAB, Pakistan	59	113	124	29.75	2.128	0.00
F.H.-682	NIAB, Pakistan	57	111	122	23.00	2.239	6.82
F.H.-685	NIAB, Pakistan	59	118	109	26.75	1.745	26.08
F.H.-679	NIAB, Pakistan	58	114	117	27.50	1.623	0.00
F.H.-672	NIAB, Pakistan	56	111	105	33.50	2.021	11.35
F.H.-673	NIAB, Pakistan	54	109	114	35.00	2.234	10.00
L.S.-1	NIAB, Pakistan	-	-	-	-	-	0.00
R.H.-1	NIAB, Pakistan	55	112	117	30.75	2.170	0.00
MACAOS	CDB, Bangladesh	54	113	107	27.25	1.958	15.91
S-71803958	BARI, Bangladesh	57	114	111	25.00	2.785	12.26
S-71803785	BARI, Bangladesh	53	111	110	26.00	2.883	4.54
398185	BARI, Bangladesh	53	113	102	30.50	2.775	83.76
CTM-13	BINA, Bangladesh	53	115	103	27.00	2.480	70.45
CTM-14	BINA, Bangladesh	58	117	104	32.50	2.119	9.90
CTM-15	BINA, Bangladesh	54	113	118	38.50	2.452	0.00
CTM-16	BINA, Bangladesh	58	109	113	28.25	2.106	15.90
CTM-10	BINA, Bangladesh	56	109	96	22.75	2.463	0.00
CTM-110	BINA, Bangladesh	56	114	107	33.25	2.746	4.76
CTM-19	BINA, Bangladesh	59	115	112	30.50	2.450	16.45
CTM-20	BINA, Bangladesh	55	112	113	31.50	2.526	13.63
CTM-21	BINA, Bangladesh	59	115	101	34.25	2.416	7.89
DPL-50 (C)	CDB, Bangladesh	58	124	96	25.00	2.724	67.85
DPL-90 (C)	CDB, Bangladesh	58	127	112	31.00	1.548	4.16
LSD (0.05)		**3.27**	**3.84**	**9.00**	**6.92**	**0.556**	**28.81**

Further assessment of the germplasm was made during 1997-98, excluding the elite mutants tested in the previous year (Tables X and XI).

Here, two new entries from Greece were also included. Of the entries, F.H.-682, F.H.-673 and L.S.-1 were the earliest to flower, with F.H.-682 being the very earliest. F.H.-682 also had the earliest boll opening. Interestingly, none of the entries had higher boll numbers and seed cotton yields than the check DPL-50. However, jassid infestation and quality traits did show significant differences (Table XI). Of the entries, NIAB-78, F.H.-87, F.H.-682 and

SINDOS-80 had significantly higher fibre length over the checks, DPL-50 and DPL-90. Only 8 and 5 entries had better fibre strength and fineness, respectively. L.S.-1, R.H.-1, F.H.-87, NIAB-78 and SAMOS showed lower jassid infestations.

TABLE X. MEAN EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF INTRODUCED GERMPLASM AND CONTROLS (C) OF COTTON GROWN AT MAGURA, 1997-98

Accessions	Earliness traits, mean No. days to			Plant height cm	Bolls/plant No.	Seed cotton yield kg/ha
	1st flowering	1st boll opening	Maturity			
KARISMA	64	128	147	63	9.2	820.1
NIAB-92	62	132	140	86	9.2	648.4
NIAB-78	60	114	138	69	7.5	826.1
F.H.-87	60	111	129	69	8.2	1005.4
F.H.-682	58	108	137	69	7.5	911.0
F.H.-685	62	113	145	78	8.7	822.0
F.H.-679	62	117	144	63	9.0	815.5
F.H.-672	63	110	140	61	9.7	1057.5
F.H.-673	61	114	144	56	6.0	677.4
L.S.-1	61	113	145	72	6.7	1199.0
R.H.-1	71	123	149	88	7.0	1262.4
MACAOS	57	107	140	65	7.5	969.4
S-71803958	65	118	146	74	5.2	882.4
S-71803785	62	114	143	60	6.0	873.3
398185	62	113	146	65	7.2	976.5
SINDOS-80	64	124	147	71	8.0	1107.0
SAMOS	67	113	142	67	7.5	1149.1
DPL-50 (C)	62	113	143	63	11.7	1369.0
DPL-90 (C)	63	114	146	72	9.7	977.7
LSD (0.05)	**8.41**	**12.29**	**9.20**	**11.46**	**4.71**	**327.4**

A final performance trial including three entries from Greece was made during 1998-99 (Table XII**)**. Days to first flowering, boll opening and maturity ranged between 80-92, 152-168 and 196-209 days, respectively. In the previous years they were only 57-71, 107-132 and 137-149 days, respectively. This discrepancy might have resulted from the additional time required for establishment of the plants in the polyethylene bags and then transplanted to the main field. The two entries, MACAOS and S-71803958 had the shortest plants of all and ranked equal with 4 others. In contrast, F.H.-679 had the tallest plants but did not differ from F.H.-682, F.H.-685 and B-432. More importantly, none of the germplasm entries produced significantly higher yield than the check cultivar, DPL-50.

TABLE XI. FIBRE PROPERTIES AND REACTION TO JASSID OF INTRODUCED COTTON GERMPLASM AND CONTROL (C) GROWN AT MAGURA, 1997-98

Germplasm	Fibre properties			Jassid
	2.5% length, cm	Strength, PSI	Fineness, μ	infestation, %
KARISMA	2.87	88.22	3.8	5.1
NIAB-92	2.89	85.54	4.0	9.4
NIAB-78	3.07	88.84	4.0	2.7
F.H.-87	2.99	87.97	4.4	2.5
F.H.-682	3.20	90.37	4.4	9.8
F.H.-685	2.94	88.80	4.0	26.1
F.H.-679	2.94	84.85	4.2	3.7
F.H.-672	2.59	84.06	4.1	70.5
F.H.-673	2.87	85.90	4.4	14.2
L.S.-1	2.74	88.56	4.2	1.0
R.H.-1	2.89	83.92	4.3	1.2
MACAOS	2.87	85.97	3.7	18.9
S-71803958	2.64	86.46	4.2	13.2
S-71803785	2.94	97.96	4.3	9.5
398185	2.92	84.20	4.4	44.1
SINDOS-80	3.04	87.84	3.9	5.9
SAMOS	2.81	87.49	3.6	2.9
DPL-50 (C)	2.76	82.95	4.1	65.7
DPL-90 (C)	2.79	86.28	3.9	24.0
LSD (0.05)	**0.11**	**2.98**	**0.22**	**14.83**

TABLE XII. EARLINESS, PLANT HEIGHT, YIELD AND YIELD ATTRIBUTES OF INTRODUCED COTTON GERMPLASM AND CONTROL (C) GROWN AT MAGURA AND ISHURDI DURING 1998-99

Accessions	Earliness traits, mean No. days to			Plant height cm	Bolls/ plant No.	Seed cotton yield kg/ha
	First flowering	First boll opening	Maturity			
KARISMA	89	167	201	118	21	893
NIAB-92	87	166	200	113	18	1005
NIAB-78	91	167	199	114	18	806
F.H.-87	90	165	199	116	19	973
F.H.-682	86	165	202	122	20	1063
F.H.-685	86	163	206	125	21	1129
F.H.-679	90	162	202	130	21	977
F.H.-672	85	162	202	112	24	983
F.H.-673	87	165	205	111	18	813
L.S.-1	89	168	209	113	23	993
R.H.-1	92	168	205	114	20	885
MACAOS	83	161	196	96	16	820
S-71803958	87	164	203	96	17	1017
S-71803785	83	158	200	106	20	1320
398185	85	152	198	107	19	1247
SINDOS-80	86	165	202	114	20	1252
SAMOS	84	157	200	104	15	953
AFRODITI	85	161	206	110	15	845
B-432	86	161	197	122	16	956
BAR	80	163	199	101	14	786
DPL-50 (C)	85	163	202	99	18	1318
DPL-90 (C)	85	162	201	105	16	977
LSD (0.05)	**4**	**7**	**7**	**9**	**4**	**299**

Of the quality traits, two entries, R.H.-1 and BAR had longer fibre than the check cultivars while F.H.-673 had higher strength. Only 1 entry had significantly higher fibre fineness (smallest value) than the controls (Table XIII). Jassid and boll worm incidences showed wide ranges of variability. Of the entries, 6 had lower jassid infestation than the check DPL-50 despite shared equal statistical ranking (Table XIII). In contrast, in boll worm infestation, none of the entries were significantly different from the check cultivars except F.H.-87, which showed the most tolerance for its least infestation (0.82%).

TABLE XIII. FIBRE PROPERTIES AND REACTIONS TO JASSID AND BOLL WORM OF INTRODUCED COTTON GERMPLASM AND CONTROL (C) GROWN AT MAGURA AND ISHURDI DURING 1998-99

Germplasm	Fibre properties			Infestation, %	
	2.5% length cm	Strength PSI	Fineness μ	Jassid	Boll worm
KARISMA	2.54	85.20	4.8	4.35	22.23
NIAB-92	2.90	85.02	4.8	11.92	19.42
NIAB-78	2.67	84.49	4.5	6.76	20.14
F.H.-87	2.97	84.89	4.7	9.16	0.82
F.H.-682	2.64	83.53	4.9	3.79	20.39
F.H.-685	2.72	84.62	4.7	2.40	19.51
F.H.-679	2.67	84.98	4.9	3.27	23.51
F.H.-672	2.67	83.21	4.9	4.44	18.05
F.H.-673	2.97	88.00	4.8	3.81	21.12
L.S.-1	2.95	84.96	4.7	2.70	21.33
R.H.-1	3.12	85.76	4.8	1.30	19.47
MACAOS	2.84	85.99	4.3	2.66	17.83
S-71803958	2.69	85.67	4.7	8.28	17.00
S-71803785	3.00	85.46	4.9	4.96	18.66
398185	2.82	86.87	4.8	3.42	22.78
SINDOS-80	2.82	85.31	4.8	2.55	21.31
SAMOS	2.95	85.34	4.9	4.24	19.84
AFRODITI	2.82	83.96	4.6	2.00	23.83
B-432	2.62	84.28	4.8	6.23	16.36
BAR	3.02	85.53	4.5	13.27	21.20
DPL-50 (C)	2.87	86.66	4.6	3.46	21.08
DPL-90 (C)	2.97	86.24	4.8	4.20	20.84
LSD (0.05)	**0.07**	**0.49**	**0.07**	**7.00**	**6.34**

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Soybean breeding for earliness and seed quality by induced mutations

M. Hajós-Novak, G.K. Hódos

Department of Genetics and Plant Breeding

F. Körösi

Department of Plant Physiology

Gödöllő University of Agricultural Sciences,
Gödöllő, Hungary

Abstract. Mutant soybean germplasm lines were developed by the pedigree method from two local varieties, Karpatalja (KA) and VL40, by 100-300 Gy doses of chronic gamma irradiation. The objective was to produce early mutant lines with an increased oil content and/or protein content and acceptable agronomic performance. Selection for earliness commenced in M_2 generation, for oil content and agronomic characteristics in the M_3 generation and for protein content in M_4 generation. Due to the limited genetic variation for earliness no mutant genotypes earlier than the control could be identified. Chronic gamma irradiation increased the genetic variability for oil content in both local varieties, and the genetic variability for protein content in VL40. Progenies with 23.9% oil content were detected among the KA 100 Gy progenies and among the VL40 250 Gy progenies. Furthermore, a VL40 250 Gy offspring with 43.6% protein content was selected. The applied chronic doses (100, 150, 200 and 300 Gy) significantly lowered the first pod height and the 1000-seed weight as compared to the check cultivar. The significant increase in the number of pods and in the number of seeds per plant led to an increase in yield per plant in KA 150 Gy lines despite the fact that the 1000-seed weight was decreased significantly. The applied doses did not change the colors of the seed, hylum and flowers. Correlation studies in M_6 generation between oil content and agronomic characteristics revealed that only the oil content and the number of seeds per plant were significantly ($p < 0.05$) associated ($r = -0.24^*$).

1. INTRODUCTION

The cultivated soybean, *Glycine max (*L.) Merr., is becoming an important oil seed crop in Hungary, where 40,000 ha were grown in 1996. The area decreased by one half in 1997 due to the restructuring of the agricultural system. Soybean production is mainly concentrated in the southern regions, thus a programme was undertaken to develop early varieties to extend the production range.

In Hungary in the last few years there has been a renewed interest in improving the oil and/or the protein content of the soybean crop, because these components have positive economic values both for the consumers and the sellers. The value of the soybeans to the processors is primarily determined by the protein and the oil content of the seed, while the value to the producer is determined by the yield. Therefore, breeders should consider how genetic modification and selection for oil and/or protein content can be combined with genetic improvement and selection for yield to maximize gross value per hectare [1].

Mutant populations produced by irradiation provide a good source for breeders to develop soybean cultivars with improved oil and/or protein content. During the past 30 years about 30 cultivars of soybean have been produced by X-rays, thermal neutron and gamma irradiation in different countries, mainly in China [2]. These released cultivars have been improved in yield, first pod height, oil and/or protein content, maturity, resistance to lodging,

tolerance to pod shattering and resistance to cyst nematode. In Turkey a mutant variety, TAEK A-3, with high oil (25.5%) and high protein content (39.2%) was registered [3]. Thus, the objective of this study was to obtain early (Type 0) soybean lines with improved oil and/or protein content and acceptable agronomic performance.

2. MATERIALS AND METHODS

Four hundred seeds from the Hungarian local variety Karpatalja (KA) and 400 seeds from a Vietnamese local variety adapted to Hungarian environmental conditions (VL40) were sown in the gamma field of the Department of Genetics and Plant Breeding, Gödöllő, in order to improve the earliness, oil and/or protein content of both varieties and resistance to lodging of VL40. The irradiation was chronic and was applied from sowing to physiological maturity with gamma rays from a ^{60}Co source at doses of 100-300 Gy.

All M_1 plants were harvested separately and all the seeds from the single plants as well as untreated seeds were sown in individual plots as M_2 generation. The materials were very carefully studied from seedling to maturity. Data on seedling emergence, seedling survival and sterility were recorded. Furthermore, pedigree selection for earliness was started. 500 plants each, from KA and VL40 and 5 from the control were selfed to allow the expression of earliness, which is a recessive characteristic, in the later generations. The most vigorous plants resistant to lodging and shattering were also chosen.

In the M_3 generation one untreated and five mutant populations were developed from the progeny rows of the plants being at different distances from the gamma source that is: 100 Gy population (18-20 m), 150 Gy population (14-15 m), 200 Gy population (11-12 m), 250 Gy population (7.5-9.0 m) and 300 Gy population (6.0-6.5 m). Selection for earliness was continued and selection of single plants for oil content, first pod height, number of pods, 1000-seed weight, yield per plant, harvest index, lodging and shattering were initiated.

The selected M_3 materials were grown in M_4 and M_5 progeny rows (from 20 to 30 plants per dose, and 50 seeds per single plant), together with the original cultivars. Then single plant selection, aiming at oil and protein content and the above mentioned agronomic characteristics, and line selection were carried out. The VL40 mutant lines, except for those from the 250 Gy treatment, were discarded due to their susceptibility to lodging.

In M_5 and M_6 two-location yield tests were conducted at a plot size of 2 m^2. The experimental design was a randomized complete block with two replications.

The oil content and the fatty acid composition were analysed by using nuclear magnetic resonance (NMR) as well as gas liquid chromatography. The crude protein content was measured by automatic Kjeldahl method.

For data evaluation one- and two-way analyses of variance and correlation analyses were done by the SPSS 8.0 software package. Hazard function was accomplished [4] using the software Statgraphic 4.0.

3. RESULTS AND DISCUSSION

3.1. M_1 generation

Due to the chronic gamma irradiation plants that were nearest to the gamma source suffered from severe growth retardation; in both KA and VL40 they had only a few pods with one seed. Other plants developed normally.

3.2. M_2 generation

Plant growth in the M_2 generation was depressed and a few plants were sterile or semi-sterile. Concerning the number of days from emergence to appearance of the first flowers only one or two days' differences were found at both treated cultivars as compared to the controls.

3.3. M_3 generation

In this generation there were no differences in earliness between the control and the irradiated plants' offspring. The oil content of the seeds in the M_3 generation of the local varieties KA and VL40 ranged from 17.6 to 23.1%, with a mean of 19.3%. The M_3 mutant lines showed a slight (± 3%) variability in oil content. Normal distribution of oil content data was proved at 10% by χ^2 test ($\chi^2 = 6.26$). Our results suggest that it was too early to start selection for oil content in the M_3 generation.

The correlation between percent oil and 1000-seed weight was negative, moderate ($r = -0.4$) and significant ($P < 0.01$). It can be predicted, by using linear regression analysis, that a 10.0 g increase in 1000-seed weight would result in a decrease in oil content of about 0.16%.

Seeds with more than 22.0% oil were analysed for fatty acid composition. The linoleic acid (C18:2) content in the seed oil ranged from 45.4 to 55.0% and the linolenic acid (C18:3) portion from 5.5 to 8.6%, respectively.

Selection for the most important agronomic characteristics was initiated in this generation. The selection criteria for the different traits were the following: plant height - > 90 cm, first pod height - 10-12 cm, number of pods - < 80, number of seeds per plant - < 150, 1000-seed weight - < 140 g, yield per plant - < 25 g, harvest index - < 45%.

Seed and/or flower color mutants were not detected.

3.4. M_4 generation

The oil content of the seeds ranged between 19.8 and 24.1% with an average of 21.4%. The highest oil content of 24.1 and 24.0% were found in the variants of KA150 Gy and VL40 250 Gy, respectively. The oil content of VL40 treated with 250 Gy was significantly increased, by 3.2%, as compared to the control. In the KA variety, the oil content of seeds was significantly increased due to irradiation (100 Gy = 23.6%; 150 Gy = 24%) by 3.3% and 4.3% as compared to the control.

Comparing the oil content of the seeds of both the M_3 and M_4 generations, it was observed that it was significantly (1.2-1.5%) higher in M_4 than in M_3: KA 1.2-1.5%, VL40 0.9-1.6%.

Regression analyses of the oil content and 1000-seed weight revealed an expected negative correlation between the two characteristics ($r = -0.4$, $P < 0.01$). This means, that an increase in 1000-seed weight by 10.0 g may cause a decrease in oil content of 0.17%.

In the M_4 generation, the seeds of KA 150 Gy and VL40 250 Gy mutant lines had the lowest linolenic acid content, i.e. 8.3 and 8.4%, respectively. Their linoleic acid contents were 54.5 and 53.5%, respectively.

M_4 plants with high and low oil content in the seeds were checked for their protein content. The crude protein content of the M_4 seeds ranged from 27.4 to 40.5% with a mean of 37.5%. Due to the negative correlation between the oil and the protein content, seeds with 24.0% oil had only 27.4% crude protein. The highest protein content (40.5%) was found in the seeds of a VL40 250 Gy line, which had the lowest oil content (19.0%) as compared to the untreated cultivar and the other treatments.

Seed and/or flower color mutants were not detected.

3.5. M_5 generation

In the M_5 generation, the mean oil content in the seeds was 21.2%, and ranged from 17.5 to 22.5%. The oil content in progenies of the superior plants with the highest oil content in M_4, e.g. KA 150 Gy (24.1%), ranged from 20.3 to 22.1% perhaps due to environmental conditions, with a mean of 21.1%. But the progeny of a superior KA 100 Gy plant of 23.1% oil content were homozygous for this character.

Seven seed samples with the highest oil content were analysed for fatty acid composition using gas-liquid chromatography. Their linoleic acid content ranged from 51.8 to 55.0%. In M_5 the linolenic acid content was very high compared to M_4. It ranged from 7.9% to 9.3%.

In this generation the protein content ranged from 31.1 to 41.9% with an average of 38.3%. The highest protein content (41.9%) was in a VL40 250 Gy line. The protein content of five plants from this line were determined, the values were: 37.5, 38.2, 39.8, 41.8 and 41.9%, respectively.

TABLE I. CORRELATION COEFFICIENTS BETWEEN 1000-SEED WEIGHT AND SEED QUALITY PARAMETERS /PEARSON R, SIG. (2-TAILED)

	Oil, %	Protein, %	1000-seed weight, g
Oil, %		-0.650**	-0.387**
Protein, %	-0.650**		0.710**
1000-seed weight, %	-0.387**	0.710**	

** Significant at 0.01 level of probability (n = 82)

Correlation studies between oil and protein content, oil content and 1000-seed weight, as well as protein content and 1000-seed weight revealed significant ($p < 0.01$) associations (Table I). Oil content exhibited a strong negative correlation with protein content (r = -0.650**) and a moderately negative one with 1000-seed weight (r = -0.387**). But there was a strong positive correlation between seed protein content and 1000-seed weight (r = 0.710**).

Seven near-homozygous mutant lines, five from KA and two from VL40, selected for earliness, high oil and/or protein content, as well as for agronomic characteristics, and the check cultivars, were evaluated for seed yield performance at two locations (Gödöllő and Szarvas). Two-way analysis of variance of seed yield/plot showed significant differences between the two locations. In Szarvas the seed yields/plot ranged from 208 g to 372 g, and there were no significant differences among them. In Gödöllő, the yields were from 41.4% to 68.2% higher than in the other location. Here the average seed yield ranged from 409.5 g to 1134 g. The highest standard deviation values at both locations (175.4 and 464.2) were shown by the VL4O 250 Gy, indicating that this mutant line is still segregating.

3.6. M_6 generation

3.6.1. Oil and protein content

In the M_6 generation the oil content showed a normal distribution. The mean oil content of the seeds was 22.4%, and ranged from 19.0 to 23.9%. The highest oil content (23.9%) was found in the KA 100 Gy, which was significantly higher than that of the control. The Hazard function, which can be obtained by dividing the density function and the survivor function (1-cumulative distribution function) predicts up to 28% oil content in the

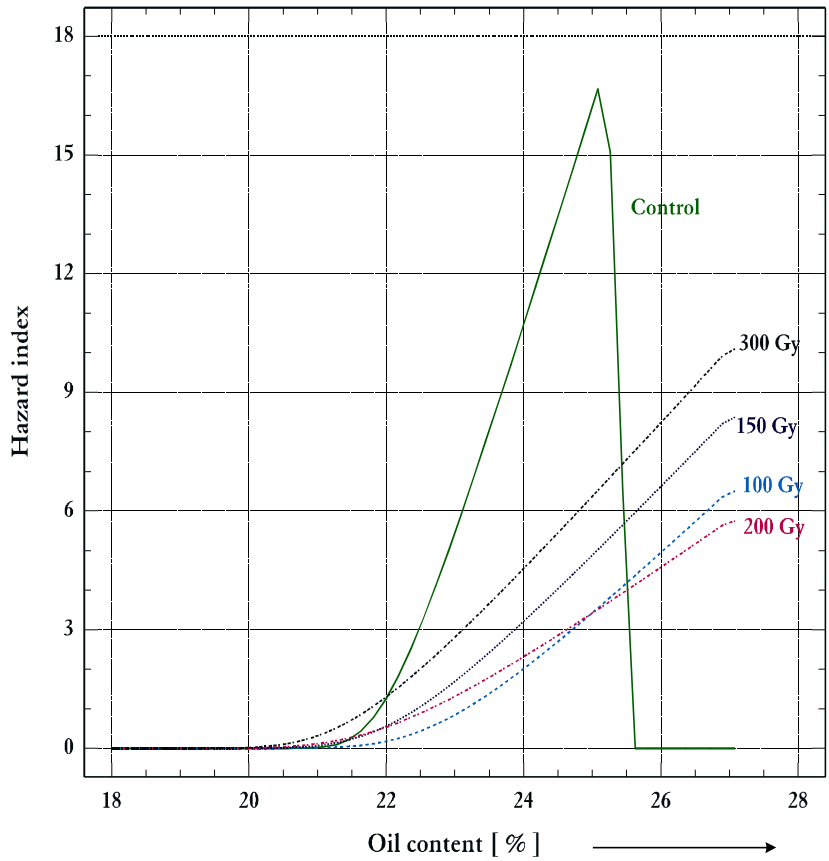

FIG. 1. Hazard Function for the oil content (%) distribution of the KA M_6 lines.

irradiated KA populations, while in the control a sharp decline can be observed at about 25.5% of oil content. This means that the genetic potential of the oil content in KA local variety is about 25.5%. It can be increased by using chronic gamma irradiation (Fig. 1). From the VL40 250 Gy population, which consists of three near-homozygous progenies, both plants with high protein (43.7%) and high oil (23.3%) content were selected.

The mutant near-homozygous soybean lines with the highest oil content had a better fatty acid composition than that of the controls (Table II). Their linoleic acid content ranged from 51.7 to 53.4%. The lowest values were achieved by the KA and VL40 controls (49.7 and 48.9%). The linolenic acid content was moderate in the mutants, and ranged from 6.7 to 7.8%. Correspondingly, the highest values were detected in the KA and VL40 controls (9.0 and 8.7%).

TABLE II. FATTY ACID COMPOSITION OF M_6 MUTANT SOYBEAN LINES WITH MODERATE AND HIGH OIL CONTENT

Population	Oil content %	Palmitate C_{16}, %	Stearic acid C_{18}, %	Oleic acid $C_{18:1}$, %	Linoleic acid $C_{18:2}$, %	Linolenic acid $C_{18:3}$, %
KA Control	21.9	11.3	5.3	22.9	49.7	9.0
KA 100 Gy	23.9	11.1	4.7	21.7	53.4	7.7
KA 150 Gy	22.9	10.1	4.9	23.2	52.9	7.4
KA 200 Gy	23.6	10.1	5.2	24.1	51.7	7.4
KA 300 Gy	21.1	11.1	4.5	21.3	53.8	7.8
VL40 Control	21.5	11.0	5.8	23.7	48.9	8.7
VL40 250 Gy	23.3	11.0	5.2	23.5	52.5	6.7

3.6.2. Agronomic characteristics

In the M_6 generation plant height ranged from 60.0 to 110.14 cm with a mean of 76.9 cm. Gamma irradiation significantly reduced the height of the plants by a range of 13.1-20.7 cm as compared to the check cultivar, except the KA 150 Gy dose.

The first pod height was significantly lowered at all of the applied doses.

Gamma irradiation significantly increased the number of pods per plant. On the control plants the mean was 46 pods/plant, the KA 150 Gy plants had 87, and the 300 Gy plants had 83 pods.

Due to the mutagen treatments and/or selection, the number of seeds per plant has increased significantly. On the control plants as many as 85 seeds were counted on average, while on the mutants from 129 to 155. The number of seeds per pod did not change. There were always two seeds in each pod.

The applied doses significantly decreased the 1000-seed weight as compared to the control population (Fig. 2).

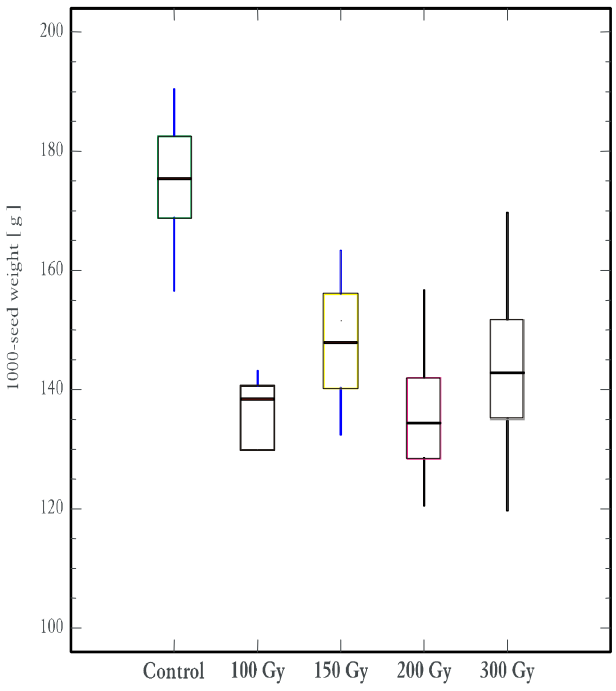

FIG. 2. 1000-seed weight (g) of the KA mutant M_6 lines.

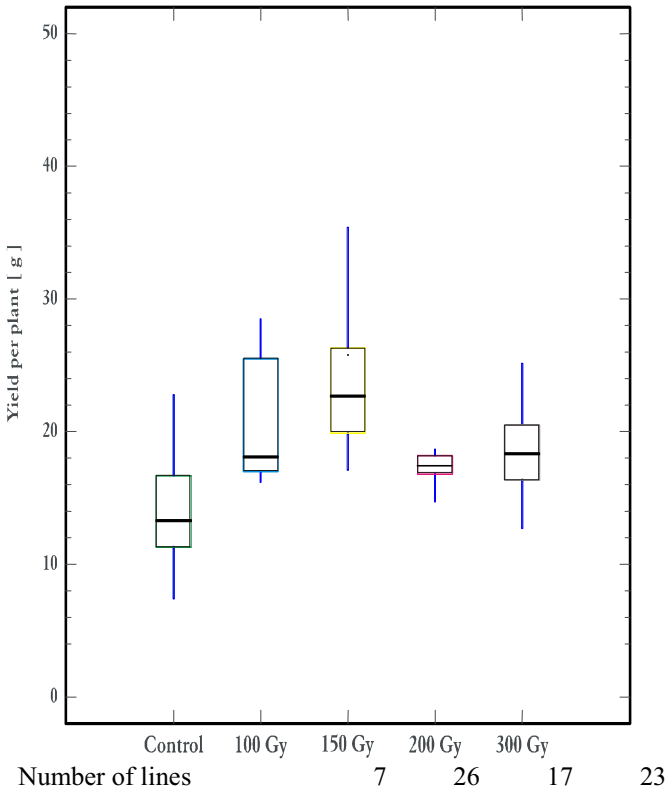

FIG. 3. Yield per plant (g) of the KA mutant M_6 lines.

Because of the increase in the number of pods and the number of seeds the yield per plant of the KA 150 Gy lines increased by 57% as compared to the control. Untreated plants yielded 14.88 g, while KA 150 Gy plants gave 23.40 g (Fig. 3).

Table III presents the means and the standard deviation values of all studied characteristics for the control and the mutant KA populations. The high standard deviation values relate to a significant environmental effect.

TABLE III. MEANS AND STANDARD DEVIATION (SD) VALUES OF THE KA MUTANT M_6 LINES SELECTED FOR AGRONOMIC PERFORMANCE

Population	Plant height cm		First pod height, cm		No. pods per plant		No. seeds per plant		Yield/plant g		1000-seed weight g	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	88.81	18.97	11.69	4.90	46	15	85	28	14.89	5.60	175.9	9.1
100 Gy	70.19	5.14	6.07	0.67	77	13	146	29	20.20	4.78	133.2	11.3
150 Gy	66.78	12.16	6.68	1.22	87	14	154	29	23.40	4.27	148.5	9.0
200 Gy	68.12	4.12	6.15	0.74	67	6	129	16	17.60	2.50	135.8	10.3
300 Gy	75.71	3.98	7.79	0.97	84	20	143	45	20.63	7.97	144.1	11.7

3.6.3. Correlation studies

The correlation of the important agronomic and quality characters in advanced KA mutant lines are presented in Table IV. According to the Pearson correlation coefficients only the oil content and the number of seeds per plant were significantly associated (r = -0.24*) (Table IV).

Plant height was negatively correlated with yield per plant (-0.43**), with number of seeds per plant (-0.47**), and number of pods per plant (-0.51**). It was positively correlated with first pod height (0.42**) and 1000-seed weight (0.33**).

First pod height was positively correlated with plant height (0.42**) and 1000-seed weight (0.42**). It was negatively correlated with number of pods per plant (-0.45**), number of seeds per plant (-0.46**) and yield per plant (-0.32**).

Number of pods per plant exhibited a positive, strong correlation with number of seeds per plant (0.92**) and yield per plant (0.88**). A negative, moderate correlation was observed with plant height (-0.51**), first pod height (-0.45**) and 1000-seed weight (-0.48**).

Number of seeds per plant was positively correlated with the yield per plant (0.93**) and number of pods per plant (0,92**). It was negatively correlated with plant height (-0.47**), first pod height (-0.46**), 1000-seed weight (-0.45**) and oil content (-0.24*).

A strong, positive correlation existed between the yield per plant and the number of seeds per plant (0.93**) and the number of pods per plant (0.88**). However, plant height, the first pod height and the 1000-seed weight were negatively correlated with yield per plant (-0.43**, -0.32**, -0.28**, respectively).

TABLE IV. CORRELATION COEFFICIENTS BETWEEN STUDIED CHARACTERISTICS

		Oil content %	1000-seed weight	Yield per plant	No. seed per plant	No. pods per plant	First pod height	Plant height
Oil content, %	Pearson Cor.	1.00						
	Sig. (2-tailed)							
	N	42						
1000-seed weight	Pearson Cor.	0.20	1.00					
	Sig. (2-tailed)	0.19						
	N	42	99					
Yield per plant	Pearson Cor.	-0.17	-0.28**	1.00				
	Sig. (2-tailed)	0.28	0.01					
	N	42	99	100				
No. seed per plant	Pearson Cor.	-0.24*	-0.45**	0.93**	1.00			
	Sig. (2-tailed)	0.12	0.00	0.00				
	N	42	99	100	100			
No. pods per plant	Pearson Cor.	-0.16	-0.48**	0.88**	0.92**	1.00		
	Sig. (2-tailed)	0.33	0.00	0.00	0.00			
	N	42	99	100	100	100		
First pod height	Pearson Cor.	0.07	0.42**	-0.32**	-0.46**	-0.45**	1.00	
	Sig. (2-tailed)	0.66	0.00	0.00	0.00	0.00		
	N	42	99	100	100	100	100	
Plant height	Pearson Cor.	0.18	0.33**	-0.43**	-0.47**	-0.51**	0.42**	1.00
	Sig. (2-tailed)	0.25	0.00	0.00	0.00	0.00	0.00	
	N	42	99	100	100	100	100	100

*, ** Significant at 0.05 and 0.01 levels of probability (2-tailed)

TABLE V. THE MOST IMPORTANT AGRONOMIC AND QUALITY CHARACTERS OF NEAR-HOMOZYGOUS M_6 MUTANT SOYBEAN LINES

Treatment	Plant height cm	First pod height cm	Number of pods/plant	Number of seeds/plant	1000-seed weight g	Yield per plant g	Harvest index %	Oil content %	Protein content %	Lodging	Shattering
KA control	87.3	14.2	46.0	83.7	177.6	14.8	40	22.2	33.7	resistant	resistant
KA 100 Gy	70.6	5.9	83.8	166.4	142.4	24.0	44	23.5	31.8	resistant	resistant
KA 150 Gy	71.5	5.4	110.2	215.7	163.4	35.4	42	23.3	31.1	resistant	resistant
VL40 control	66.8	9.5	43.9	78.8	143.5	11.5	42	21.3	35.5	susceptible	resistant
VL40 250 Gy	64.6	6.9	56.4	97.3	125.9	12.4	39	19.0	43.7	moderately resistant	resistant
VL40 250 Gy	73.8	5.5	66.7	112.7	144.3	16.5	37	23.3	32.3	resistant	resistant

1000-seed weight was positively associated with plant height (0.33**) and first pod height (0.42**), but negatively with the yield components and the yield per plant (-0.48**, -0.45** and 0.28**).

The results demonstrate that radiomutation induction and selection could be successfully used in the two soybean cultivars to improve the quality of the seeds and some of the agronomic characters, e.g. number of pods per plant, number of seeds per plant and yield per plant. Soybean mutants with high oil (23.9%) and high protein (43.6%) content, and favourable agronomic traits were selected from two adapted varieties (Table V). Using radiation techniques, similar improvements in the oil and protein contents of soybean were achieved [2,3,5,6].

Genetically improved soybeans with 40% protein content are assumed to produce a new type of soybean meal with 48% protein content and 85% crushing yield [7]. Taking into consideration the market value of soybeans today, it would seem that mutant soybean lines with about 40% protein content would provide the expected economic benefits for soybean producers; in addition, their lysine and methionine content is also favourable. Developing a mutant soybean variety with more than 40% protein content in the seeds and no farm-yield reduction would produce about a 800 kg/ha protein yield in Hungary, and in the case of a 10% farm-yield reduction, this figure would come to about a 720 kg/ha protein yield. Growing higher oil soybeans (23.9%), even with a loss of farm production yield, theoretically, farmers could achieve an oil yield of about 460 kg/ha.

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Combining radiation mutation techniques with biotechnology for soybean breeding

L.Z. Wang, L. Wang, R.J. Zhao
Y.L. Pei, Y.Q. Fu, Q.S. Yan, Q. Li

Crop Breeding and Cultivation Institute,
Chinese Academy of Agricultural Sciences (CAAS),
Beijing, China

Abstract. Through the ^{60}Co irradiation of adapted Chinese cultivars and selection for high yield, good quality, resistance to diseases and insects, and early maturity, several lines were obtained that were better than the control Zhonghuang 4, an important variety of the Beijing area. After ten generations of individual selection and quality analysis, we got five high yielding soybean mutants including Zhongzuo 965, Zhongzuo 962, Zhongzuo 973, Zhongzuo M-5028 and Zhongzuo M-17. From these mutants one or two were to be released in 2000. We selected three mutants with the crude oil content exceeding 22%. The crude oil content of 397-2 is 23.52%. The crude protein of plant 348-4 is 47.67% (mean of two years). Furthermore, mutant lines and varieties were evaluated for their genetic transformation ability; an improved commercial variety - Heinong 35 - has been identified as being very suitable for genetic transformation.

1. INTRODUCTION

Soybean is an important crop. In China it is the fourth largest crop after rice, wheat and corn. Soybean meal contributes 60% of the world protein consumption. Its oil contributes about 20-30% of the total vegetable oil production in the world. In China soybean production reached about 16 million tons in 1994, 13.5 million tons in 1995, 13.2 million tons in 1996, 14.7 million tons in 1997 and 15 million tons in 1998. In soybean production the main problems are drought in the spring, low yield and some diseases and insects. Factors reducing germination can decrease the yield; and also the pod borers, cyst nematode (*Heterodera glycine*) and weeds cause serious damage in some areas. The objectives of this project were: 1) To develop high yield mutants of soybean, with high oil or high protein content and/or resistance against cyst nematode, pod borer, and soybean mosaic virus; 2) To screen soybean germplasm including mutants for molecular markers and transformation ability.

2. MATERIALS AND METHODS

Mutation treatment of soybean was done in 1994 using three doses of ^{60}Co irradiation: 100, 120 and 150Gy. The M_1 generation of ten cultivars was grown at Aicheng Experimental Farm of the Cotton Research Institute, CAAS, including: Linzhen No.1, F5 Sidou 11×Jilin 22, F5 Sidou 11×Kefeng No. 6, F4 Youdou 8×D90, Juifeng, Youchou No. 4, Hanying No. 1, CG661×91-1, Zakang F6×Ludou 4, Thailand soybean and H.P.F6×2285-095.

Two mutant populations were planted on the experimental farm near CAAS in May 1995 (M_2-populations) and 1996 (M_3-population) to select for suitability as spring sown soybean. All other mutant populations were planted at the Changping Experimental Farm of CAAS in the suburb of Beijing in June 1995 (M_2), 1996 (M_5) in order to select the lines which are suitable for the summer sown crop. M_3 and M_4 populations were grown in the winter from October 1995 to May 1996 at Hainan Island. Selection for semi-dwarf types and early maturity was started in M_2 and continued in subsequent generations. From M_3, plants with high protein content and high oil content were tested and selected. From M_4 onwards

populations were selected for improved resistance to soybean mosaic virus. The M_5, M_6, M_7 and M_8 generations were grown in different locations. Individual selection was practiced for desirable mutants.

3. RESULTS AND DISCUSSION

3.1. Seed yield

Through 11 generations of mutation breeding and selection during 1994 to 1999, 34 soybean mutants were selected. In 1997 according to the regional yield tests, the following soybean mutants were good lines in Northern China: Zhongzuo-M17 (yield 2234 kg/ha), Zhongzuo 962 (2310 kg/ha), Zhongzuo 965 (2429 kg/ha), Zhongzuo 96-M5028 (2432 kg/ha), Zhongzuo 973 (2621 kg/ha). Yield increments from 12% to 25% in comparison to Zhonghuang 4 (2100 kg/ha), an important variety of the Beijing area, used as check (CK), were achieved. These mutant lines are now under national testing.

TABLE I. PROMISING SOYBEAN MUTANTS IN NORTHERN CHINA, REGIONAL YIELD TESTS, 1997, BEIJING

Line	Yield kg/ha	Increase over CK, %	Maturity	Lodging
Zhongzuo 973 (M5003)	2622	25.02	Oct.10	0
Zhongzuo 96- M5028 (Nangyin)	2433	21.81	Oct.10	1
Zhongzuo 965 (M55-56 Youdou 8×D90)	2492	21.75	Oct.12	0
Zhongzuo 962	2310	19.75	Oct.28	0
Zhongzuo M-17	2234	12.00	Oct.8	0
Zhonghuang 4 (check)	2100	0	Oct.5	0

These soybean mutants were evaluated in regional tests and production tests in ten provinces. Some of them were to be released by 2000 or soon after. These mutants have very good characteristics (improved yield, large seed and disease resistance). However, according to rules of seed management, regional tests need 2-3 years, and production test needs 1-2 years. According to the yield tests, the following mutant lines are good lines of soybean.

3.1.1. Zhongzuo 965

This line yielded 21.75% over the control, plant height is 80 cm with many pods per plant. It proved adapted in the northern part of Henan Province after winter wheat. In 1999, the yield of Zhongzuo 965 exceeded Longchung No. 1 by 11.2% in Gangsu Province.

3.1.2. Zhongzuo 962

The yield of Zhongzuo 962 was 3218 kg/ha in Teiling Soybean Institute, Liaoning Province and was 12.9% higher than Teifeng 27 (CK) in 1999. This line has early maturity and it is suitable for growing two crops per year near Beijing, Tianjin and the northern part of Hebei Province. Plant height is about 65-70 cm, it is semidwarf and requires high density stands.

3.1.3. Zhongzuo 973 (96-M-5003)

The yield of this line was 2622 kg/ha, 25% above the control. It is suitable for growing in the northern parts of Henan and Shandong Provinces. It is suitable for two crops per year and is resistant to some diseases.

3.1.4. Zhongzuo 96-M-5028

The yield of this line was 2433 kg/ha, 21.8% above the control. It is suitable for growing in the southern part of Shanxi Province and the northern part of Shandong Province. It is resistant to lodging. This mutant was obtained following treatment of Hanying No. 1.

3.1.5. Zhongzuo M 17

It is a good line with high protein content of 45.11% and 19.15% oil. It has large seeds. One hundred seeds weight is about 26-28 grams. It is resistant to lodging.

3.1.6. Additional promising mutants

In 1998, the following mutants showed promise in a yield test.

TABLE II. PROMISING SOYBEAN MUTANTS IN 1998, BEIJING

Line	Yield increment over CK, %
Zhongzuo M 5037	25.31
Zhongzuo M 5042	15.71
Zhongzuo 97 M 4397	14.59
Zhongzuo 965	13.64

3.2. Seed quality

3.2.1. Performance of some mutants and cultivars

Some soybean mutant lines were selected following crude oil and protein content analyses. The results are shown in Table III

TABLE III. CRUDE OIL AND PROTEIN CONTENT OF SELECTED MUTANTS

Lines	Crude oil, %	Crude protein, %
Zhongzuo 965(M 55-56)	18.20	43.55
M-53	16.30	43.98
M-19	18.55	46.07
M-17	19.15	45.11
Zhaoshu 18(CK)	18.78	43.98

Different cultivars were also tested. Their crude oil content and crude protein content are shown in Table IV.

TABLE IV. OIL AND PROTEIN CONTENT OF DIFFERENT CULTIVARS

Cultivar	Crude oil, %	Crude protein, %
Hanying No.1	20.89	34.96
CG661×91-1	21.16	37.66
Zakang F6×Ludou 4	19.01	42.18
H.P.F6×22 85-095	18.89	41.19
Sidou 11×Jilin 22	20.57	38.68

3.2.2. Results of mutagenic treatment of H.P. F6×2285-095

In this population two lines with higher protein content were identified. The results are shown in Table V; the 2-year mean crude protein content of 348-4 was 47.67%, and of 349-4 - 47.09%.

TABLE V. VARIATION OF CRUDE OIL AND PROTEIN CONTENT IN M_3 (1995) AND M_4 (1996) PROGENIES OF H.P. F6×2285-095

	M_3 (1995)		M_4 (1996)	
Plant No.	Crude oil, %	Crude protein, %	Crude oil, %	Crude protein, %
348-1(CK)	18.89	41.19		
348-3	17.58	45.31	17.84	43.48
348-4	18.22	45.81	16.18	49.54
349-2	17.30	45.68	16.18	48.84
349-3	17.15	47.08	16.52	44.23
349-4	16.30	47.00	17.04	47.18
351-3-1	18.32	44.27	17.20	45.95
351-4	18.41	44.28	17.73	41.61
351-5	16.42	44.55	15.26	46.58
351-6	17.30	44.42	16.35	49.33

3.2.3. Mutagenic treatments of Zakang F6×Ludou 4

TABLE VI. VARIATION OF CRUDE OIL AND PROTEIN CONTENT IN M_3 (1995) AND M_4 (1996) PROGENIES OF ZAKANG F6×LUDOU 4

	M_3 (1995)		M_4 (1996)	
Plant No.	Crude oil, %	Crude protein, %	Crude oil, %	Crude protein, %
423-1(CK)	19.01	42.18		
424-5	18.94	44.29	18.83	40.68
426-1	17.95	44.27	17.61	41.01
426-5	17.60	44.31	17.09	44.07
427-1	18.90	45.13	16.81	44.25
427-2	17.73	47.06	17.96	42.32
427-9	18.33	45.24	18.08	42.82

3.2.4. Mutagenic treatments of Sidou 11×Jilin 22

The finding in M_3 and M_4 are shown in Table VII. The mean 2-year crude protein content of line 388-2 was 46.02%. The crude oil content of 375-3 was 21.00%.

TABLE VII. VARIATION OF CRUDE OIL AND PROTEIN CONTENT IN M_3 (1995) AND M_4 (1996) OF SIDOU 11×JILIN 22

Plant No.	M_3 (1995)		M_4 (1996)	
	Crude oil, %	Crude protein, %	Crude oil, %	Crude protein, %
CK	-	38.68		
375-3	21.00	37.56	16.77	44.28
387-3	19.18	44.14	17.95	45.76
387-9	19.11	44.80	18.48	45.02
388-2	19.06	46.16	17.97	45.88
388-3	18.72	44.40	17.34	45.57
388-4	18.59	44.83	16.20	46.03
389-4	17.24	46.87	18.44	43.90
392-4	20.19	45.23	19.01	43.16

We analysed crude oil and crude protein content of 145 plants of (Sidou 11×Jilin 22) M_3. The crude oil content of the check (Sidou 11×Jilin 22) was 20.06%. We found that 6 lines had crude oil content above 21.5%, and plant 386-2 has crude oil content of 21.93%. Among 145 lines, we have found 19 whose crude protein content exceeded 43%, nine over 44%, and three over 45%. The crude protein content of line 389-4 (M_3) was the highest - 46.87%.

3.2.5. Mutagenic treatments of CG661×91-1

The findings are shown in Table VIII. The crude oil content of the check (CG661×91-1) was 21.16%. In M_3 we found that crude oil content of seven plants exceeded the control: with three plants over 22.00%. The crude oil content of 397-2 is 23.52%. It is a plant with high oil content. Crude protein content of five plants of M_3 (CG661×91-1) was higher than the content of the check. The crude protein content of plant 398-2 was over 40.87%.

TABLE VIII. VARIATION OF CRUDE OIL AND PROTEIN CONTENT IN M_3 OF CG661×91-1, 1995, BEIJING

Plant No.	Crude oil, %	Crude protein, %
397-1 (CK)	21.16	37.88
397-2	23.52	36.15
397-3	21.61	36.95
397-4	21.04	38.72
397-5	22.64	37.82
397-6	22.27	38.01
398-1	21.88	39.03
398-2	20.72	40.87
398-3	21.44	36.29
398-4	21.94	36.39

3.2.6. Mutagenic treatments of Hanying No. 1

Plants exceeding the check in crude oil content were not found, but seven plants exceeded the check in crude protein content (Table IX). This cultivar has low crude protein content.

TABLE IX. VARIATION OF CRUDE OIL AND PROTEIN CONTENT IN M_3 OF HANYING NO. 1, 1995, BEIJING

Plant No.	Crude oil, %	Crude protein, %
321-1(CK)	20.89	34.96
321-2	19.21	36.26
321-3	20.76	33.68
321-4	19.00	36.73
323-1	20.66	34.37
323-2	19.01	35.64
323-3	20.01	36.52
323-4	19.05	38.40
323-5	20.01	34.12
323-6	18.26	37.95
323-7	19.22	35.61

3.2.7. Overview

From 220 plants of different M_3 populations analysed for crude oil and crude protein content 3 plants had oil content above 22% (high oil sample) and 7 plants had crude protein content above 46% (high protein sample). Selection of genotypes to be treated to improve quality is very important. For example, M_3 of CG661×99-1 is very good material to obtain higher oil content because oil content of the cultivar was already high with 21.16%. We got 3 plants with crude oil above 22% by this treatment. M_3 and M_4 of H.P F6×2285-095 are good material in this treated population to obtain higher protein content. In M_3 we found 2 samples with more than 47% protein; in M_4 there were 4 samples. Analysis of quality in early generations is another important factor affecting success of mutation breeding for improved quality.

FIG. 1. Early maturing mutant (left) and Lingzhen No. 1 (check right).

3.3. Mutation of growing period

Mutations can shorten the growth period of soybean. After treatment of Lingzhen No. 1 with ^{60}Co, we got a lot of early maturing mutants. Some mutants matured 15-20 days earlier than the control.

3.4. Resistance to cyst nematode, *Heterodera glycines* race 4

Soybean cyst nematode (SCN) is a very serious pest of soybean in China. It can damage soybean production very severely.

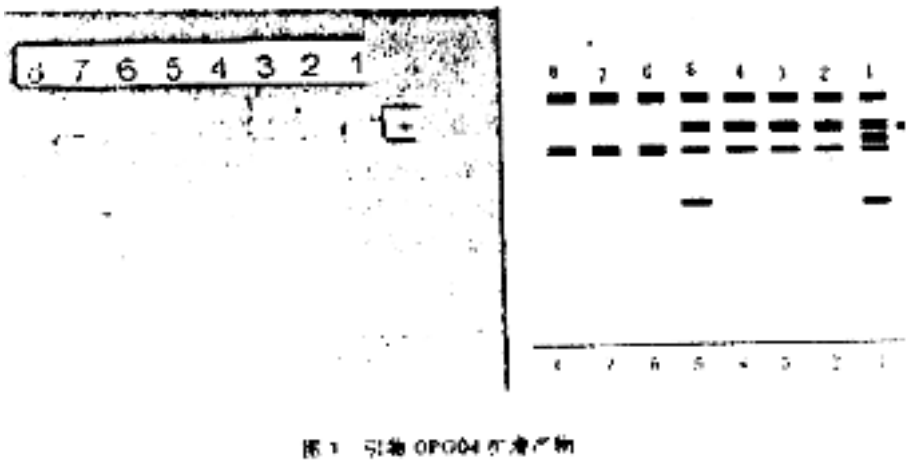

FIG. 2. RAPD products amplified by primer OPG04 of nematode resistant lines (1-5) and susceptible ones (6-8).

Applying the randomly amplified polymorphic DNA (RAPD) technique, we analysed eight soybean cultivars and lines. They included two resistant ones, 1259Y with yellow seed coats and 1259B with bicolor seed coat, and their resistant parent Huipizhiheidou, susceptible parent-Jinyi 9, two other resistant varieties, Yuanboheidou and PI 437654, two other susceptible cultivars, Ludou 1 and Ludou 7. Among 33 primers, one primer, OPG 04 (Operon Company), amplified a DNA fragment which specifically existed only in the products of all five resistant varieties and lines (lanes 1-5), and was not found in those of the three susceptible cultivars, including the susceptible parent, lanes 6-8 (Fig. 2). Lanes 1-8 show (right to left) the RAPD products of PI 437654, Yuanboheidou, Huipizhiheidou, 1259Y (yellow colour seed coat), 1259B (Bicolour seed coat), Jinyi No.9, Ludou No.7 and Ludou No. 1. The fragment specifically amplified in the resistant cultivars or lines is indicated. The nematode resistant lines 1259 Y and 1259 B, show the same fragment as their resistant parent huipizhiheidou, which cannot be found in their susceptible parent Jinyi No. 9.

3.5. Transformation of mutants using *Agrobacterium* mediated gene transfer

During the past 15-20 years, genetic engineering made great progress in important crops, such as rice, cotton, wheat, corn, tomato, soybean and others. Regeneration of soybean is known to be difficult, but it is an essential requirement for the successful transformation. Experiments were conducted to optimize plant regeneration from protoplast, anther and tissue cultures of soybean and to identify genotypes with good regeneration capacity. Some soybean cultivars, e.g. Heinong35 (commercial variety developed through cross with mutant) and Ludou10, proved to be good genotypes for regeneration.

We started the transformation of soybean by using the *Agrobacterium*-cotyledonary node transformation system. Under guidance of Dr. Thomas H. Clemente from Biotechnology Centre at the University of Nebraska Lincoln, NE, USA we tested transformation capability of newly developed soybean mutant lines Zhongzuo 962, Zhongzuo 965, Zhongzuo M17, and officially released varieties Heinong35, Ludou 10, Heinong37 (mutant), Thorne and other genotypes using different strains of *Agrobacterium tumefaciens*. Successful transformation was measured by GUS expression of regenerated plants. After co-cultivation of cotyledonary node explants with *A. tumefaciens* strain EHA101 carrying the binary vector pPTN140 with the GUS gene, GUS expression was found in regenerated plants of various mutants and varieties, such as Heinong 35 (good transformation capacity), Zhongzuo 965, Zhongzuo M – 17, Heinong 37 and Thorne.

4. DISCUSSION AND CONCLUSIONS

— Following mutagenic irradiation treatments variation for growth period, plant height, seed size and resistance to disease was found in M_2 and M_3 generation.
— Variation was found also in crude oil and crude protein content. High crude oil contents were found in selected plants/lines up to 23.5% and protein contents up to 47.7% and high crude protein content up to 47.7%.
— Through use of mutation techniques we can select and obtain stable lines by the M_5-M_6 generations, sometimes by M_4.
— Selected soybean lines, treated with Co^{60} are promising due to their good yield, large seed and resistance to lodging.
— In soybean cross breeding and induced mutations can be combined to develop improved germplasm. In cross breeding, progenies are often obtained with late maturity; they can be mutagenized to create variability from which to select the best lines with best comprehensive characteristics and early maturity.
— The breeding process can be shortened by using off-season propagation and selection in winter time on Hainan Island, southern China, and the greenhouse in northern China.
— It is possible to transfer foreign genes to soybean mutants and to use this techniques to develop mutants resistant to diseases and nematodes.
— It is difficult to breed soybean cultivars with yields higher than four tons per hectare. According to our experience superyielding plant types of soybean should have the following characteristics: (a) 3-10 branches with convergence. (b) Determinate or semi-determinate types, which can use apical dominance under best conditions; such plants have many pods, especially at the branch tops. (c) Plant height 70-90 cm with a strong stem, short internode and without lodging. (d) Much more dry matter with a harvest index over 5.5. (e) Many pods on each internode with large seeds. (f) Many small leaves with low shade density but high photosynthesis. (g) High resistance to diseases and insects.
— Identification of RAPD marker associated with resistance to race 4 of the cyst nematode *H. glycines* is very useful.

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Domestication of *Oenothera* as a new oil plant

Q.X. Liu, M. Wel, L.Q. Zhu, G.M. Me, Y. Vang

Laboratory of Rape Crop Research,
Southwest Agricultural University,
Chongqing Municipality, China

Abstract. Plant oils rich in gamma linolenic acid (GLA) have been widely used as essential ingredients of medicines, healing products, cosmetics etc. Although GLA is widely distributed in many plant families, the *Oenothera* genus has been shown to be the most promising to be used as a new oil plant in the future. In the past, evening primrose was occasionally planted in China as an ornamental, remaining unimproved, without any valuable agronomic characters. In Chongqing municipality in the South of China, the whole growing season of *Oenothera* spans 320-340 days, which is too long. We collected a number of *Oenothera* species accessions from China and abroad and irradiated their seeds with ^{60}Co gamma rays. From the resulting induced mutants, we selected and characterized an early-maturing line termed R5-3 that matured 35 days ahead of the control. GLA and oil content in seeds of R5-3 were also higher than those of the control, and its seed yield was 1800 kg/ha. Meanwhile suitable cultural practices were successfully established. Another new line, H8-1, with high density of capsules was obtained by using interspecific hybridization.

1. INTRODUCTION

The fatty acid composition of the oil determines its physical and chemical properties, physiological activities and utilization prospects. Oils used in industry, medicine and foodstuff have some special requirements. In order to meet the requirements and the needs, researchers have been engaged in the development of novel oil sources with special fatty acid contents [1-6]. Their efforts included research on plants rich in gamma linolenic acid (GLA). Many investigators have studied the structure, pharmacology, toxicity, clinical uses etc. of GLA [7-11] since it was described in 1919. In fact, GLA is considered an essential fatty acid because it was found to be closely related to the normal function of human cells, the transformation and utilization of cholesterol as well as the synthesis of prostaglandins. GLA is a common precursor of dihomo-T-linolenic acid, arachidonic acid and prostaglandins (PG) such as PGE_1 and PGE_2 in humans [8-9]. These catalytic products are important physiologically. PGE_1 was found to have some effects on the regulation of the immune system, anti-inflammation, anti-thrombus, inhibition of cholesterol synthesis, hemangiectasia and reducing blood sugar, reducing blood-fat. Therefore, GLA is used in health agents for hyperlipidemia, hypercholesterolemia, hyperglycemia, coronary disease, enriching the brain, anti-senility, slimming, anti-ulcer as well as menopathy, mastopathy, schizophrenia, depression and arthritis [10-16]. Thus, vegetable oils rich in GLA, have become more and more important raw materials in medicine, health products and cosmetics.

Although GLA was found in various families, e.g. Onaraceae, Boraginaceae, Acerdaceae, Scrophulariacea, Rannuculaceae and Liliaceae, the *Oenothera* genus (Onagraceae) is considered at present to be the best source and can be a promising new oil plant (17-28].

In China, in the past *Oenothera* was planted occasionally in gardens without any improvement in its poor traits, such as its long growing season (320-340 days in Chongqing, South China), highly uneven capsules' maturation, small seeds, low content of oil and GLA in the seed. The purpose of our research is to domesticate *Oenothera* and improve some of its

agronomic and quality traits by using radiation treatments and interspecific hybridization in order to select and obtain new lines adapted to the conditions of the south of China such as Chongqing municipality. The corresponding optimal cultivation practices were also designed and tested.

2. MATERIALS AND METHODS

2.1. Collection and characterization of *Oenothera* germplasm

Twelve species of *Oenothera* from China and abroad were collected and characterized, viz. *O. biennis, O. villosa, O. lamarckiana, O. odorata, O. flova, O. biennis* var. *grandiflora, O. muricoys, O. glazioviana, O. erythrosepala, O. ammphila, O. parriflora,* and *O. speciosa.* The materials were planted in the autumn of 1994 at the experimental farm of the Southwest Agricultural University and their agronomic traits were recorded and analyzed. In the following year, we collected the seeds and characterized their principal quality traits, i.e. the oil content (using ether extracts) and the fatty acid composition (using gas chromatography).

2.2. Pollen viability studies

In order to facilitate the correct timing of the flowering of the male and female parents, the viability of evening primrose pollen grains under natural conditions was studied. The pollen grains of *O. biennis* were used to test the viability by the peroxidase method. Pollen grains were collected from the newly opened flowers (6:30 p.m.) and stored in glass germination dishes. The viability was tested daily.

2.3. Radiation treatment

Seeds of *O. biennis, O. glazioviana* and *0. erythrosepala* were irradiated in 1994 with ^{60}Co-gamma rays. The doses used were 0, 87, 130, 174, 261 and 435 Gy; the dose rate was 1.74 Gy/min.

Moreover, pollen and seedlings of these species were also irradiated. The pollen treatment doses were 1.74, 3.48, 5.22 and 6.96 Gy with a dose rate of 0.29 Gy/min. Seedling treatment doses were 8.7, 17.4 and 26.1 Gy with a dose rate 0.435 Gy/min.

2.4. Selection and characterization of M_1 and M_3

The irradiated seeds (M_1) were sown in the experimental farm of Southwest Agricultural University (Chongqing) on September 5, 1994. The M_1 populations of each treatment by species were about 500 plants. The size of the corresponding M_2 populations was about 3000 plants. Their agronomic traits were characterized and certain plants were selected. The quality traits were further characterized, those with desired agronomic traits were selected, and the M_3 seeds were harvested. The experimental protocols were the same as previously described in the characterization of *Oenothera* germplasm.

2.5. Cultivation practices in the trials

The two-year trial of *Oenothera* cultivation was conducted in the experimental farm of the Southwest Agricultural University, under optimal conditions of sowing date, planting density, rate of fertilizer application and care.

3. RESULTS AND DISCUSSION

3.1. Characterization of accessions of *Oenothera*

Of the *Oenothera* materials sown in the autumn in Chongqing, accessions from North America and Europe bloomed late and had a long growing period (up to 340 days), thus they were not suitable for release in southern areas of China. The reason is that the *Oenothera* materials from those high-latitude sources are typical long-day plants, not flowering and bearing in short-day environments. This is so not only in Chongqing, but also in Jilin province in northern China where Wu et al. [29] found that Gong-Xuan No. l, a mid-season variety of *Oenothera,* cannot flower normally even with 15 h sunshine. Combining and comparing with other experimental results, the critical day length of Gong-Xuan No. l may be between 15-16.5 h of daylight [29]. Thus, typical long-day plants, such as *Oenothera,* cannot flower and have long growth period duration in Chongqing in South of China.

The oil contents in the seeds and the main fatty acids' composition of several species of *Oenothera* are shown in Table I.

TABLE I. THE SEED OIL CONTENTS AND THE MAIN FATTY ACIDS CONTENT OF SEVERAL SPECIES OF *Oenothera*

Species	Oil contents %	Fatty acid, %		
		Oleic acid	Linolenic acid	GLA
0. biennis	28.20	8.90	75.50	9.50
0. glazioviana	21.10	7.50	69.51	8.50
0. biennis var. *grandiflora*	20.10	7.10	77.21	7.45
0. acaulis	20.50	14.10	68.23	8.25
0. parriflora	28.01	6.15	73.40	8.60
0. erythrosepala	20.53	5.35	74.10	9.50

3.2. Pollen viability

The results (Table II) indicated that within one day after blooming the pollen grains had the highest viability (97.2% viable). After two days there was a slight drop in the viability of

TABLE II. VIABILITY OF STORED POLLEN GRAINS OF *O. biennis*

Days after anthesis	Pollen grains tested, No.	Viable pollen grains	
		No.	%
1	254	247	97.2
2	279	249	89.2
3	261	112	42.9
4	328	127	38.7
5	214	70	32.7
6	181	37	20.4
7	251	50	19.9
8	332	72	21.6
9	225	32	14.2
10	281	10	3.5

the pollen grains (<90%). The viability dropped markedly, to 42.9%, after three days of storage. From the 4th day of storage the viability dropped rapidly (Table II). These results suggest that it is suitable to pollinate with pollen grains that have been stored for one or two days. A special storage method must be devised to preserve the pollen grains for longer periods.

3.3. Selection and breeding of early-maturing lines of *Oenothera*

The room germination tests showed that the germination rate of *Oenothera* seeds was enhanced when treated with ^{60}Co-gamma rays, with doses of 87 or 174 Gy (Table III). When treated with 261 or 435 Gy, the germination rate of the seeds dropped to that of the control.

TABLE III. EFFECT OF ^{60}CO GAMMA-RAY DOSES ON SEED GERMINATION RATE

Dose (Gy)	0	87	174	261	435
Germination rate (%)	60.5	72.0	75.5	65.0	64.5

The elongation of the hypocotyls was seriously inhibited. In field sowing seeds treated with 435 Gy did not germinate at all. Plants from the 87 to 261 Gy seed treatments flowered and produced seeds normally.

Two early-maturing mutants, R5-3 and R5-4, were selected and characterized in 1997 in the M_2 generation of *O. erythrosepala,* whose seeds were treated with 261 Gy ^{60}Co-gamma rays in 1995. They began blooming respectively, 20 and 15 days ahead of the control; they matured respectively, 35 and 30 days ahead of the control. The early-maturing trait, in the M_3 generation of both mutants, was stable in 1998. The oil content and GLA concentration of mutant R5-3 were nearly the same as those of the control (Table IV).

TABLE IV. OIL CONTENTS AND FATTY ACID COMPOSITION OF SEEDS OF THE EARLY-MATURING MUTANT LINES OF *O. erythrosepala*

Lines	Oil contents	Fatty acid, %				
	%	16:0	18:0	18:1	18:2	18:3(GLA)
Control	21.0	8.1	3.1	5.3	74.0	9.5
R5-3	22.0	7.8	3.2	4.8	74.1	10.1
R5-4	20.5	7.7	4.0	6.3	73.5	8.5

In the 1999 yield comparison test, the yield of the early maturing mutants, R5-3 and R5-4 were respectively, 2.7 kg /15 m^2 (~1,800 kg/ha) and 2.4 kg /15 m^2 (~1,600 kg/ha), in contrast to the control 2.6 kg/15 m^2 (~1,733 kg/ha). The yield of mutant R5-3 exceeded that of the control.

The R5-3 line is very promising for release and dissemination because it matures 35 days ahead of the control.

3.4. Selection for a higher number of capsules

Line H8-1 with a higher number of capsules was selected from the offspring of the interspecific hybrids, *O. erythrosepala* x *O. acaulis.* In a 10 cm segment of average main rachis the mutant set 25-30 capsules in contrast to 14-17 capsules in the control.

3.5. Cultivation practices suitable for early maturing line of *Oenothera*

The cultivation practices for the early maturing line have been established in the experimental farm of Southwest Agricultural University in the last two years. The optimal seeding time in the climatic conditions of Chongqing municipality in South of China is early September (1-10). With moderate plant density fertilizer needs are N 35-40 kg/ha, P_2O_5 10-15 kg/ha and K_2O 25-35 kg/ha where the soil is of medium fertility.

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Breeding and genetic engineering of *Cuphea* and meadowfoam

S.J. Knapp

Centre for Gene Research and Biotechnology,
Department of Crop and Soil Sciences,
Oregon State University,
Corvallis, Oregon, United States of America

1. THE *CUPHEA* PROJECT

Saturated short- and medium-chain fatty acids (SCFAs and MCFAs, respectively) are produced in high concentrations in the seed oils of *Cuphea*. The goal of our laboratory has been to develop commercial sources of *Cuphea* oil by domesticating *C. viscosissima* and developing elite cultivars for machine planting and harvesting. *Cuphea* is a widely adapted, temperate, summer annual and can be grown throughout much of the USA. The first experimental cultivars of *Cuphea* were developed for field testing and commercial production by combining partial non-shattering with non-dormancy and non-sticky hairs in self-pollinated genetic backgrounds. These cultivars can be machine planted and harvested and are being increased for field tests. The field performance of non-sticky lines has not been assessed yet. Seed shattering has been and still is the primary constraint to commercial production. Our present research is focused on developing completely non-shattering lines and enhancing the performance of elite lines and experimental cultivars.

Chemically induced mutations have played an important role in the domestication of *Cuphea*. The non-sticky phenotype and several fatty acid profile changes were produced by induced mutations. We have initiated a new project to induce mutations affecting the development of the dehiscence zone in *Cuphea* capsules. If such mutations are produced and isolated, then fully non-shattering lines can be developed.

C. viscosissima is one of several *Cuphea* species with SCFA and MCFA concentrations greater than 900 g/kg. Wildtype *C. viscosissima* lines produce 1 g/kg caproic acid (6:0), 170 g/kg caprylic acid (8:0), 700 g/kg capric acid (10:0), 30 g/kg lauric acid (12:0), and 10 g/kg myristic acid (14:0). We have developed several *C. viscosissima* lines with novel fatty acid profiles: high capric acid lines producing 850 g/kg 10:0; high caproic acid lines producing 80 g/kg 6:0, 380 g/kg 8:0, and 300 g/kg 10:0; and high myristic acid lines producing 250 g/kg 10:0, 385 g/kg 14:0, and 140 g/kg 16:0. Caproic acid is rare in nature and is found only in low concentrations in a few seed oils. High caproic acid *Cuphea* oil is the only known source of 6:0 from a crop plant. *Cuphea* was primarily developed to supply oils to the surfactant industry. Edible, cosmetic, and medical markets and additional industrial markets have not been developed for *Cuphea* oil thus far.

2. THE MEADOWFOAM PROJECT

Meadowfoam (*Limnanthes alba* Benth.) oil has unique physical and chemical characteristics. Three novel unsaturated long-chain fatty acids (20:1 D5, 22:1 D5, and 22:2 D5, D13) and erucic acid (22:1 D13) are found in meadowfoam oil in high concentrations. The oil and oil derivatives are presently sold to industrial markets [1]. The goal of our research is to increase the productivity of meadowfoam by developing superior cultivars, discovering and developing novel phenotypes, and advancing our understanding of the

genetics of economically important traits. Our breeding work has concentrated on increasing seed yield, seed oil concentration, lodging resistance, *Scaptomyza* resistance, developing self-pollinated lines and cultivars, and developing novel oils. A fuller discussion of our breeding and cultivar development research activities is presented in a recent review [2]. Our research on the development of low erucic acid meadowfoam oil is described in this report.

Because erucic acid has been shown to pose health risks, we initiated a project to develop low erucic acid meadowfoam lines. We developed low erucic acid (22:1 D13) lines by exposing seeds of the cultivar 'Mermaid' to methanesulfonic acid ethyl ester (EMS) and selecting for reduced erucic acid concentration among manually self-pollinated M_1, M_2, and M_3 progeny. One M_1 (LE78) produced less erucic acid than the wildtype. The erucic acid concentration was 3.1% for the selected M_2 line (LE78-1) and 2.4% for the selected M_3 line (LE78-1-3) versus 9.4% for the untreated control (Mermaid). LE78-1-3 produced less 20:1 D5 (51.4 vs. 65.5%) and more 22:2 D5, D13 (37.8 vs. 17.3%) than the wildtype. The M_3 seeds produced 1.3 to 3.7% erucic acid. The narrow phenotypic ranges for erucic acid and other fatty acids among the M_3 progeny showed that the induced mutation(s) are probably fixed. We have begun introgressing the low erucic phenotype from LE78-1-3 to elite open-pollinated populations (OMF78 and OMF86) with different genetic backgrounds and investigating the genetic basis for the low erucic phenotype. The erucic acid distribution among LE78-1-3 x OMF86 F2 progeny was continuous and normal. The low erucic mutation(s), in the OMF86 background, produced more 20:1 D5 (70.7 vs. 65.5%) than the wildtype, but had no effect on 22:2 D5, D13 concentration; thus, the phenotypic effect of the low erucic mutation(s) seems to vary across genetic backgrounds. Progeny from other crosses have not yet been assayed. LE78-1-3 and other low erucic acid lines open the way to the development of pharmaceutical, nutraceutical, medical, and food markets for meadowfoam oil.

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A procedure to minimize the linkage drag in backcross breeding of rapeseed (*Brassica napus*) through irradiation

Y. Zhou, S. Shi, Z. Wei, J. Wu, Q. Nie, G. Li

Institute of Crop Genetics and Breeding,
Huazhong Agricultural University,
Wuhan, China

Abstract. Backcross is an efficient breeding procedure in transferring traits governed by one or a few major genes. However, undesirable genomic segments linked to the target genes (so-called linkage drag) often prevent backcross projects' products from reaching practical utilization. This study aimed to explore approaches to minimize linkage drag in backcross breeding in rapeseed (*Brassica napus)*. Plants of BC_6F_1, which were derived by crossing two rapeseed varieties, a double low x a double high (erucic acid, glucosinolate), were exposed to various doses of gamma rays (0, 15, 25, 35, 40, 50, 100, 150, 200, 250, 300, 350 and 400 Gy) in order to irradiate the young buds (2-3 mm in length). The effects of irradiation on the growth and development of the floral organs were investigated, and microspores were isolated and cultured *in vitro* to produce doubled haploids. Buds were seriously damaged and finally died on plants treated with 100 Gy and higher doses while those on plants treated with 50 Gy and lower doses survived at reduced flower size and delayed growth and development. Pollen viability from plants irradiated by 50 Gy and higher doses decreased (as assessed by staining), but the percentage from plants irradiated with 40 Gy and lower doses had a slight increase. Siliques developed on plants treated with 50 Gy and lower doses, were only 58-71% as long as control and seed number per silique dropped to 56-23% of the non-irradiated control. Severe chromosome damage and distortion were observed at various developmental stages in pollen mother cells from plants irradiated with 350 or 400 Gy gamma rays. Microspore-derived embryos were obtained from 40 Gy and lower doses treated plants but no embryo was obtained from plants irradiated with 50 Gy and higher doses. Leaf color and fertility mutants were identified among the progeny population derived from 50 Gy plants while the majority of them grew and developed normally. A few normal double low plants were identified in the populations. Two lines had a similar morphological performance as the recurrent parent but the yield differed significantly. One of them had the same performances in both seed yield and *Sclerotinia sclerotiorum* infection as the control. Thirteen out of 311 doubled haploid (DH) plants were recovered with double low quality and RFLP analysis with these lines is under way.

1. INTRODUCTION

Rapeseed (*Brassica napus*) is the most important oil crop in China. The priority of rapeseed breeding in China is to develop new cultivars with low glucosinolate and low erucic acid contents (double low) in order to improve the edible oil quality and increase animal feed supply [1]. Numbers of double low cultivars have been developed since the late 1980s but the yield potential and disease resistance of those still need to be improved further as compared to conventional double high varieties [1]. The strategies for breeders to improve seed yield of double low cultivars in China generally fall into two categories. One is to develop hybrids with improved qualities, in which heterosis in seed yield can compensate for the penalty from quality improvements. Another is to explore novel breeding procedures to obtain cultivars with competitive yield capacity and disease resistance. The latter is as important as the former approach because of its advantages over hybrids with regard to the cost of seed production and disease resistance. Based on this consideration a backcross breeding programme to transfer the double low traits to Zhongyou 821, a conventional variety widely cultivated in the Yangtze River Valley - the main rapeseed production area in China - was initiated in Huazhong Agricultural University (HAU).

It is well known that repeated backcrossing allows segregation to remove donor parent chromosomes unlinked to target genes, and recombination to remove linked donor parent segments. Theoretical and practical experiments show that it is more difficult to remove the segments linked to target genes than unlinked ones. Theoretically, even after 20 backcross generations, a linked donor segment of 10 cM flanking a target gene is expected to persist [2]. In many cases such a "linkage drag" could preclude the backcross product from reaching practical application and release. In our backcross program mentioned above, for example, seed yield of a double low rapeseed line in BC_5, was still 5-8% lower than its recurrent parent, Zhongyou 821.

Two approaches can be used to increase the probability of obtaining backcross products with minimum linkage drag in a given backcross breeding period. One is to apply some external induction factors, such as ionizing radiations, in order to enhance the chances of chromosome/chromatid breaks and recombination between chromosome segments linked to target genes from donor parent. Another is to identify efficiently recombinants with minimum linkage drag at the DNA level by using molecular markers, such as restriction fragment length polymorphisms (RFLPs) [3]. This project explored the application of the two measures in our backcross breeding for development of double low rapeseed; the results gained so far are presented here.

2. MATERIALS AND METHODS

2.1. Plant materials

BC_6F_1 plants of *Brassica napus* derived from a backcrossing program in HAU were used for irradiation. In this programme, the double low line #707 was crossed to Zhongyou 821, a double high winter variety with superior yield and resistance performance in the Yangtze River Valley. F_2 progenies from the cross were selected for double low quality and backcrossed to Zhongyou 821. The process was repeated until the BC_6F_1 seeds were obtained in 1996.

2.2. Irradiation treatments

Plants were cultured in 22 cm diameter pots. When most of plants started blooming, buds on the plants that were most likely at meiosis (less than 2mm in length, based on previous cytological observation) were exposed to a ^{60}Co source for gamma rays irradiation. Doses used in the first project year (1996-97) were 0 (control), 50, 100, 150, 200, 250, 300, 350, and 400 Gy (at the rate of 2.5 Gy/min). The treatments were decreased to 0 (control), 150, 250, 350 and 400 Gy (at the rate of 1.0 Gy/min) in the second project year (1997-98) based on the first year data. Ten plants were irradiated for each dose, and 4-6 inflorescences on each plant were kept after irradiation.

2.3. Pollen vitality and cytological observation

Buds were collected immediately after irradiation and fixed over night in a solution of acetic acid and 95% alcohol (3/1,v/v). The fixed buds were transferred to 70% alcohol and stored in a refrigerator. Observation of meiosis in pollen mother cells was done following routine smear technique.

Buds ready to open were taken from all plants and pollen grains were stained with acetic carmine and observed in a microscope. At least 500 pollen grains per treatment were counted to calculate the percentages of staining.

2.4. Microspore culture

Buds were collected from irradiated plants as soon as they were ready for microspore isolation, i.e. when buds were 3-4 mm in length and most of the microspores were at late uninucleate to early binucleate stage based on cytological check. Isolated microspores were cultured by using the procedure established in our laboratory [4, 5]. Plants obtained from microspore culture of irradiated buds were multiplied for quality analysis and preliminary agronomic observation. Double low DH lines were selected for RFLP analysis.

2.5. Field evaluation of plants derived from radiation

Seeds produced by 50 Gy treated plants were planted in the fall of 1997. Ten progeny populations, each consisting of about 350 plants, were sown. Seeds from untreated plants were sown as a control. Morphological observations were made during the course of plant growth and development. The harvested seeds were screened for double low individuals using established protocols [6,7]. Two double low lines selected from the irradiated progenies were submitted to a yield trial in the 1998-99 season. The trial was conducted in a complete randomized block design with 4 replications. Zhongyou 821, the recurrent parent used for backcross, and a double low line derived from backcrossing (BC_5) with Zhongoyu 821 were used as controls. Morphological characters and plant growth and development stages were observed throughout the growing season. The rate of *Sclerotinia sclerotiorum* infection, the most serious disease of winter rapeseed in this area, was investigated before the harvest and seed yield was assayed.

3. RESULTS AND DISCUSSION

3.1. Effects of irradiation on plant growth and development

3.1.1. Effects of high doses (50 Gy or higher)

During the first three days after radiation, all of the irradiated plants did not differ in appearance from the non-irradiated plants (control). However, green buds on the irradiated plants became yellow 4 days after radiation. Some of the buds changed color earlier than others among individuals irradiated with the same dose, and the change mostly started from the apical inflorescence and the larger buds. The color change of the buds occurred almost on all treated plants, and the amount of yellowed buds and their severity increased on each individual with higher irradiation doses.

The plants with yellowed buds showed changes in two directions 6 days after radiation: some of the buds on upper inflorescences continued yellowing and gradually became withered while others recovered their green color. At this time buds on secondary inflorescences grew larger with normal green color. It was quite common that green buds and yellowed ones existed on the same inflorescence.

Secondary inflorescences extended on 50 Gy treated plants and normally appearing buds on the inflorescences grew larger at two weeks after radiation. A few new buds from secondary inflorescences on plants treated by higher doses were also observed, but many buds

developed abnormally, either becoming yellowish or cracked before blooming. The proportions of buds that developed to flowers on the treated plants varied, but the size of the flowers was reduced sharply with the higher irradiation doses.

3.1.2. Effects of low doses (40 Gy or lower)

As with 50 Gy-irradiated plants and higher doses, plants irradiated with lower doses also showed obvious retardation in their growth and development. Irradiated buds turned yellow about five days after irradiation. With the higher irradiation doses the growth of inflorescences and flowers became slower than in the control and the petal sizes were smaller (Table I). A few flowers on 25 Gy and higher doses-treated plants died gradually, 1-3 weeks after irradiation while the majority of flowers developed further to bloom. Surviving buds were morphologically normal but smaller.

TABLE I. MEAN AND STANDARD ERROR (SE) OF PETAL WIDTH (MM) OF IRRADIATED PLANTS

Dose, Gy	0	15	25	35	40
Petal size	10.3±0.04	8.7±0.078	7.9±0.11	7.1±0.095	5.7±0.12

3.2. Effects of irradiation on pollen viability

Pollen viability, expressed as the percentage of acetic carmine stained pollen grains, decreased with the rising irradiation doses from 50 to 400 Gy, according to observations on mature buds taken from plants during 9-15 days after irradiation (Table II). However, the acetic carmine staining percentage from plants irradiated with 40 Gy and lower doses was even higher (96.12%-96.84%) than control (87.83%).

TABLE II. PERCENTAGE OF STAINED POLLEN GRAINS FROM IRRADIATED PLANTS

Treatment, Gy	0	50	100	150	200	250	300	350	400
Stained pollen %	91.8	87.0	84.5	81.6	75.5	71.2	68.3	62.75	49.0

3.3. Effects of irradiation on silique development

The vegetative growth of plants treated at 50 Gy or lower doses, as well as new buds derived from secondary inflorescences returned to normal 3 weeks after irradiation and the buds flowered normally. The ovaries on those plants finally developed to siliques. In contrast, very few normal buds survived on the plants treated with higher doses and the flowering dates were delayed. A few ovaries on 100-200 Gy treated plants grew into very short siliques but no seeds were formed at all. Buds on plants treated with 250 Gy and higher doses wilted gradually and finally died.

Leaves and stems of all irradiated plants grew normally during the first 40 days after irradiation. After that time, leaves on plants treated with 100 Gy and higher doses became red and wilted, and finally shed. Plants irradiated with lower doses (50 Gy and lower) continued growing although their development lagged behind the control to a certain extent.

Irradiation obviously affected silique growth and seed development on plants treated with 50 Gy and lower doses. The plants had shorter siliques (Table III) and fewer

seeds/silique than the control (Table III). The seed number was affected more markedly than silique length, e.g. silique length on 50 Gy treated plants was reduced to 57.7% of the control whereas seed number was reduced to 23% (Table III).

TABLE III. EFFECTS OF LOW IRRADIATION DOSES ON MEAN SILIQUE LENGTH AND SEED NUMBER (% OF CONTROL)

Trait	Irradiation dose, Gy					
	0	15	25	35	40	50
Silique length	100	69.2	71.0	65.5	66.2	57.7
Seeds/silique	100	56.4	45.9	32.4	27.9	23.0

3.4. Effects of irradiation on meiosis in pollen mother cells

An evident cytological abnormality in meiosis of pollen mother cells (PMC) on treated plants was that chromosome structure was distorted and completely destroyed. Severely damaged chromosome structures were observed at various developmental stages in PMCs from the plants treated with 350 or 400 Gy gamma rays.

3.5. Culture of microspores from irradiated plants

Microspores were isolated from all plants treated. The culture was repeated 3-6 times for each treatment. Unfortunately, no embryos were obtained from plants irradiated with 50 Gy and higher doses. After lowering the doses microspore-derived embryos and plants were obtained. The embryo yield declined as the irradiation dose increased (Table IV). For example, embryo yield reduced to 40.3% of the control when the dose was increased to 40 Gy. A similar tendency was also observed for plant regeneration.

There was no visible difference in embryo morphology between those that developed on control plants and those irradiated at 15 Gy and 25 Gy, respectively. But some abnormal development was observed in embryos produced from higher doses, like root extension at 2-3 week embryo stage, and irregular cotyledons. Those types of embryos were difficult to regenerate. In this study also the control had a low embryo yield (0.67-0.76 embryo/bud, Table IV), which was consistent with our previous results that Zhongyou 821 was very poor in initiating embryogenesis in microspore cultures of *B. napus* [8,9].

TABLE IV. EFFECTS OF IRRADIATION ON EMBRYOGENESIS IN MICROSPORE CULTURES OF *Brassica napus*

	Spring 1998					Spring 1997								
Dose, Gy	0	15	25	35	40	0	50	100	150	200	250	300	350	400
No. buds sampled	185	300	310	195	160	335	650	275	229	301	225	319	301	256
No. embryos obtained	124	172	152	92	43	254	0	0	0	0	0	0	0	0
No. embryos/bud	0.67	0.57	0.49	0.47	0.27	0.76								

3.6. Field evaluation of plants derived from 50 Gy radiation

The majority of the grown plants did not show obvious morphological variation from the control (recurrent parent). However, some abnormal plants and mutants were observed in

treated populations, such as leaf color and male sterile mutants. In addition, some plants showed unusual growing vigor during vegetative growing and flowering. For example there was one plant with many more branches than the control and other plants within the treated population and the control. Mutations for male sterility seemed particularly frequent.

3.7. Evaluation of double low lines selected from 50 Gy irradiated population

Two double low lines were obtained from the 50 Gy irradiated plant populations. Their morphological characteristics as well as growth and developmental behaviour resembled their recurrent parent, Zhongyou 821. However, the seed yield of the two lines was very different as was their disease resistance (Table V). Line 2 had the same performance as the recurrent parent, Zhongyou 821, in both seed yield and disease resistance (Table V). The line is now under seed multiplication in order to conduct further multiple location yield trials.

TABLE V. YIELD AND DISEASE RESISTANCE OF TWO DOUBLE LOW LINES[a]

	Yield, kg/ha		Index of *S. sclerotiorum* infection	
Zhongyou 821	2145	(100)	9.31	(100)
Non-irradiated CK	1927.5	(89.9)	8.56	(91.9)
Line 1	1732.5	(80.8)	13.36	(143.5)
Line 2	2122.5	(99.0)	8.41	(90.3)

[a] Numbers in brackets are in percent of Zhongyou 821.

3.8. Screening of double low DH lines for RFLP analysis

Thirteen out of 311 doubled haploid (DH) plants were identified with low erucic acid content (<2%) and low glucosinolate content (<25 μmol/g seeds). Those plants are under observation in nursery plots in order to provide tissues for DNA preparation and to multiply seeds for seed yield trial. The RFLP analysis with these lines will be carried out subsequently. In addition, a number of DH plants with low erucic acid or glucosinolate content were also identified, which could be used as breeding germplasm after field tests and laboratory analyses.

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Improvement of rapeseed and mustard by induced mutations and in vitro techniques

A.S. Syed, I. Ali, S.J.A. Shah, K. Rahman, M. Ahmad

Nuclear Institute for Food and Agriculture (NIFA),
Tarnab, Peshawar, Pakistan

Abstract. Research on the improvement of rapeseed (*Brassica napus* L.) and mustard (*Brassica juncea* Czern & Coss.) was initiated during 1995-96 with the objective to develop genotypes with high yield potential, high oil content, canola quality and improved polyenoic fatty acid composition or high erucic acid for industrial applications, and tolerance to different stresses. The results of the M_1 and M_2 generations have been reported in the second RCM at Giessen, Germany. Stable mutants (M_5) of a photoperiod sensitivity exotic *juncea* genotype (DLJ-3) were assessed in preliminary yield trials (PYT) in the field for yield and other agronomic characteristics in comparison with the parent (P) variety. The mutants performing better in PYTs were further assessed in advanced yield trials (AYT) in comparison with P and a local commercial cultivar (LC). Fatty acid composition of promising mutants and interspecific hybrid lines was analysed by gas chromatography (GC) and total seed glucosinolates by spectrophotometry. The quality analyses showed that 50 mutants had low erucic acid contents and 10 of them showed 0%. Two mutants were isolated for high oleic acid (~50%), 16 mutant plants had more than 35% linoleic acid and one mutant had the desirable low linolenic acid content, 3%. No mutant with more than 60% erucic acid for industrial uses was found. The yield tests indicated that significant variability existed among the mutant lines with regards to different traits. 58 mutants flowered significantly earlier, reducing their growing period by 17 to 55% as compared to P (control), 43 were significantly shorter than P (7-30% reduction in height), and 32 mutants outyielded the parent line significantly by a margin of 26-71% during two years of field testing. Some of the mutants exhibited better performance than P and LC under local short photoperiod environment, indicating adaptability and great potential to replace the indigenous non-canola genotypes. Induced mutations appear to have been successful in breaking the photoperiod sensitivity in exotic *juncea* genotypes. However, some of the promising mutants will be further assessed in field trials at NIFA and other locations for ascertaining their adaptability and genetic stability under diversified climates of Pakistan. It is hoped that some of the early maturing and high yielding canola quality mustard mutants may lead directly to the development of improved canola quality cultivars of mustard in near future.

1. INTRODUCTION

Pakistan continues to meet the domestic edible oil requirements through huge imports at the cost of scarce foreign exchange. More than 33.8 billion rupees (US$ ~ 620 million) were spent on the import of 1.19 million tons of vegetable oil for edible and industrial uses during 1997-98 [1]. Out of the total import, about 5,320 tons were for industrial uses, costing 220.4 million rupees (US$ ~ 4 million) to the national exchequer. The huge oil import bill can only be reduced by increasing the domestic oilseed production both for food and non-food uses. Brassica oilseeds were grown on 339,500 ha with an average yield of 859 kg/ha during 1997-98 [2]. Brassica oilseeds contribute about 30% to the domestic oilseed production. Although rapeseed and mustard have been grown traditionally in Pakistan for centuries their average yield is still very low compared to other countries.

High erucic acid rapeseed has significant potential uses in local industries such as in detergents, soap, lubricants, plastics, nylon, carriers for printing ink, surfactants, and bio-diesel. Such products are environmentally friendly, biodegradable and readily renewable. Brassica oil with high oleic acid and low linolenic acid content will enhance the shelf life and

stability of the oils and their products and will be suitable for use in deep frying, in dairy products and as salad oil.

The number of modifications in oil quality of oilseed Brassica is increasing rapidly, as the technology for producing the necessary variation and the ability to select more efficiently for the desired genotypes are being refined. Induced mutation is one of such techniques to create useful genetic variations in characters of great economic importance.

Keeping these facts in view and the importance of rapeseed and mustard in our national economy, research on the improvement of these crops for industrial applications, both for edible as cooking oil and solid fat (shortening) and non-food uses, was initiated at NIFA.

This paper reports results of promising mustard canola mutants assessed in different yield trials during 1997-99 and of the quality analyses and *in vitro* experiments carried out during the project period.

2. MATERIALS AND METHODS

2.1. Field evaluation experiments

These studies included stable M_5/M_4 mutants of mustard (*B. juncea*), an exotic juncea canola quality line DLJ-3 (received from Canadian scientists) and J0 obtained from Brassica research centre, Norwich, UK, local commercial cultivars, resynthesized lines provided by Prof. W. Friedt, Giessen University, Germany and local rapeseed mutant lines developed at NIFA. The stable 72 M_5/M_4 mutants of mustard were evaluated in different replicated preliminary yield trials (PYTs) at NIFA during 1997-98. Each PYT was laid out according to a randomized complete block (RCB) design with four replications. Each replication was laid out as a square to overcome soil heterogeneity. Each entry was planted on an area of 4.8 m^2, comprising four rows, 30 cm apart and 4m long. Data on flowering were recorded when 50% of the plants in a plot flowered. Plant height was recorded from ground surface to terminal inflorescence, at the time of maturity. The two central rows were harvested at maturity to determine yield per plot, which was converted to yield per ha. The data was statistically analyzed using MSTAT-C software.

Forty-six promising mutant lines showing better field performance under local conditions in different PYTs were further assessed in five advanced yield trials (AYTs) at NIFA during 1998-99. The same experimental procedure was followed for conducting AYTs, except that each entry was planted on 9 m^2 comprising six rows 5 m long and 30 cm apart. The AYTs data were analyzed using the SAS statistical software.

2.2. *In vitro* experiments

2.2.1. Regeneration of interspecific hybrids

The interspecific recombinants were developed from crosses of a Canadian double low line of *Brassica juncea* (J0-7) with a very early but non-canola type local cultivar "Chaliar" of *Brassica campestris* and stable mutants of *Brassica napus* (RM-182 & RM-159-2) respectively. Reciprocal crossing was achieved by removing the stamens from maternal parents' buds with fine sterile forceps and dusting fresh pollen collected from the paternal flowers on the stigmas. Developing siliques from the terminal/auxiliary raceme of the maternal parents were collected within 15 days of crossing and were surface sterilized by 10% sodium hypochlorite solution and Tween bleach for 10 min on a slow shaker. The siliques

were then rinsed by sterile distilled water three times. 2-4 siliques of the same hybrid combination were immersed in 100% ethanol and placed on Nitsch's medium with 30% sucrose in an upright position in deep petri dishes (50 ml) and kept in light at 25^{o}C for 10 days in a growth room. After 7 to 10 days the siliques were dissected under a microscope in sterile manner in a flow bench and young developing embryos were transferred to Gamborg's B_5 medium. These young embryos were incubated in a growth room at 25^{o}C for plantlet development. After about 6 weeks young plantlets were transferred to fresh Gamborg's B5+1% sucrose medium. Rooted plantlets were transferred to sterile "Jiffy 7's" in magentas and after about 2 weeks the young plants were transplanted to sterile soil in pots. The young hybrids were kept in the propagator for at least 20 days in a net house.

2.2.2. Isolated microspore culture

Two heterotic F_1 plants of inter-mutant crosses, Abasin-95 x RM-182 and Abasin-95 x RM-159-2, were used for the microspore culture. Buds approximately 2-4 mm in size were collected from the terminal and the first two axillary racemes of each F_1 plant were surface sterilized by immersion in 10% bleach for 10 min. The buds were then rinsed 3 times in sterile distilled water. The microspores were isolated by macerating the buds with a glass rod in a few drops of Gamborg's B5-13 medium, filtered through a nylon membrane and the filtrate made up to 10 ml with B5-13. The filtrate was centrifuged at 900g for 3 min and the supernatant was discarded. Fresh B5-13 was added to re-suspend the microspores and centrifuged as before, and was repeated once more. The supernatant was discarded and the pellet resuspended in Nitsch and Nitsch NLN-17 medium, and placed in the dark for 24 hr at 32^{o}C. At the end of the incubation period the microspore suspension was centrifuged, the supernatant discarded and the pellet resuspended in fresh NLN-17 medium. The microspore suspension was diluted to 6 buds per 5 ml of medium, which is a concentration of approximately 2 x 10^4 microspores/ml. The suspension was aliquoted into small petri dishes, sealed with micropore tape and kept at 32^{0}C in the dark for 48 hr. When the embryos had grown large enough to be seen by the naked eye, they were placed on a slow shaker at 25^{0}C in the dark for 4-6 weeks or till the embryos were large enough to be placed on a solid medium.

2.2.3. Regeneration of embryos

The developing embryos of 3-4 mm in size were transferred from their liquid medium to a solid B5 medium and kept at 20^{o}C under a 16 hr photoperiod. When the embryos turned green they were transferred to fresh B5 medium and repeatedly sub-cultured until they regenerated into single apex plantlets with roots. If no shoots were present the embryos were placed on Murashige Shoot Induction Medium to encourage shoot production. Rooted plantlets were transferred to sterile "Jiffy 7's" in magentas until large enough to be transferred to the net house to grow in pots. A propagator was necessary for the first 2 weeks in the net house.

2.3. Oil quality analysis

Brassica genotypes were screened through gas chromatography for desirable modified fatty acid (FA) composition. The FA composition in extracted and methylated lipids from composite seeds was determined using ATI UNICAME model 610 gas chromatograph equipped with a flame ionization detector (FID) and a UNICAME 4815 integrator. A DB-wax (polyethylene glycol) 30m X 0.53-mm megabore column was used.

2.4. Glucosinolates

Seeds and leaves of the promising breeding materials of the *Brassica* species were analyzed for total glucosinolates through spectrophotometry on UV 160 Spectrophotometer (Shimadzu).

3. RESULTS AND DISCUSSION

3.1. Evaluation of mustard canola mutants for yield and other agronomic traits in different PYTs

The results of the trials (Table I) indicated that 65 mutants were significantly early maturing and the reduction in maturity period was in a range of 17 to 55 days as compared to the parent (control). Fifty-five mutants were significantly shorter than the parent i.e. 7-30% reduction in height was induced, and 46 mutants significantly outyielded the parent by a margin of 26-71%. However, 19 mutants produced less than the controls. Some of the mutants exhibited better performance than LC under local short photoperiod environs, indicating adaptability and great potential to replace the indigenous non-canola cultivars or genotypes. Induced mutations appear to have been successful in breaking the photoperiod sensitivity in exotic juncea genotypes. Some of the promising mutants were further assessed in field trials at NIFA for ascertaining their stability and confirmation of these findings.

TABLE I. SUMMARY OF RESULTS OF 6 PRELIMINARY YIELD TRIALS, 1997-98

Trait	No. of mutants	% of control (range)	Control (range)
Earliness	65	-17.3 to -55.8	127-147 days
Height reduction	55	-7 to -30	273-303 cm
Increased yield	46	+26.3 to +71.2	950-1330 kg/ha

3.2. Performance of promising mustard canola mutants in different AYTs

These trials showed that 58 mutants were significantly earlier flowering, 43 were significantly shorter than the parent and 32 mutants significantly outyielded the parent line. The results of 14 outstanding mutants along with the parent line (DLJ-3) and BM-1, a local commercial cultivar (LC), are presented in Table II. It is clear from the results that all mutants flowered significantly earlier than the parent, showing induced genetic variability in genes controlling this trait. Heavy selection pressure for earliness resulted in the development of morphological mutants exhibiting this trait. Only one mutant (MM-1201) flowered earlier than the LC. Similarly all mutants were significantly ($P<0.05$) shorter than the parent, however, only MM-1297 was shorter than LC. Nevertheless, most of the entries produced plants above the ideal level under irrigated conditions. The yield data indicated that all but one mutant significantly outyielded the parent line. Similarly all mutants except MM-1276 produced higher yields than LC, while five of them (MM-1266, MM-1238, MM-1200, MM-1293-1, MM-1288-1) significantly outyielded it. Induction of mutations for yield and seed quality improvement in oilseed Brassica has also been reported by other researchers [3-6].

The quality analysis revealed that only MM-1266 had a high level of erucic acid content, the rest of the mutants fell well within the canola standard. The parent also contained

a permissible level of this fatty acid. However, the LC possesses a very high amount of erucic acid. Keeping in view the high yield potential and improved level of polyenoic fatty acid pattern of MM-1266, a cross breeding programme will be devised to introgress canola quality trait into this mutant line.

It can be inferred from the results (Table II) of the preliminary and advanced yield trials that irradiation did induce significant genetic variability in the treated exotic mustard line (DLJ-3) with regard to maturity, plant height and seed yield. This line is very sensitive to photoperiod and is adapted to areas having long photoperiod. Under Pakistan's short photoperiod during winter, it grows very tall and hence matures very late, i.e. it has very long vegetative growth phase but very short reproductive phase. The yield is, therefore, very low due mainly to short photoperiod. Induction of mutations through gamma rays and fast neutron irradiation was therefore used to develop mutants insensitive to photoperiod. The superior performance of canola quality mutants such as MM-1200, MM-1201, MM-1293-1, MM-1238, MM-1285 etc. under the local environment indicated that these mutant lines are well adapted locally. These mutants will be extensively tested in diversified climatic conditions throughout Pakistan to ascertain their adaptability and genetic stability over years and locations. It is hoped that some of the promising mutants will lead directly to the development of improved canola quality mustard varieties in Pakistan in the near future.

TABLE II. MEAN VALUES OF DIFFERENT TRAITS OF PROMISING JUNCEA MUTANTS/CV TESTED IN ADVANCED YIELD TRIALS AT NIFA, 1998-99[a]

Entry	Days to 50% flowering, No.	Plant Ht. cm	Yield kg/ha	$C_{22:1}$ %	Glucosinolates μ mol/g
MM-1266	96.2 C	211.5 EF	2041.8 A	14.2	17.0
MM-1238	108.7 BC	230.9 CDE	1812.3 AB	1.8	20.2
MM-1200	84.0 CDE	225.5 DE	1764.0 AB	0.4	19.3
MM-1293-1	82.7 CDE	228.8 DE	1750.0 AB	1.7	18.0
MM-1288-1	88.3 CDE	263.7 B	1722.3 AB	0.1	17.5
MM-1297	96.5 C	203.1 F	1614.5 BC	2.0	20.0
MM-1285	102.0 BC	250.5 BC	1597.3 BC	0.2	12.0
MM-1305	112.2 B	233.9 CDE	1528.0 BC	0.0	17.0
MM-1201	63.7 F	221.9 DEF	1513.7 BCD	1.5	11.8
MM-1289	77.0 DEF	240.1 CD	1513.7 BCD	2.0	15.0
MM-1284-1	95.7 C	232.5 CDE	1500.0 BCD	2.8	23.3
MM-1313	100.0 BC	212.0 EF	1496.6 BCD	1.8	14.1
MM-1267	78.7 DEF	228.9 DE	1416.7 CD	1.3	18.5
MM-1276	102.3 BC	257.1 B	1097.0 E	0.0	9.5
BM-1 (LC)	75.0 DEF	205.0 F	1260.0 DE	45.0	58.2
DLJ-3 (P)	140.7 A	289.7 A	1104.5 DE	1.0	18.4

[a] Means are of 4 replications, those with the same letter are not significantly different at $P<0.05$, using Duncan New Multiple Range Test (DNMRT).

3.3. *In vitro* experiments

3.3.1. Interspecific hybridisation

Out of 100 pollinations per hybrid combination, about 80% of the ovaries were enlarged in crosses of *B. juncea* × *B. napus* and *B. juncea* × *B. campestris*. However, 50% and 30% of the rescued embryos developed on Gamborg's B-5 solid media, respectively. Only plants from

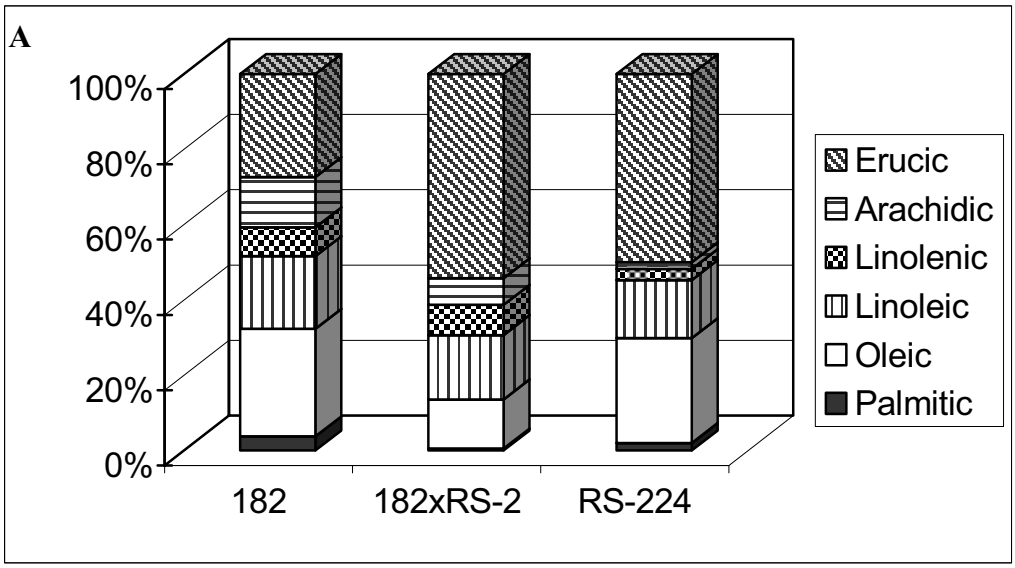
the crosses of J0-7 x RM-182 and J0-7 x RM-159-2 (both *B. juncea* x *B. campestris*) were recovered and four hybrid plants survived in the soil. The oil quality of the F_1 hybrids was determined through gas chromatography for fatty acid profile while total glucosinolates were assayed via spectrophotometry. The hybrids were intermediate in their fatty acid composition. JO-7 has 45% oleic acid and 0% erucic acid while mutants of *B. napus* (RM-182 and RM-159-2) have 30% oleic and 33% erucic acid (Figs. 1, 2). The F_1 hybrid (JO-7 x RM-182) seeds (F_2 embryos) had 33% oleic and 14% erucic acid (Fig. 1). The total glucosinolates in the seeds and leaves of the hybrid plants was also intermediate between the two parents as shown in Figs 1 and 2. Although hybrids with desirable fatty acid composition were not developed yet the whole procedure worked perfectly well. It is expected that desirable hybrids will be developed in the future through this technique by carefully selecting suitable parental material.

3.3.2. Isolated microspore culture

Plants No. 24 and 25 of the F_1 Abasin-95 x RM- 182, which exhibited earliness and robust vigour, were selected for haploidy breeding through isolated microspore culture. A number of developing plantlets of both crosses expressed F_1-performance regarding the vigour and growth during *in vitro* culture. Agronomic data of doubled haploid (DH) lines regarding flowering, plant height, number of branches per plant, number of roots per plant, root length, siliques per plant, silique length, seed per plant, seed weight, total oil content and yield per plant were recorded at maturity. The fatty acid composition and total glucosinolates in the seeds were also analysed, using techniques mentioned earlier in this paper. Plant F_1 DH_1 No. 99001 produced more branches, roots, siliques and seed yield as compared to the parents and to 'Tower', the source variety of both mutants (Table III). Plant F_1 DH_1 No. 99005 produced 7.8% more oil than both parents. Polok et al. [7] reported similar results in barley F_1 DH lines heterosis. The DH lines will be further evaluated in the next generation for confirmation of these findings.

TABLE III. HETEROSIS MANIFESTATION IN F_1DH_1 OF SELECTED PLANTS IN RAPESEED MUTANT CROSSES - % INCREASE OVER THE BETTER PARENT (a) AND THE SOURCE VARIETY TOWER (b)

Plant	Branches No.	Roots No.	Siliques No.	Seeds/plt	Oil content	Yield/plt
MF_1 DH_1-99001	a- 28.5	a- 43.2	a- 44.4	a- 7.4	a- 1.5	a- 25.0
	b- 36.2	b- 43.2	b- 54.2	b- 8.5	b- 6.4	b- 27.6
MF_1 DH_1-99004	a- 10.0	a- 3.2	a- 18.0	a- 6.0	a- 1.0	a- 8.4
	b- 10.0	b- 7.3	b- 25.5	b- 3.0	b- 6.3	b- 9.4
MF_1 DH_1-99005	a- 20.0	a- 11.7	a- 13.6	a- 9.9	a- 6.7	a- 6.6
	b- 20.0	b- 3.1	b- 27.5	b- 9.9	b- 7.8	b- 8.4



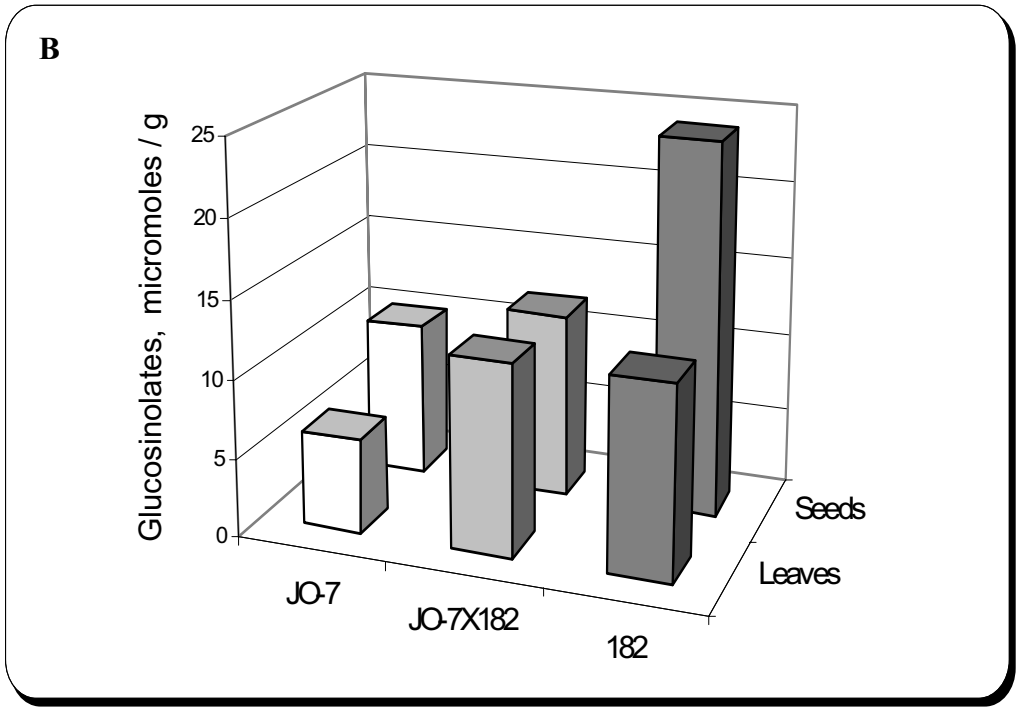


FIG. 1. Fatty acid (A) and total glucosinolate (B) content in seeds and leaves of the F_1 hybrid (J0-7x182) and parent plants.

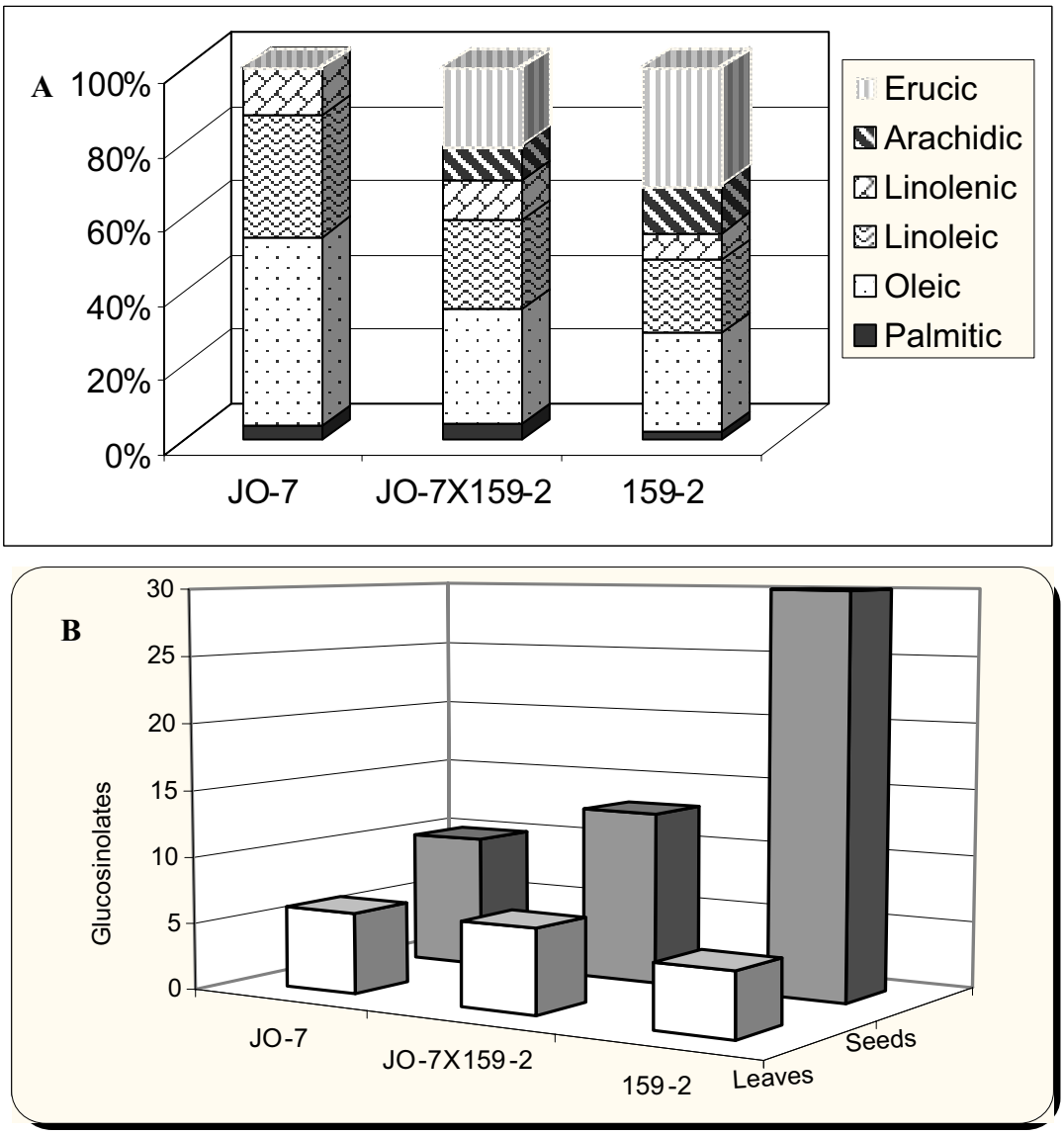


FIG. 2. Fatty acids composition (A) and total glucosinolates (B) in seeds and leaves of F_1 hybrid (J0x159-2) and parent plants.

3.4. Quality assessment of Brassica genotypes

The screening for erucic acid in bulk samples of M_5 seeds harvested from M_4 plants resulted in detection of 50 low erucic acid mutants/recombinant plants and 10 of them had 0% erucic acid. Twenty genotypes produced oil with up to 45% erucic acid. Two mutants were isolated for high oleic acid (~50%). More than 35% linoleic acid was recorded in 16 mutant plants while one mutant with a desirable low concentration of 3% linolenic acid was observed. Significant variability in fatty acid profile was observed in the M_3 seeds harvested from M_2 selections of rape and mustard. Regarding erucic acid, 3.29 to 12.47% variability was noted and 2.34 to 7.23% variability was recorded in oleic acid composition. Comparatively fewer genetic modifications were induced for polyunsaturated fatty acids. One mutant plant was isolated from a 2% EMS mutagenised population with 0% erucic acid. In the F_2 generation, 7 recombinants of *Brassica napus* were selected with 40 to 50% oleic acid, 8 plants with low erucic acid (< 5%) while only one recombinant produced oil with 41%

erucic acid. No mutant/recombinant plant could be isolated with more than 50% of this fatty acid for industrial uses. Polyunsaturated fatty acids in all F_2 selections ranged from 5 to 27%.

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Modification of flax (*Linum usitatissimum*) by induced mutagenesis and transformation

G.G. Rowland, R. Wilen, Y. Hormis

Crop Development Centre, University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

G. Saeidi

College of Agriculture, Isfahan University of Technology,
Isfahan, Islamic Republic of Iran

C. Ntiamoah

Sun Valley Research Station,
Carman, Canada

Abstract. The modification of flaxseed (*Linum usitatissimum* L.) for the purpose of developing new end uses for the crop was the objective of this project. Ethyl methane sulphonate (EMS) was used to induce mutations in flax with the main aim of producing seed oils with altered fatty acid composition. Mutations affecting both linolenic and palmitic acid levels were found and have been introduced in combination with seed colour mutations, into stable breeding lines. In addition, *Agrobacterium* mediated transformation of flax with a medium chain thioesterase gene from *Cuphea wrightii* was used to further modify a high saturated fatty acid genotype.

1. INTRODUCTION

For thousands of years *Linum usitatissimum* (linseed, flax or fibre flax) has been used for both its fibre and oil. In recent centuries the oil (linseed oil) has been used to produce industrial products such as paint and linoleum. The straw is processed for linen fibre, paper or, more recently, for various types of panels. The whole seed is also increasingly being used in human foods and nutraceuticals. It is a versatile crop that is an excellent alternative in many crop rotations. However, world production of linseed has been declining in the latter half of the 20th century. New fatty acid profiles of the oil are required to supply new industrial and edible demands for the oil. Therefore, the objective of this project was to develop altered fatty acid profiles of linseed through the use of chemical mutagenesis and biotechnology. These alterations are being incorporated into elite breeding lines.

Linseed is regarded as an industrial oilseed crop because of the very high levels of alpha-linolenic acid found in its oil (ca. 50%). Thus, linseed oil is classified as a drying oil which is used for coatings such as paint, inks and linoleum flooring. The alpha-linolenic acid oxidizes when it is exposed to air. As a result, linseed oil has limited uses as an edible oil because it quickly becomes rancid when it is expelled from the seed during the crushing process.

In order for flax to become an edible oil the high (50%) alpha-linolenic acid level in the oil should be eliminated or nearly eliminated. Green [1] described an EMS (ethyl methane sulphonate) derived mutant genotype of flax whose seed oil contained less than 2% linolenic acid. Unable to obtain this fatty acid profile through conventional breeding or these mutants from Green, a mutation program was initiated at the Crop Development Centre. In 1987,

20,000 seeds from the linseed cultivar 'McGregor' were treated with EMS [2]. A large number of mutations were identified from this population and two fatty acid mutant lines were of particular interest and formed the basis of the research reported here. E67 was a mutant line with very elevated levels of palmitic acid and E1747 was a line with extremely low levels of linolenic acid (Table I).

TABLE I. FATTY ACID COMPOSITION (%) OF THE M_5 SEED OF TWO MUTANT LINES OF MCGREGOR AND THE SOURCE VARIETY MCGREGOR

Line	Fatty acid					
	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic
E67	27.8	4.8	1.8	17.5	6.0	42.0
E1747	9.5	Trace	4.6	15.6	65.3	2.1
McGregor	9.4	Trace	5.1	18.4	14.6	49.5

2. FURTHER RESEARCH

2.1. Low linolenic acid mutant line E1747

Green [1] found two independent mutations that affected linolenic acid levels in linseed. When combined into a single genotype these produced a seed oil with less than 2% linolenic acid. It seemed unlikely that the low linolenic (<2%) line E1747 would have two separate mutations affecting linolenic acid level, but crosses with the parent McGregor [3] showed this to be the case (Table II). These two mutant genes showed independent inheritance and would appear to affect the same two genes reported by Green [1]. The homozygous recessive mutant genotype gives an oil profile that is very similar to traditional sunflower oil.

TABLE II. GOODNESS OF FIT FOR A 15:1 RATIO OF LINOLENIC ACID LEVELS OF INDIVIDUAL F_2 SEED OF RECIPROCAL MCGREGOR (MCG) × E1747 CROSSES.

Cross	n	Linolenic acid class, %	Observed No.	Expected No.	χ^2	P (1df)
McG x E1747	419	<7.5	29	26.2		
		>12.5	390	392.8	0.2	0.64
E1747 x McG	419	<7.5	23	26.2		
		>12.5	396	392.8	0.3	0.59
Pooled	838	<7.5	52	52.4		
		>12.5	786	785.6	0.01	0.99

2.2. High palmitic acid mutant line E67

The appearance of the high palmitic acid mutant was interesting because this saturated fatty acid is important in the production of margarine, shortening and other fat products. This is the highest level of palmitic acid that has been reported in a temperate oilseed crop. To understand the inheritance of this character and its relationship to the low linolenic acid

genes, crosses were made between E67 and McGregor and E67 and E1747 [4]. The high palmitic phenotype is the result of a single gene with an additive affect (Table III). The gene affecting palmitic acid levels is inherited independently of the two genes controlling linolenic acid levels (Table IV) and thus a genotype with a high palmitic and low linolenic acid phenotype can be bred (see HPED6 in Table XI).

TABLE III. GOODNESS OF FIT FOR A 1:2:1 SEGREGATION RATIO OF PALMITIC ACID LEVELS OF INDIVIDUAL F_2 SEED OF RECIPROCAL MCGREGOR × E67 CROSSES

Cross	n	Palmitic acid class, %	Observed No.	Expected No.	χ^2	P (2df)
McG x E67	420	<11	97	105		
		11-19	206	210		
		>19	117	105	2.06	0.36
E67 x McG	419	<11	83	104.75		
		11-19	217	209.5		
		>19	119	104.75	4.78	0.09

TABLE IV. GOODNESS OF FIT OF PALMITIC AND LINOLENIC ACID LEVELS TO INDICATED SEGREGATION RATIOS IN INDIVIDUAL F_2 SEEDS OF CROSSES BETWEEN E67 AND E1747

Fatty acid	Fatty acid class, %	Observed No.	Expected No.	χ^2	P
Palmitic				1:2:1	(2df)
	<11.5	201	210		
	11.5-18.5	428	420		
	>18.5	211	210	0.54	0.76
Linolenic				1:15	(1df)
	<7.0	44	52.5		
	>11.0	796	787.5	1.3	0.25

2.3. Seed vigour and agronomic performance of solin

In Canada the low linolenic, edible oil type of linseed is known as solin and in order to distinguish it from regular linseed it must have a yellow seed colour. Both seed colour and fatty acid composition can affect seed germination and vigour [5, 6] and seed emergence problems of solin have been noted in Canada. Near isogenic populations of flax differing in seed colour or linolenic acid level were used to study the relationship of these characters to seed germination and vigour [7]. The results presented here are Petri dish germination tests averaged from three germination temperatures (5, 10 and 15°C) and a vigour test which consisted of germinating seeds which had been lightly dusted with soil and incubated at 5°C for 7 days followed by 4 days at 20°C.

Seed colour had no effect on germination in any of the four populations (Table V). However, there were significant differences in seed vigour between yellow seed and brown seed in each of the populations. In every instance yellow seed had much poorer seed vigour than brown seed.

TABLE V. MEAN GERMINATION RATES (%) OF SEEDS SOWN IN THE GERMINATION (G) AND VIGOUR (V) TESTS FROM DIFFERENT POPULATIONS OF FLAX NEAR ISOGENIC FOR SEED COLOUR

Seed colour	Population 1		Population 2		Population 3		Population 4	
	G	V	G	V	G	V	G	V
Brown	79.0	71.4	77.4	75.9	77.2	62.2	74.2	69.8
Yellow	74.9	31.1	74.1	41.6	71.3	29.5	84.0	44.3
LSD (P=0.05)	**9.1**	**12.6**	**8.6**	**7.6**	**11.2**	**16.0**	**10.7**	**14.5**

The results indicated (Table VI) that linolenic acid concentration could have an affect on germination and vigour. In Population 2 the low linolenic acid lines had a lower germination than the high linolenic lines. In Population 1 the low linolenic acid lines displayed seed vigour that was lower than that of the high linolenic lines.

TABLE VI. MEAN GERMINATION RATES (%) IN THE GERMINATION (G) AND VIGOUR (V) TESTS FOR SEEDS DIFFERING IN LINOLENIC ACID LEVELS IN DIFFERENT POPULATIONS OF FLAX

Linolenic acid level	Population 1		Population 2		Population 3		Population 4	
	G	V	G	V	G	V	G	V
Low	73.4	43.9	71.3	55.1	75.1	44.4	76.7	54.4
High	80.6	58.7	80.2	62.4	73.4	47.3	81.6	59.7
LSD (P=0.05)	**9.1**	**12.6**	**8.6**	**7.6**	**11.2**	**16.0**	**10.7**	**14.5**

The field emergence of brown seeds was much better than that of yellow seeds and high linolenic acid seeds germinated better than low linolenic acid seeds (Table VII). While the effect of the lower emergence of yellow seed was reflected in a lower seed yield the

TABLE VII. MEANS OF FIELD EMERGENCE, SEED YIELD, DAYS TO MATURITY, OIL CONTENT, VISIBLY DAMAGED SEED, AND SEED SPLITTING AND CRACKING FOR BROWN (B) AND YELLOW SEED (Y) AND FOR HIGH (H) AND LOW (L) LINOLENIC ACID LINES OVER TWO YEARS

	Seed colour		LSD (0.05)	Linolenic acid		LSD (0.05)
	B	Y		H	L	
Emergence, No. seedlings/m^2	399	328	25	378	348	25
Yield, kg/ha	1546	1439	75	1453	1532	75
Days to maturity, No.	101.3	101.5	1.2	100.8	101.9	1.2
Oil, %	41.47	42.15	0.60	41.84	41.78	0.60
Damaged seed, wt. %	0.08	0.11	0.04	0.10	0.08	0.04
Seed splitting, wt. %	1.79[a]	2.73	0.33	2.23	2.29	0.33
	(3.1)[b]	(7.9)		(5.9)	(5.3)	

[a] Means based on transformed data.
[b] Means within parentheses are based on original data.

opposite was the case with linolenic acid where the poorer emerging low linolenic acid seed had a higher seed yield. The higher oil concentration of yellow seed compared with brown seed is consistent with the literature (8). Yellow seed suffered much greater seed damage during harvest than did brown seed and this may partly explain the poorer seed vigour and field emergence of yellow seed.

These results show that any variety development program of solin will have to take note of germination and stand establishment. Selection for good seed vigour and resistance to harvester damage is essential.

2.4. Alternate seed colour for high palmitic acid solin

Since brown seed is used in Canada to identify regular linseed and yellow seed is used to identify solin it was felt that another seed-based marker would be useful for the identification of high palmitic acid solin. In the original EMS derived mutant population of McGregor a variegated seed coat mutation was found (9). The recessive variegated phenotype is controlled monogenically (Table VIII). The variegated seed mutation is inherited independently of the high palmitic acid mutation (Table IX).

TABLE VIII. GOODNESS OF FIT FOR A 3:1 SEGREGATION RATIO FOR SEED COLOUR OF INDIVIDUAL F_2 PLANTS FROM A CROSS OF BROWN × VARIEGATED SEED

Seed colour	Observed No.	Expected No.	χ^2	P (1df)
Brown	123	114.75		
Variegated	30	38.25	2.1	0.15

TABLE IX. GOODNESS OF FIT FOR SEGREGATION OF SEED COLOUR AND OF PALMITIC ACID LEVELS IN 163 F_2 SEEDS FROM A CROSS BETWEEN A BROWN SEED, LOW PALMITIC LINE AND A VARIEGATED SEED, HIGH PALMITIC LINE

Seed Colour	Palmitic acid level	Observed No.	Expected No.
Brown	Low	32	28.69
Brown	Intermediate	54	57.38
Brown	High	37	28.69
Variegated	Low	4	9.56
Variegated	Intermediate	16	19.13
Variegated	High	10	9.56
χ2 (3:6:3:1:2:1)			6.8
P (5df)			0.24

Since yellow seed has been associated with reduced seed vigour there was concern that variegated seed might also have seed vigour problems. However, there was no reduction in either germination or seed vigour associated with variegated seed (Table X).

TABLE X. MEAN GERMINATION RATE (%) IN THE GERMINATION AND VIGOUR TESTS FOR HIGH PALMITIC, LOW LINOLENIC ACID SOLIN LINES DIFFERING IN SEED COLOUR.

Genotype	Seed colour	Germination (%)	
		Germination test	Vigour test
Line 2115	Brown	85.2	59.6
Line 2113	Variegated	81.6	62.0
LSD (0.05)		7.8	18.5

2.5. Transformation of high palmitic acid solin

In an attempt to develop linseed with even higher levels of saturated fatty acids and with medium chain fatty acids, a medium chain thioesterase gene, BFAT2, from *Cuphea wrightii* was obtained from Dr. Steven Knapp, Oregon State University, Corvallis, OR, USA. This gene was used to transform the high palmitic solin line HPED6 by *Agrobacterium tumefaciens*-mediated transformation. This line was selected because it already had elevated saturate levels and was a reasonably good agronomic line. Five series of inoculations were made and a total of 51 plants tested positive for both kanamycin resistance and GUS activity, which were the selectable markers included along with the thioesterase gene. Of the original 51 transgenic plants, 17 showed significant alterations in the fatty acid profile. Total saturates were greatly elevated in some of the seed and there was the appearance of both myristate (C14:0) and lauric (C12:0) fatty acids (Table XI).

TABLE XI. FATTY ACID PROFILE AND TOTAL SATURATED (TS) FATTY ACIDS OF LINSEED, SOLIN, HIGH PALMITIC SOLIN AND SELECT SEED FROM HIGH PALMITIC SOLIN PLANTS TRANSFORMED WITH THE MEDIUM CHAIN THIOESTERASE GENE (BFAT2) FROM *Cuphea wrightii*

Line	Fatty acid, %								TS
	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	
Linseed	-	-	9.4	-	5.1	18.4	14.6	49.5	14.5
Solin	-	-	9.5	-	4.6	15.6	65.3	2.1	14.1
HPED6	-	-	17.0	2.0	3.0	16.0	60.0	2.0	20.0
1-5	1.5	6.0	39.0	2.0	2.5	9.0	39.0	1.0	49.0
4C3	1.0	3.0	28.0	2.0	2.0	11.0	50.0	2.0	34.0
C38	3.0	8.0	39.0	2.0	2.0	5.0	40.0	2.0	52.0

3. CONCLUSION

It has been shown that *L. usitatissimum* can be easily manipulated by chemical mutagenesis and transformation. Apart from the changes described here in the fatty acid profiles and colour of the seed, other changes in the chemical makeup of the seed have been found. Mutations affecting the cyanogenic glucosides, lignans and mucilage have been identified and are being characterized. Molecular markers are being developed to identify genes for wilt tolerance and seed colour. These changes should lead to a much greater demand for the linseed crop.

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Use of biotechnology in flax germplasm development

R. Wilen

Crop Development Centre,
University of Saskatchewan,
Saskatoon, Saskatchewan, Canada

Andro, CDC Normandy, CDC Triffid, and Linola 989 are examples of flax varieties that have been developed through the application of biotechnology. Somaclonal variation, cell selection, haploid breeding, mutagenesis, genetic engineering and molecular marker development are all being employed in flax germplasm development. Tissue culture techniques such as somaclonal variation (CDC Normandy) and cell selection (Andro) have been successful, but the greatest potential for the use of tissue culture methodology is the application of haploid breeding. While a number of groups worldwide have had limited success in producing doubled haploid plants from anther and/or microspore culture [Chen et al. 1998, Plant Breeding 117: 463; Friedt et al. 1995, Plant Breeding 114: 322; Nichterlein & Friedt 1993, Plant Cell Rep. 12: 426], the frequency of regeneration has limited its application in variety development. Several groups are currently using anther culture within their breeding programs, but the ultimate success of haploid breeding will undoubtedly depend on developing an efficient microspore-derived system for doubled haploid production.

Perhaps the most successful technique to date, in terms of germplasm development, has been the use of mutagenesis. The Linola types [Dribnenki et al. 1996, Can. J. Plant Sci. 76:329; Dribnenki & Green 1995, Can. J. Plant Sci. 75: 201], have already been released, and other modified oil types are currently being developed [Saeidi & Rowland 1997, J. Hered. 88: 466; Ntiamoah et al. 1995, Crop Sci. 35: 148]. Additional traits being investigated in mutagenized populations include seed colour, reduced levels of antinutritional factors in seed, increased nutraceutical content, and traits of agronomic interest. For example, screening of an EMS-treated population of McGregor flax at the Crop Development Centre identified three mutant lines which had greatly reduced levels of cyanogenic glucosides.

Flax has proven to be amenable to genetic modification via *Agrobacterium*-mediated transformation [Besairan et al. 1987, Plant Cell Rep. 6: 396; Jordan & McHughen 1988, Plant Cell Rep. 7: 281; Mlynarova et al. 1994, Plant Cell Rep. 13: 282]. Early work using this technology focused on input traits such as herbicide resistance [McHughen 1989, Plant Cell Rep. 8: 445]. CDC Triffid is the only transgenic flax variety released to date and is resistant to the chlorosulfonylurea herbicides [McHughen et al. 1997, Can. J. Plant Sci. 77: 641]. Recent efforts on the production of transgenic flax have targeted seed quality traits and value added traits. Several groups are involved with the genetic modification of flax for altered fatty acid content including the production of γ–linolenic acid, long chain polyunsaturated fatty acids and highly saturated fatty acids. The reduction of cyanogenic glucosides in flax has also been targeted by genetic engineering [Trummler et al. 1998, Plant Sci. 139: 19]. The use of flax as a vehicle for molecular farming - the introduction of novel high value compounds into the seed and/or vegetative tissue - has also been promoted [Abenes et al. 1997, Plant Cell Rep. 17: 1]. The crop offers the advantage of being a self-pollinated, high yielding, oilseed that is not generally used as a food crop, which exhibits little outcrossing.

The last biotechnology approach is the use of molecular markers for marker-assisted selection. This technology is just being developed in flax. Early work has been successful in

identifying markers for rust resistance [Luck et al., 1998 Plant J. 16: 365: Anderson et al. 1997, Plant Cell 9: 641] and ploidy determination [Chen et al. 1998, Plant Cell Rep. 18: 44]. Various techniques such as RAPD and AFLP have been used and recently two microsatellite libraries were constructed from flax DNA at the Crop Development Centre. Research is now focused in various laboratories on the development of markers for a wide range of traits including cadmium accumulation, fatty acid biosynthetic genes, and wilt resistance.

Mutation breeding in sunflower (*Helianthus annuus* L.) for disease resistance and oil content

M. Fernandes de Oliveira, R.M. Villas Bôas de Campos Leite
V.B. Rodrigues Castiglioni, A. Lopes de Farias Neto
C.A. Arrabal Arias, J.M. Gontigo Mandarino

Brazilian Agricultural Research Corporation,
National Soybean Research Center (EMBRAPA Soja),
Londrina, Brazil

A. Tulmann Neto

Agricultural Atomic Energy Center (CENA-USP),
Piracicaba, Brazil

Abstract. Genetic variability for resistance to Alternaria disease (*Alternaria helianthi*) can be induced in cultivated sunflower by radiations or chemical mutagens. The objectives of this study were to develop sunflower germplasm resistant to Alternaria with good oil content. In the first experiment, sunflower seeds of the genotype HA BR 104 were irradiated with 150 and 165 Gy of gamma rays. Seeds were sown in the field at the EMBRAPA Soja experimental station, in Londrina, PR and M_1 plants were harvested in bulk. M_2, M_3 and M_4 plants were screened for disease resistance, under natural infection in the field. Self-pollinated plants and open pollinated plants from the 150 Gy and 165 Gy populations with no or mild disease symptoms were selected. Regrettably, all the selections proved susceptible in M_4 and were discarded. In the second experiment, sunflower seeds of the genotypes HA 300 and HA BR 104, were treated with ethyl methanesulfonate (EMS) at 0.015 M and the M_1 and selected M_2 and M_3 were grown and screened at the above Station. From the EMS treatment 300 M_3 plants were recovered. Their agronomic characteristics, oil content, disease resistance and combining ability are under evaluation in the offspring.

1. INTRODUCTION

Sunflower is an oilseed crop with wide adaptation, due to its relative tolerance to lower temperatures and drought. The seeds have high oil content with very good quality. This crop represents a new option for Brazilian farmers, considering both agronomic and market aspects. Diseases are one of the major limiting factors of sunflower production worldwide. The plant is a known host of more than 35 infectious microorganisms, mostly fungi, which may, under certain climatic conditions, significantly reduce yield and quality. One of the most important diseases occurring in sunflower in Brazil, especially in the south, is Alternaria (*Alternaria helianthi*) leaf and stem spots, seedling blight and head rot. This pathogen produces dark brown lesions on the leaves, petioles, stems and flower parts; when numerous, they form large necrotic areas. Chemical control is not recommended because it is difficult to obtain complete foliage coverage by aerial application of fungicides. Therefore genetic control of the disease through breeding for resistance, is a highly desirable objective. However, the genetic base of cultivated sunflower is narrow and resistance genes are scarce. Resistance to Alternaria diseases has been found in some species of *Helianthus*, e.g. *H. tuberosus*, *H. hirsutum* and *H. resinosus*. The use of these species as sources of resistance requires interspecific hybridization with *H. annuus*. This is particularly difficult because these species are tetraploid or hexaploid, while cultivated sunflower is diploid. Wider variability within *H. annuus* would be very useful in order to identify sources of resistance to Alternaria diseases. One way to create genetic variability in cultivated sunflower is to induce mutations

by irradiation with gamma rays or with chemical mutagens. Seed treatments with gamma radiation or with chemical mutagens, such as ethyl methanesulfonate (EMS) have been used extensively in sunflower to increase variability for several characteristics, such as days to flowering, seed weight and oil content.

The objectives of the present work are: to create genetic variability in cultivated sunflower; to select lines resistant to Alternaria diseases; and to evaluate these lines for oil content. Advanced resistant mutant lines will be multiplied for field trials and selected for hybrid production. The best hybrids will be distributed rapidly to sunflower farmers.

2. MATERIALS AND METHODS

2.1. Mutations induced by radiation

A preliminary experiment was performed at the Radiation Genetic Section of the Agricultural Atomic Energy Center (CENA), in collaboration with EMBRAPA Soja, to define the level of gamma radiation that should be used for treating sunflower seeds. Seeds of the line HA BR 104, from EMBRAPA Soja, were irradiated with gamma rays at 100, 200, 300, 400 and 500 Gy. Each treatment consisted of 10 seeds, with 3 replications. The irradiated seeds were tested in the greenhouse, with non-irradiated seeds as control. The seedlings were evaluated for survival, hypocotyl and epicotyl length and plant height.

Subsequently, four thousand sunflower seeds of the line HA BR 104 were irradiated with the desired gamma ray doses for mutagenic treatment, at 30 cm of distance during 14.4 min. The irradiated and non-irradiated seeds were sown in the field, in order to examine the M_1 plants.

A second trial was conducted in June 1996, when two lots of eight thousand seeds of the same line were irradiated, with 150 and 165 Gy of gamma rays. The M_1 seeds were sown in the field to get at least four thousand plants, which were self-pollinated. About 300 non-irradiated seeds were sown as controls. Seedlings and plants were evaluated for survival, chlorosis, plant height at harvest, sterility and number of seeds per head. All M_1 plants were harvested in bulk.

In January 1997, the next generation (M_2) was sown in a dense stand of about 70000 plants/ha, in order to provide conditions for high incidence of Alternaria leaf spot. The plants were irrigated twice a week, to increase the humidity required by the pathogen to spread. The plants were evaluated for Alternaria leaf spot symptoms, using a disease scale of 0 (no symptoms) to 5 (most severe). Before flowering, plants showing no symptoms of Alternaria leaf spot (grade 0) or less than 5% diseased leaf area (grade 1) were bagged for self-pollination. These plants were evaluated periodically, to observe the manifestations of the disease. The plants with low disease scores were harvested individually, to produce M_3 lines. At harvest time, open pollinated plants that showed few symptoms were collected, in order to form M_3 half-sib families.

In January 1998, M_3 lines from the self-pollinated or open pollinated plants from both radiation levels' populations were sown for evaluation of disease resistance and to get the M_4 seeds, at the EMBRAPA Soja station, in Londrina, PR.

The M_4 lines from self-pollinated plants of 150 and 165 Gy radiation levels were sown in two periods in Londrina, PR (December 8, 1998 and February 2, 1999), and on February 2,

1999 in Planaltina, DF for evaluation of disease resistance, agronomic characteristics and oil content, compared to the control plants. These two locations represent different regions for sunflower production in Brazil.

2.2. Mutations induced by chemical mutagens

Chemical mutagens have been used to increase variability for several characteristics in cultivated sunflower [1]. The mutagen selected was ethyl methanesulfonate (EMS).

An initial experiment was conducted to determine the appropriate concentration range of EMS for mutagenesis of sunflower. Sunflower seeds of the genotypes HA 300, from USDA, and HA BR 104, from EMBRAPA Soja, were treated with EMS at 0 (control), 0.010 M, 0.015 M and 0.020M. Each lot consisted of 13 seeds, with four replications. The seeds were pre-treated with potassium phosphate buffer, pH 8.0, for 8h. They were then treated with EMS solution for 16h, rinsed and planted under greenhouse conditions. Plants of genotype HA 300 were evaluated for vigor and plant height two weeks after emergence.

A second experiment was conducted using the selected EMS concentration for mutagenic treatment of sunflower seeds. Ten thousand seeds of line HA BR 104 and fourteen thousand seeds of line HA 300 were treated with EMS at 0.015 M for 16 hours. Untreated seeds were used as control. After treatment, seeds were sown in the field on July 9, 1997, at the EMBRAPA Soja experimental station, in Londrina, PR, in order to get the M_1 plants.

The seedlings and plants were evaluated for survival, chlorosis, plant height at flowering time and harvest, sterility and number of seed per head. All M_1 plants were harvested in bulk in November, 1997.

In January 1998, the M_2 generation was planted at a density of about 60000 plants/ha, in order to provide conditions for high incidence of Alternaria leaf spot. In January, the plants were irrigated twice a week, to raise the humidity to that required by the pathogen to spread. The plants were evaluated for Alternaria leaf spot symptoms, using a disease scale of 0 (no symptoms) to 5 (maximum severity). Before flowering, plants showing no symptoms of Alternaria leaf spot (grade 0) or less than 5% diseased leaf area (grade 1) were bagged for self-pollination. These plants were evaluated periodically, to observe the evolution of disease. The plants with low disease grades were harvested individually on May 8, 1998. From these plants the M_3 lines were obtained.

The M_3 lines from self-pollinated plants of EMS treatment, were sown on two sowing dates (December 8, 1998 and February 2, 1999) in Londrina, PR, and on February 2, 1999 in Planaltina, DF, for evaluation of disease resistance, agronomic characteristics and oil content, compared to the control plants. These two locations represent different regions for sunflower production in Brazil.

3. RESULTS AND DISCUSSION

3.1. Mutations induced by radiation

The preliminary experiment performed in the greenhouse for determination of the level of the desired gamma irradiation doses for sunflower seeds is summarized in Table I. The seeds survived up to the 200 Gy level treatment. However, there was a marked reduction in

plant height (about 37%). Based on these results, a dose of 180 Gy was chosen for further treatments.

In M_1, seedlings from the 180 Gy treatment evaluated in the field experiment, had low germination rate (lower than 50%) and there was a high level of plant sterility, indicating that this dose was still high. Thus, more seeds were treated with 150 and 165 Gy radiation doses and their effects were evaluated in another field trial.

TABLE I. MEANS OF GERMINATION RATE, HYPOCOTYL AND EPICOTYL LENGTH AND HEIGHT OF SEEDLINGS FOLLOWING DIFFERENT GAMMA RAYS' TREATMENTS[a]

Dose	Hypocotyl length	Epicotyl length	Seedling height	Germination
Gy	mm	mm	mm	%
0	22.7	36.0	59.7	86.7
100	19.8	32.9	52.7	90.0
200	10.9	16.5	37.4	100.0
300	5.7	-	-	77.0[b]
400	-	-	-	
500	-	-	-	

[a] Means of three replications with 10 plants.
[b] Seedlings without leaves.

A reduction in the M_1 seedlings emergence rate and survival was observed for 150 and 165 Gy radiation treatments (Table II). No plants showed chlorosis but sterility was markedly increased by the 165 Gy dose. There was also a reduction in the plants' height and in the number of seeds per head for both radiation levels. Most control plants were 60-90 cm tall while M_1 seedlings from treated seeds (150 and 165 Gy) were considerably shorter (35-60 cm). The number of seeds/head was reduced in most of the treated plants (the range was 1-51 seeds/head). These results indicated that the radiation levels used for sunflower seeds were still high, and studies on the determination of the best radiation level should be continued.

TABLE II. MEANS OF SURVIVAL, PLANT HEIGHT, STERILITY AND SEEDS PER HEAD OF SUNFLOWER M_1 SEEDLINGS AND PLANTS AT 15 AND 30 DAYS AFTER EMERGENCE AND AT HARVEST

Dose	Survival[a], %			Sterility[b]	Plant height	No. seeds/head
Gy	15 DAE	30 DAEs	At harvest	%	cm	
0	73.8	74.5	67.1	7.3	67.80	88
150	48.4	43.9	28.5	9.6	48.72	35
165	50.4	50.2	34.3	41.4	47.89	29

[a] Means of 283, 3395 and 3401 plants for 0, 150 and 165 Gy, respectively. DAE = days after emergence.
[b] Means of 190, 967 and 1166 plants for 0, 150 and 165 Gy, respectively.

In the M_2 generation, the plants were evaluated for Alternaria leaf spot using a disease scale from 0 (no symptoms) to 5 (maximum severity). Plants showing no symptoms or less than 5% diseased leaf (grade 1) were bagged before flowering and, at harvest time, open pollinated plants that showed light symptoms were also harvested. Seeds of some open

pollinated plants showing head deformations were harvested in order to evaluate the nature of this character in the next generation. The selected plants are listed in Table III.

The M_3 lines, from the self-pollinated and open-pollinated disease resistant plants from both radiation levels were planted and evaluated for Alternaria leaf and stem spot under natural high disease conditions, in Londrina, PR. The disease was evaluated using the same scale as in the M_2 generation. Agronomic characteristics were not evaluated and the trial was not conducted in Planaltina, DF, as previously planned, because the lines were still heterogeneous. In the M_3 lines some plants were selected for Alternaria disease resistance (Table IV).

TABLE III. NUMBER OF EVALUATED M_2 PLANTS, AND NUMBER OF SELF- AND OPEN-POLLINATED PLANTS HARVESTED FOR DISEASE RESISTANCE AND HEAD DEFORMATIONS

Dose Gy	Evaluated plants No.	Disease resistant plants harvested		No. open-pollinated plants with head deformations
		Self-pollinated No.	Open-pollinated No.	
150	8406	2	25	10
165	5552	3	28	10

TABLE IV. M_3 LINES TESTED AND PLANTS SELECTED FOR ALTERNARIA DISEASE RESISTANCE

Dose Gy	Self-pollinated, No.		Open-pollinated, No.		Open-pollinated, with head deformations, No.	
	Evaluated lines	Selected plants	Evaluated lines	Selected plants	Evaluated lines	Selected plants
150	2	1	25	10	10	6
165	3	1	28	29	10	4
Total		2		39		10

In the tests in Londrina and Planaltina the environment allowed a greater disease potential and all the M_4 lines showed very severe levels of Alternaria, i.e. they were susceptible and not useful for the breeding program.

3.2. Mutations induced by chemical mutagens

Despite their reduced height (12.5%), plants of the 0.015M EMS treatment showed good development (Table V). Seeds of HA BR 104 emerged poorly and only the physiological aspects were observed. Based on these results, the chosen concentration for EMS treatment was 0.015 M.

In the second trial, the EMS treatment reduced the M_1 survival for both genotypes (Table VI). Few seedlings showed chlorosis, and died three days after emergence. The mutagenic agent did not cause sterility effect; the sterility observed in plants of the genotype HA BR 104 is natural, because the untreated plants showed the same frequency of sterility as the EMS-treated population (Table VI). Plant height of HA 300 treated with EMS was

reduced. For the genotype HA BR 104, this difference was not significant: most HA BR 104 control plants were 65-95 cm high while the M_1 plants were 70-95 cm high. For HA 300, the control plants' height was 120-140 cm, and the M_1 plants' height was lower, 95-135 cm. The number of seeds per head was assessed only with M_1 plants, because the untreated control seeds were mixed after harvesting.

TABLE V. MEAN HEIGHT OF SEEDLINGS FROM SEEDS OF HA300 SOAKED WITH DIFFERENT CONCENTRATIONS OF EMS

EMS concentration	Height[a], cm	Height reduction, %
0 (control)	11.2	-
0.010 M	10.8	2.6
0.015 M	9.8	12.5
0.020 M	8.3	25.9

[a] Means of 4 replications with 13 plants.

TABLE VI. MEANS OF SURVIVAL, STERILITY, PLANT HEIGHT AND SEEDS SET OF SUNFLOWER M_1 SEEDLINGS AND PLANTS FROM SEEDS TREATED WITH EMS

Genotype	EMS	Survival %, days after emergence[a]			Sterility[b] %	Plant height, cm[c] at		Seed No/head[d]
		5	21	31		Flowering	Harvest	
HA BR 104	0	52.00	52.00	52.00	2.00	73.39	72.35	-
HA BR 104	0.015 M	41.17	41.63	41.40	1.74	79.38	81.80	50.00
HA 300	0	82.00	83.00	82.00	0.00	123.70	123.03	-
HA 300	0.015 M	63.95	65.66	63.37	0.00	108.50	107.87	58.56

[a] Means of 200, 7800, 200 and 12800 seeds, respectively for HA BR 104 (0 and 0.015M) and HA 300 (0 and 0.015M).
[b] Means of 52, 3229, 82 and 8111 plants, respectively for HA BR 104 (0 and 0.015M) and HA 300 (0 and 0.015M).
[c] Means of 300 plants.
[d] Means of 200 plants.

In the M_2 generation, the plants were evaluated for Alternaria leaf spot using a disease scale from 0 to 5. Plants showing no symptoms or less than 5% diseased leaf (grade 1) before flowering were bagged and harvested individually. The remaining open-pollinated plants, that showed light disease symptoms after flowering, were discarded because of the low genetic gain showed by this procedure in the previous experiment of inducing mutation by gamma radiation. The selected plants are listed in Table VII.

TABLE VII. M_2 PLANTS SELECTED FOR ALTERNARIA DISEASE RESISTANCE FOLLOWING EMS TREATMENTS

Genotype	EMS	Evaluated plants, No.	Self-pollinated plants selected, No.
HA BR 104	0.015 M	22080	16
HA 300	0.015 M	6720	393

In the next generation, M_3 lines from self-pollinated resistant or tolerant M_2 plants from both genotypes were evaluated for Alternaria leaf and stem spot in the field under natural high disease conditions. The disease was evaluated using the same 0-5 scale used in the M_2 generation. M_3 plants were selected for Alternaria disease resistance in Londrina and Planaltina.

The best results were obtained with the chemical mutagen, which showed a total of 300 M_3 plants with no disease. The agronomic characteristics and oil content were not evaluated because the number of seeds per selected plant was not sufficient. The next step will be to verify the disease reaction of these materials and to test them for combining ability. The best lines will be used for hybrid production.

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AYT	advanced yield trial
BAC	bacterial artificial chromosome
BAP	benzyl-aminopurine
CLCuV	cotton leaf curl virus
CMS	cytoplasmic male sterility
DAE	days after emergence
DAP	days after planting
DAS	days after sowing
DH	doubled haploids
EMS	ethyl methanesulphonate
EST	expressed sequence tag
FA	fatty acids
FISH	fluorescence *in situ* hybridisation
GCA	general combining ability
GISH	genomic *in situ* hybridisation
GLA	gamma linolenic acid
GMO	genetically modified organisms
HEAR	high erucic acid rapeseed
IAA	indole-acetic acid
IPM	integrated pest management
KCS	β-ketoacyl-CoA synthase
LFM	late flowering mutant
LPA-AT	lysophosphatidic acid acyltransferase
MCFA	medium chain fatty acid
NAA	naphtaline-acetic acid
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PMC	pollen mother cell
PYT	preliminary yield trial
QTL	quantitative trait locus
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
SCA	specific combining ability
SCFA	short chain fatty acid
SD	standard deviation
SE	standard error
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TAG	triacylglycerol
TGW	thousand grain weight
VLMFA	very long chain monosaturated fatty acid
YAC	yeast artificial chromosome
ZYT	zonal yield trial

LIST OF PARTICIPANTS

Fernandes de Oliveira, M.	EMBRAPA-CNPSO, Rodovia Carlos Joao Strass-Aceso Orlando, Amaral, CX. Postal 231, 86001-979 Londrina, Paraná, Brazil E-mail: marcelo@cnpso.embrapa.br
Friedt, W.	Institute of Crop Science and Plant Breeding, Justus-Liebig-University, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany E-mail: wolfgang.friedt@agrar.uni-giessen.de
Hajós-Novak, M.	Gödölló Agricultural University P.O. Box 303, H-2103 Gödölló, Hungary E-mail: hajos-novak@fau.gau.hu
Hamid, M.A.	Bangladesh Institute of Nuclear Agriculture, Plant Breeding Division, P.O. Box 4, Mymensingh 2200, Bangladesh E-mail: bina@bdmail.net
Jambhulkar, S.J.	Bhabha Atomic Research Centre, Nuclear Agriculture and Biotechnology Division, Trombay, Mumbai 400 085, India E-mail: nad@magnum.barc.ernet.in
Kechagia, U.F.	National Agricultural Research Foundation, Cotton and Industrial Plants Institute, Cotton Breeding Department, 574 00 Sindos, Thessaloniki, Greece E-mail: ok31944@compulink.gr
Knapp, S.J.	Centre for Gene Research and Biotechnology, Department of Crop and Soil Sciences, Oregon State University, Corvallis, OR 97331-3002, United States of America E-mail: steven.j.knapp@orst.edu
Rowland, G.	University of Saskatchewan, Crop Development Centre, Saskatoon, S7N 5A8, Canada E-mail: rowland@sask.usask.ca
Stewart, J.McD	Crop, Soil, and Environmental Sciences Dept., University of Arkansas, Fayetteville, AR 72701, United States of America E-mail: jstewart@comp.uark.edu
Syed, A.S.	Nuclear Institute for Food and Agriculture, Tarnab, P.O. Box 446, Peshawar-25000, Pakistan E-mail: nifa@paknet2.ptc.pk *or* anwarshah@netscape.net

Wang, L.
Crop Breeding Institute – CAAS,
Soybean Department,
30 Bai Shi Qiao Road,
Hai Dian District,
10081 Beijing, China
E-mail: lanwang13@hotmail.com

Xanthopoulos, F.
National Agricultural Research Foundation,
Cotton and Industrial Plants Institute,
Cotton Breeding Department,
574 00 Sindos,
Thessaloniki, Greece
E-mail: ok31944@compulink.gr

Zhou, Y.
Institute of Crops Genetics and Breeding,
Huazhong Agricultural University,
Wuhan 430070, China
E-mail: znhsjtw@public.wh.hb.cn

INTERNATIONAL ORGANIZATIONS

Griffee, P.
Plant Production Division,
Crop and Grassland Service,
Food and Agriculture Organization,
Viale delle Terme di Caracalla,
00100 Rome, Italy
E-mail: peter.griffee@fao.org

Nichterlein, K.
Plant Breeding and Genetics Section,
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture,
P.O. Box 100,
A-1400 Vienna, Austria
E-mail: K.Nichterlein@iaea.org