Concurrent Session 1

Mutation Enhancement of Genetic Diversity and Crop Domestication

Mutational Events in a Homeobox Gene *Vrs1* that Created a Six-Rowed Spike in Barley Domestication

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Abstract

Early cultivators of barley (*Hordeum vulgare* ssp. *vulgare*) selected a phenotype with a six-rowed spike that stably produced three times the usual grain number during domestication. *SIX-ROWED SPIKE 1* (*Vrs1*) isolated from barley encoded a homeodomain leucine zipper I–class protein (HD-ZIP I), a potential transcription factor. *Vrs1* is expressed only in lateral spikelet primordia of the early developmental stage. Fifty-four six-rowed mutant lines showed mutational events at the *vrs1* gene except for five mutant lines, which suggested mutational events at the regulatory regions of *Vrs1*. We found three haplotypes among six-rowed barley revealing loss-of-function mutation of the homeobox gene Vrs1, while another allele showed no DNA changes throughout the coding region of the *Vrs1* gene indicating another origin of the six-rowed barley.

Introduction

Throughout the process of cereal domestication started about 10,000 years ago [1-3], humans have deliberately selected individuals of wild species to emphasize seed recovery [4-6] and improved seed yield [1, 7]. The appearance of six-rowed spikes during the domestication of barley (*Hordeum vulgare* ssp. *vulgare*) is one of the most conspicuous instances of this process. The barley spike is composed of "triplets" (each with one central and two lateral spikelets) arranged alternately at rachis nodes. All three spikelets of six-rowed barley are fully fertile and develop into grains, but the lateral spikelets of two-rowed barley are reduced in size and sterile. Wild barley (*H. vulgare* ssp. *spontaneum*), the progenitor of cultivated barley [1, 8], is two-rowed, and its arrow-like triple spikelets are an adaptive specialization that ensures the seeds will bypass stones and pebbles and reach soil when they fall to the ground [9]. Spontaneous six-rowed mutants are eliminated naturally from wild barley population, thus, six-rowed barley occurs primarily as cultivars or weeds [8].

The development of a six-rowed spike is controlled by a single allele, *vrs1* (formerly *v* for *vulgare*), that is recessive to the dominant allele responsible for the two-rowed spike (*Vrs1*) [10, 11]. *Vrs1* has been the primary target of mutation during the evolution of six-rowed barley. It has been assumed that six-rowed barley developed from domesticated two-rowed barley by means of spontaneous mutation [1, 12], but the origin of six-rowed barley has not yet been confirmed. Recently, map-based cloning of the *vrs1* gene revealed that *Vrs1* encodes a member of the homeodomain-leucine zipper (HD-ZIP) I class of transcription factors [13, 14]. Transcription of *Vrs1* was abundant during the early developmental stages of the immature spike, and *Vrs1* was expressed only in the lateral spikelet primordia. The dominant nature of *Vrs1* suggests VRS1 protein represses directly or indirectly the expression of genes for the development of lateral spikelets. In this paper, mutational events at the homeobox gene *Vrs1* in mutant lines were characterized to infer the function of the *Vrs1* gene.

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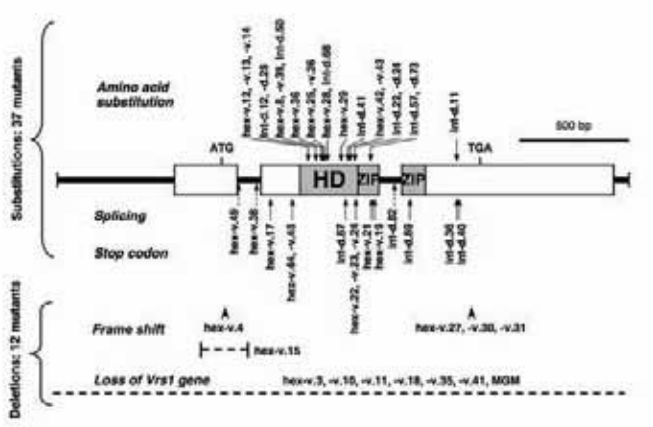

Figure 1 Analysis of mutants allelic to *vrs1*. Lesions at *Vrs1* detected in 49 mutants. Arrows pointing down indicate amino acid substitutions, arrows pointing up with a solid line indicate new stop codons, and the 3 arrows pointing up with a dotted line indicate single nucleotide substitutions in the introns with a changed splicing. The arrowheads and horizontal broken lines indicate deletions, where five mutants have a partial deletion and seven mutants have a complete deletion of *Vrs1*. After [13] with slight modifications.

Variable mutational events at *vrs1* in mutant lines

DNA sequences of a total 57 mutants, which were derived from five two-rowed cultivars mainly by the Swedish mutation research group [11], were analyzed [13]. The *hexastichon* (*hex-v*) mutants have six-rowed spikes with fully fertile, well-developed, and long-awned lateral spikelets [15], and thus resemble normal six-rowed barley. The *Intermedium spike-d* (*Int-d*) mutants produced sterile or partially fertile lateral spikelets with variable awn length. Allelism of these mutations with *vrs1* was documented in a previous study [16]. Lesions in *Vrs1* were correlated with morphological changes in 49 mutant lines. Twenty-two mutant lines revealed a single amino acid substitution, where most of their mutations were located at the homeobox (**Fig. 1**). Twelve mutant lines revealed truncation of the protein by a new stop codon. Amino acid substitution and creation of new stop codon at the 3' region downstream the HD-ZIP region also resulted in the change of row type indicating there are some additional functions at this region (Fig. 1). Three mutant lines showed a single nucleotide substitution in the conserved GT-AG splicing sites of introns (**Fig. 2A**). These mutants showed modified mRNA splicing of their *Vrs1* transcripts by deleting or replacing the conserved GT-AG splicing sites (**Fig. 2B**).

Five mutant lines had a frame shift mutation caused by a deletion, and seven revealed complete deletion of the *Vrs1* region [13]. These deletions (>182 kb), which were generated by means of irradiation, always resulted in *hex-v*–type six-rowed spikes under a range of growing conditions [13]. The phenotypes observed in mutants that consistently exhibited six-rowed spikes support our hypothesis that complete deletion of *Vrs1* occurred. Since the 7 deletion mutants did not show any developmental lesions, *Vrs1* appears to be dispensable in barley.

We detected five mutants that did not show any DNA changes throughout the coding region of *Vrs1* (I*nt-d.80, hex-v.7, hex-v.16, hex-v.33,* and *NGM13*). Expression analysis showed that all of the five mutants exhibited no transcripts of *Vrs1*. This result suggests the occurrence of the mutational events in regulatory regions for *Vrs1*. The regulatory elements may correspond to 5' up-stream cis-elements of the *Vrs1*, but it was also noticed that the *hex-v.33* mutant has a small deletion of a DNA sequence corresponding to *e34m13-260S* marker at the 3' down-stream of *Vrs1* (Supporting Information of Table 2 in [13]).

hex-v.46, *hex-v.47* and *hex-v.48* were also included in this category because no DNA changes were detected, but gene expression was detectable. Further allelism test, crossing these mutant lines with *hex-v.23* (stop codon) and *hex-v.49* (splicing modification) as testers indicated that these mutants do not have genes allelic to the *vrs1* locus because lateral spikelets of the F_1 plants were very poorly developed (data not shown). In a separate allelism test, all three *hex-v* mutants (*hex-v.46, 47* and *48*) got crossed to *hex-v.3* and *hex-v.4*. In F_1 all of them were noticed as a very weak six-rowed spike or only S plants (pointed lateral spikelets with very short awns), suggesting that *hex-v.46*, *47* and *48* are no six-rowed mutants (U. Lundqvist, personal communication). It was noted that the three mutants were not typical six-rowed spikes and irregular, and hex-v.48 looks similar to *intermedium* (*int-e.26*) mutant. Therefore the three mutants must be excluded from *hex-v* mutant lines. Since the *hex-v.46* showed a transcription level of Vrs1 the same as two-rowed barley.

hex-v.08 was scored without any DNA changes throughout the coding region of *Vrs1* [13], but detailed analysis of the DNA sequence and resequencing revealed that the mutant had a single amino acid substitution at the homeodomain of *Vrs1* as did *hex-v.39* and *Int-d.50*. Therefore, data concerning *hex-v.08*, *46*, *47* and *48* reported previously [13] were corrected in this paper.

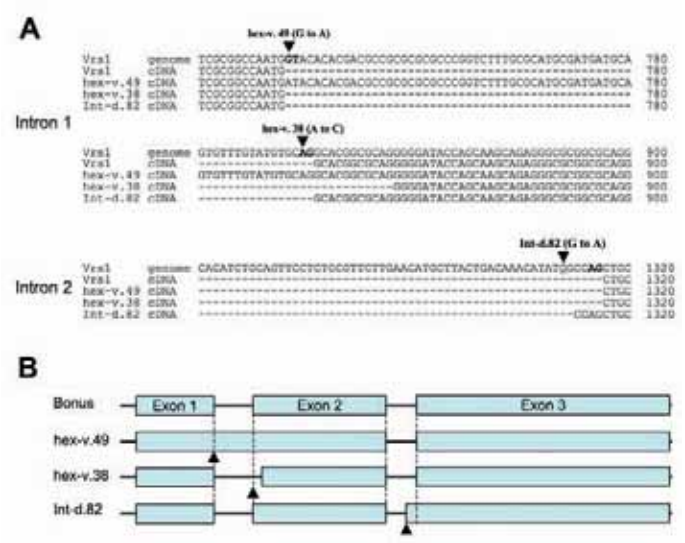

Figure 2 Analysis of mutants with a single nucleotide substitution in the conserved splicing sites of introns in *Vrs1*. (A) Splicing changes in first intron and second intron results from nucleotide substitutions. Arrows pointing up indicate the position of a single nucleotide change in Bonus. (B) Scheme of cDNA for the three mutants through the expression analysis. Broken lines indicate the original splicing site in functional *Vrs1*.

Mutational events at *vrs1* in cultivated six-rowed barley

Survey of barley cultivars and wild barley identified *Vrs1.b2* and *Vrs1.b3* alleles in two-rowed barley and *vrs1.a1, vrs1.a2*, and *vrs1.a3* alleles in six-rowed barley [13]. *vrs1.a1* and *vrs1.a2* have a deletion and insertion of one nucleotide respectively, which results in a frame shift of the deduced amino acid sequence of VRS1. The *vrs1.a3* allele has a substitution of one nucleotide in which an amino acid at a highly conserved position in the DNA-binding domain was substituted. The DNA sequences were identical among each allele except that the *vrs1.a1* had three sub-haplotypes due to SNPs at the non-coding region. A direct descent of *vrs1.a2* from *Vrs1.b2* and *vrs1.a3* from *Vrs1.b3*, as a result of point mutation were deduced. The progenitor of the *vrs1.a1* allele remains to be identified.

In addition, *vrs1.c* allele was found in "Arlington Awnless"(awnless) and "Hayakiso 2" (lateral spikelets awn-reduced), but all the spikelets are filled with grains producing six-rowed spikes. These six-rowed forms occur in East Asia, most dominantly in Tibet and Nepal. These cultivars did not reveal a change of amino acid sequence at VRS1 from two-rowed. The progenitor of the *vrs1.c* allele remains also to be identified.

Discussion and Outlook

Deletion mutants are powerful tools for the initial gene targeting by map-based cloning. Gene identification and biological function of genes could be confirmed by analysis of mutant lines revealing single nucleotide substitutions and insertion/deletions. Our study indicated that mutant lines are extremely useful for the identification not only of coding regions of target genes but also of their non-coding regions such as splicing sites and cis-regulatory elements. Secure storage of mutant lines and their systematic documentation are essential for sustainable molecular genetics in plants, especially cereal plants in which analysis of biological gene function by transformation remains difficult.

The creation of six-rowed spikes in the loss of function nature is similar to the gigantism that occurs during domestication [17]. The dominant nature of *Vrs1* and the potential DNA-binding activity of HD-ZIP I proteins suggest that VRS1 is a repressor protein that may bind to the DNA of genes that regulate the development of lateral spikelets. Further investigation of the subcellular localization of VRS1 proteins will be necessary to test this hypothesis. Expression analysis of genes downstream of *Vrs1* could link the *vrs1* mutations and morphological changes in barley spikes.

The inflorescence architecture in the Poaceae could be a continuous story of reduction from a more original "panicle" (as seen in rice and oats) to a "spike" [18]. Spikes contain a single sessile spikelet per node in wheat and rye and three sessile spikelets per node in barley. In two-rowed barley, strict temporal and spatial regulation of *Vrs1* expression leads to reduction and sterility of the lateral spikelets. We speculate that either strong alleles or differential regulation of *Vrs1* orthologs could lead to complete repression of lateral spikelet formation at inflorescence nodes found in wheat and rye. A Poaceae-wide assessment of variability and regulation of *Vrs1* orthologs would be an exciting and productive way to improve our understanding of plant development and of the evolution of grass species.

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Irradiation-Induced Wheat-Alien Translocation Lines and their Application in Wheat Breeding

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Abstract

Wild relatives are rich gene resources for wheat improvement. Transfer of useful alien genes to wheat through development of wheat-alien translocations, especially small alien segment translocations, is important for wheat breeding. Wheat-alien genetic stocks such as amphiploid, addition or substitution lines were irradiated for translocation induction. Mature male or female gametes before flowering on the spikes were irradiated by ^{60}Co-Gamma-rays at doses ranging from 800 to 2240 rad. Chromosome C-banding and genomic *in situ* hybridization (GISH) was used to identify chromosome translocation. Backcross of M_1 plants using normal fresh pollen of common wheat was employed to enhance the transmission rate of various structural changes in their progenies. The results showed that a dose of 800~1200 rad was suitable for pollen irradiation while 1500~2000 rad was suitable for female-gamete irradiation. Irradiation treatment just before gamete maturation is advantageous to acquire more M_1 hybrids with a high frequency of chromosome structural variation. The frequency of plants with at least one translocation chromosome in M_1 could be increased up to 70% through pollen irradiation of *Triticum durum-Haynaldia villosa* amphiploid. More than 100 translocated chromosomes have been identified in the BC1 and BC2. Translocations with small alien chromosome segments, 57 terminal and 80 intercalary, were induced through female gamete irradiation conducted on *T.aestivum-H.villosa* 6VS/6AL translocation line. For the 2240 Rad dosage treatment, the induction frequencies of interstitial translocation, terminal translocation and deletion were 21.02%, 14.01%, and 14.65%, respectively, which were much higher than those previously reported. The *T.aestivum-H.villosa* 6VS/6AL translocation has been used in wheat breeding and many elite cultivars, such as Nannong 9918, Neimai 9, Shimai 14, etc. have been developed and released.

Introduction

Wild relatives of crops are rich in gene resources, such as resistance or tolerance to biotic and abiotic stresses, as well as high yield and good quality. However, because of their distant genetic relations, it is difficult to introduce these useful genes into cultivars by normal crossing, chromosome pairing and recombination between homologous chromosomes. The gene transfer can be achieved by chromosome manipulation, i.e. developing amphiploid, alien addition, and substitution and translocation lines. The amphiploid contains a complete set of the alien chromosomes, and the addition or substitution lines contain a whole chromosome. In these materials, many redundant genes would be introduced into cultivated species along with the target genes. Therefore, the best way for gene transfer should be the production of translocation lines, especially interstitial translocation with a small alien chromosome segment.

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Spontaneous alien translocation could be observed as a result of occasional chromosome breakage and re-union in the process of wide hybridization, but the frequency is extremely low and the breakpoint usually occurred near the centromere and produced whole arm translocation. Ionizing-irradiation is a popular method for the induction of chromosome translocation. Irradiation of dry seeds is most convenient, but the frequency of chromosome structure rearrangement is very low. Sears (1956) developed a common wheat-*Aegilops umbellulata* translocation line through irradiating pollen of a *Triticum aestivum- Ae.umbellulata* addition line by X-ray, followed by pollinating the irradiated pollen to common wheat cv. Chinese Spring, and successfully transferred leaf rust resistance of *Ae. umbellulata* into common wheat [9]. Irradiation was also used successfully in the transfer of Fusarium head bright resistance from *Leymus racemosus* into common wheat in the form of chromosome translocation [3, 6, 7].

Haynaldia villosa Schur. (syn. *Dasypyrum villosum* Candargy, 2n=14, VV), a related species of wheat, has been reported to be resistant to powdery mildew, rusts, take all and eyespot diseases, and tolerant to drought and cold stresses. The powdery mildew and spindle streak mosaic virus resistances of *H. villosa* have been introduced into common wheat through development of alien addition, substitution and whole-arm translocation lines (4VS/4DL and 6VS/6AL) in the Cytogenetics Institute, Nanjing Agricultural University (CINAU) [1, 2, 5, 8, 10]. For further fine mapping and better utilization of useful genes of *H. villosa*, it is urgent to develop more translocation involved in different chromosomes, different regions and with various fragment sizes of *H.villosa*.

Materials and Methods

Plant materials

Triticum durum-Haynaldia villosa amphiploid and *T.aestivum-H.villosa* 6VS/6AL translocation were developed by CINAU and used as basic materials for irradiation. *T. aestivum* cv. Chinese spring was used as the recurrent parent.

Irradiation treatment

Irradiation of mature pollens

Flowering spikes of *T.durum-H.villosa* amphiploid were cut off with flag leaves, maintained with their cut lower ends in water, and irradiated with ^{60}Co-Gamma-rays (800 ~ 1600 Rad) at a dose rate 100 Rad/min. Fresh matured pollen harvested from irradiated spikes at 1 ~ 3 days after irradiation was pollinated to emasculated florets of *T.aestivum* cv. Chinese Spring. Matured hybrid seeds were harvested and sown to set up a M_1 population. Pollen collected from untreated *T. durum-H.villosa* amphiploid plants was used to pollinate Chinese Spring as a control.

Irradiation of mature female gametes

The mature female gametes, two to three days before flowering, on the plants of 6VS/6AL translocation line 92R137 were irradiated by ^{60}CO Gamma- ray using the dosages of 1600 Rad, 1920 Rad or 2240 Rad.

Q.Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, 2009, **74-76**

The irradiated spikes were emasculated the same day and pollinated with normal fresh mature pollen of common wheat cv. Chinese Spring after two to three days, and the produced hybrids were named as M_1. Irradiation treatments were carried out at the Jiangsu Academy of Agricultural Sciences.

Cytogenetics and molecular analysis The chromosome translocation between wheat and alien species were detected by genomic *in situ* hybridization (GISH)[11] and C-banding [4].

Results

Induction of chromosome translocation by irradiating mature pollen of *T.durum-H.villosa* amphiploid Fresh mature pollen harvested from irradiated spikes at about one to three days after irradiation with 800~1600 Rad was pollinated to emasculated florets of *T.aestivum* cv. Chinese Spring. The hybridization seed-set rate was 87.2~93.1%, which was similar to the control (89.6~96.0%)(**Table 1**). In 719 of 1009 M_1 plants, at least one translocation chromosome between wheat and *H.villosa* was detected by chromosome *in situ* hybridization using labeled genomic DNA of *H.villosa* as probe. The average induction frequency was 71.2%. The highest induction frequency of 96.0% was observed in the treatment of 1600 Rad (**Table 2**). The translocation chromosomes consisted of whole arm, terminal and interstitial translocations with different sizes of alien chromosome segments. These translocation chromosomes could be transmitted to the next generation by backcrossing with normal fresh pollen of common wheat Chinese Spring, and the transmission rates of the translocation chromosomes were 72.9% in the M_1 to BC_1 and 100% in the BC_1 to BC_2, respectively (**Table 3**). The transmission rate of translocation chromosome through female gametes was higher than through male gametes (**Table 4**).

Induction of chromosome translocation and deletion involved in the small segment of 6V short arm by irradiating mature female gametes of translocation line 6VS/6AL The structurally aberrant translocation chromosome (TC) and transmission ratio (TR) involved in the short arm of 6V of *H.villosa* were detected by GISH. Among the 534 M_1 plants, 97 plants with 192 structurally changed chromosomes of 6VS were identified, including 57 terminal translocation, 80 interstitial translocation and 55 deletion chromosomes (**Fig. 1**). The frequency of plants with small fragment structural changes of 6VS was as high as 18.3%. The highest induction frequency of terminal translocation (14.0%), interstitial translocation (21.0%) and deletion (14.7%) was observed in the treatment of 2240 Rad dosage (**Table 5**). The backcross seed-set rate using fresh pollen of common wheat Chinese Spring was 70.2%~ 82.5%. Most of the structural changed chromosomes observed in the M_1 were rediscovered in the M_2. These lines are potentially useful materials for chromosome-based physical mapping. Two heterozygous interstitial translocation lines with a segment of 6VS (FL0.40-FL0.70), which showed high powdery mildew resistance, were obtained.

Utilization of *T.aestivum-H.villosa* translocation 6VS/6AL with *Pm21* The powdery mildew resistance of *H.villosa* has been transferred into common wheat through the development of 6V addition and 6V(6A) substitution lines, and the resistance gene was located on chromosome 6V [1, 8, 5]. *T.aestivum-H.villosa* translocation line 6VS/6AL with powdery mildew resistance was produced by irradiation of the F_3 dry seed derived from the cross of T.aestivum cv.Yangmai 5 / *T.aestivum-H.villosa* substitution 6V(6A) in CINAU. The powdery mildew resistance gene was further located on the short arm of 6V and designated as *Pm21* [2]. Up to now, using the translocation lines as parents, new varieties including Nannong 9918, Neimai 8~10, Shimai 14, Shimai 15, Zhongyu

Table 1. Hybridization seed-set rates in different treatment dosages.

Treatment	2006			2007		
	No. of florets pollinated	No. of hybrids obtained	Percentage of seed set	No. of florets pollinated	No. of hybrids obtained	Percentage of seed set
0R	317	284	89.6	227	218	96.0
800R	855	759	88.8	612	570	93.1
1200R	763	665	87.2	843	781	92.6
1600R				1107	1003	90.6

[OR]control

Table 2. Effect of different dosages on the production of intergeneric translocations.

Treatment	2006					2007				
	No. of plants observed	No. of plants with TCs	Occurrence frequency of TCs	No. of total TCs	No. of TCs per plant	No. of plants observed	No. of plants with TCs	Occurrence frequency of TCs	No. of total TCs	No. of TCs per plant
800R	98	54	55.1	112	1.14	93	46	49.5	83	0.89
1200R	98	75	76.5	165	1.68	93	70	75.3	177	1.90
1600R						100	96	96.0	239	2.39

[TCs] translocation chromosomes

Table 3. Recovery analysis of translocation chromosomes in different generations.

Generation	No. of plants investigated in the former generation	No. of TCs investigated	No. of plants investigated in the later generation	No. of TCs recovered	Recovery frequency %
M_1-BC_1	18	48	65	35	72.9
BC_1-BC_2	22	39	370	39	100.0
BC_2-BC_3	18	20	247	20	100.0

[TCs] translocation chromosomes

9 and Yuanzhong 175 etc., have been developed and released from different breeding institutes of China, and a number of elite lines have been selected for national regional tests.

Table 4. Analysis of translocation transmission by male and female gametes from BC2 to BC3 generation.

	No. of plants investigated	No. of plants detected with translocation	Transmission rate (%)
Female gametes	221	75	72.9
Male gametes	221	56	100.0

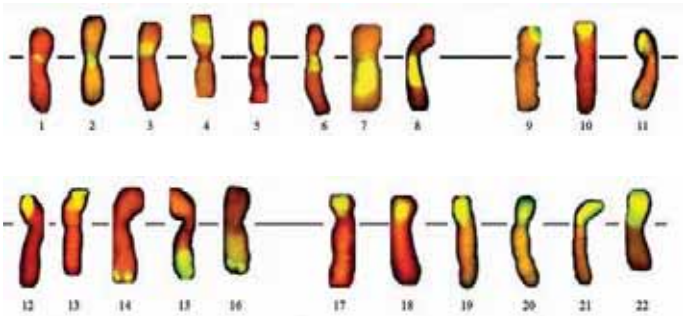

Figure 1 Part of structurally changed chromosomes involving the short arm of 6V chromosome of *H.villosa* detected by GISH in M_1 plants. 1-8: interstitial translocation chromosomes with small fragments, 9-16: terminal translocation. chromosomes, 17-22: deletion of chromosome 6VS.

Table 5. Effect of different irradiation dosages on the induction frequency of small fragment structural changes of 6VS.

Treatments	Inducing frequencies of small fragment structural changes	Inducing frequencies of interstitial translocation	Inducing frequencies of terminal translocation	Inducing frequencies of chromosome deletion
2240Rad	24.84	21.02	14.01	14.65
1920Rad	21.30	18.93	12.43	10.06
1600Rad	10.58	7.21	6.73	7.21
Control	0.00	0.00	0.00	0.00

Discussion

For mass production of various translocation lines involving different alien chromosomes or chromosome fragments, the *T.durum-H.villosa* amphiploid containing a complete set of *H.villosa* chromosomes were used for irradiation and at least one translocation chromosome was observed in 72% M_1 plants. The identified translocation chromosomes involved different fragments and regions of chromosome 1V to 7V of *H.villosa*. These translocation lines could be further used for the construction of translocation pools and were useful genetic resources for the introgression and further utilization of alien genes as well as physical mapping of the target genes.

In order to improve the efficiency for creation of interstitial translocations, the whole arm translocation 6VS/6AL was used in the present research for irradiation. In this case, one breakage in the alien chromosome can generate small fragment interstitial or terminal translocations, or deletions of the alien chromosome. We found that not only small fragment terminal translocations and deletions but also intercalary translocations were observed at a high frequency.

The increase of dosage and dosage rate will significantly increase the frequency of breakage-reunion events, including double breakage-reunion events, hence producing more chromosome structural changes, especially interstitial translocation. We used mature female gametes of 6VS/6AL for irradiation because female gametes were less lethal-sensitive and could endure higher dosages and dosage rates. The female gametes were irradiated just before fertilization and were pollinated with normal fresh pollen after irradiation. Like this, the structural aberrations had more chances to be involved in the fertilization process before restoration or elimination and transmitted to the next generation. These irradiated female gametes could be pollinated with mature and fresh pollen of normal wheat. This avoided the elimination of structurally aberrant chromosomes due to the fertilization competition mainly occurring in the male gametes, and a high proportion of the chromosome aberrations could be saved in the M_1 plants. A small fragment interstitial translocations with high powdery mildew resistance were identified in the backcrossing progenies by GISH and powdery mildew resistance evaluation.

As more disease resistance genes were cloned from model or other plant species, it was found that resistance genes were often present as a gene cluster in a specific chromosome region. By the development of alien translocation lines, more than one single useful gene can be introduced simultaneously without any safety issues brought by genetic modification. The translocation lines are genetically stable and their resistance is more durable compared with single-gene transfer. The wheat-rye 1RS/1BL translocation has been successfully utilized in breeding programmes worldwide, one important reason is that several useful genes, especially disease-resistant genes, are located in 1RS. More and more translocation lines, especially intercalary translocations with multiple useful traits will be used in modern wheat breeding with the accelerated development of various translocation lines involved in different alien species.

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Induced Mutation in Narrow-Leafed Lupin Improvement: An Example of Herbicide Tolerance

P Si[1,*], B Buirchell[2] & M Sweetingham[2]

Abstract

Spontaneous mutation has been discovered and utilized in domestication of narrow-leafed lupin (*Lupinus angustifolius* L.). As a result of domestication, lupin has become a dominant grain legume crop in Western Australia. Facing the new challenge of developing herbicide tolerant cultivars, chemical mutagenesis has been used to create new tolerance to herbicide. This paper reports the characterization of two lupin mutants (Tanjil-AZ-33 and Tanjil-AZ-55) that are highly tolerant to metribuzin herbicide. A dose response study of over eight doses revealed that Tanjil-AZ-33 was six times more tolerant to metribuzin than the original parental cultivar Tanjil, as measured by LD_{50}. This mutant Tanjil-AZ-33 is the most tolerant germplasm in narrow-leafed lupin. Both mutants also maintain the high resistance to the disease anthracnose as in cv Tanjil. Seed yield based on small field plots (3.6 m^2) under irrigation was 4.2 t/ha for Tanjil-AZ-33 and 1.9 t/ha for Tanjil when the seedlings were subjectted to 300 g/ha metribuzin at the six-leaf stage. Seed yields of both Tanjil–AZ-33 and Tanjil-AZ-55 were similar to Tanjil in absence of the herbicide. These facts indicate that the mutation process has created tolerance to metribuzin in Tanjil, without altering its yield capacity and anthracnose resistance. The mutant Tanjil-AZ-33 has been used as a parent in the lupin breeding programme and we expect future lupin cultivars to have increased metribuzin tolerance. Induced mutation proved to be an effective tool in lupin improvement.

Introduction

Narrow-leafed lupin (*Lupinus angustifolius* L.) is a wild native species of the Mediterranean region that has become a major grain legume crop in Australia since the release of the first sweet cultivar Uniwhite in 1967. Domesticating the plant for modern agriculture involved discovery and utilization of spontaneous mutants of several key domestication genes [1]. For example, Uniwhite contains natural mutants of non-shattering, low-alkaloid and soft-seed genes. The early flowering mutant *Ku* gene, present in cv Unicrop released in 1973, brought flowering forward by two to five weeks and allowed narrow-leafed lupin to be grown as a successful commercial crop in the Mediterranean climate of Western Australia [2]. Most cultivars released after Unicrop have disease resistance in addition to these domestication genes.

Under the current lupin production system in Australia, the top agronomic issues are weed and disease management control. Cultivars with increased tolerance to herbicides are needed to expand weed management options in the minimum tillage farming systems. The anthracnose resistant cultivar Tanjil has been widely used as a parent in the lupin breeding programme in Australia as a source of anthracnose resistance. Unfortunately, it was found to also be sensitive to metribuzin herbicide [3]. Therefore, improving tolerance to metribuzin in progenies containing Tanjil parentage has become very important.

One approach to improve tolerance to metribuzin in Tanjil is to create it through chemical mutagenesis. Mutants induced from cv Tanjil have been selected for metribuzin tolerance and several mutants were found to be highly tolerant to metribuzin [4]. This paper reports the characterization of two highly tolerant mutants in terms of LD50, seed yield in the presence and absence of metribuzin application, and anthracnose resistance in the disease nursery.

Materials and Methods

Two metribuzin tolerant mutants Tanjil-AZ-33 and Tanjil-AZ-55 [4], along with the metribuzin-sensitive cv Tanjil (the original parent of the mutants) and the metribuzin tolerant cv Mandelup were examined in a dose response study involving eight doses, four replicates and 20 plants per replicate. Experimental procedures were the same as reported in [3].

The two mutants were compared with cultivars Tanjil and Mandelup for seed yield when 300 g/ha metribuzin was applied to seedlings at the six-leaf stage with boom spray fitted on a motorbike with output of 72 L/ha. Metribuzin at 300 g/ha was applied on July 7, 2006 to six to eight leaf stage lupin plants. Herbicide damage was scored at three weeks after spray. Plants were grown in 3.6 m^2 (1.5 x 2.4) plots sown at 50 seeds/m2. Plants of each plot were harvested by hand at maturity and seed yield obtained.

For seed yield measured in the absence of metribuzin at regional sites, trial preparation and management was the standard of field evaluation of breeding materials by the lupin breeding programme at the Department of Agriculture and Food of Western Australia.

Assessment of anthracnose resistance was conducted in the irrigated disease nursery at the Medina Research Station, Western Australia in 2006 and followed the same protocols as used for all the breeding materials from the lupin breeding programme.

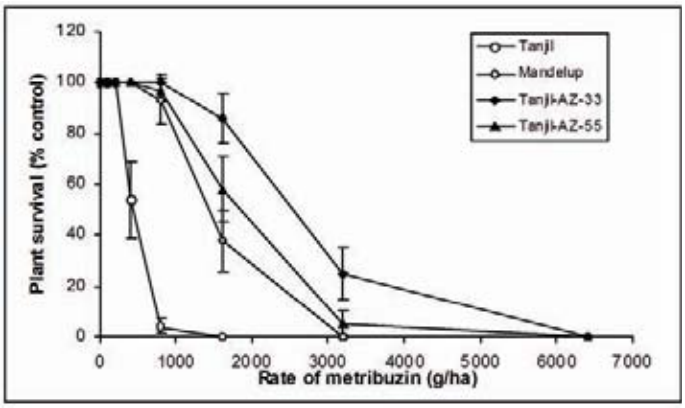

Figure 1 Percentage of plant survival of induced mutants Tanjil-AZ-33 (♦), Tanjil-AZ-55 (▲) compared with original parent cv Tanjil (○) and a tolerant cv Mandelup (◊) in metribuzin dose responses with plants grown in a 20/12°C (day/night) phytotron.

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Results

Dose response

At high herbidice rates (**Fig. 1**), mutants Tanjil-AZ-33 and Tanjil-AZ-55 had a greater percentage of survival than the wild type Tanjil. At the metribuzin rate of 800 g/ha, all Tanjil plants died whilst the mutants survived at 100%. The difference by LD_{50} between the mutant Tanjil-AZ-33 and Tanjil was six-fold. The mutant Tanjil-AZ-33 had much higher survival than Mandelup at rates greater than 1600 g/ha, suggesting a tolerance even greater than that of cv Mandelup.

Seed yield in the presence of metribuzin

Seed yield of mutant Tanjil-AZ-33 was 4.23 t/ha under irrigation, more than twice that of Tanjil when they were subject to metribuzin at 300 g/ha during the six to eight leaf stage, but very close to that of the tolerant cultivar Mandelup (**Table 1**). Both mutants showed no symptoms of leaf damage from metribuzin at three weeks after application whilst Tanjil plants were severely damaged.

Table 1. Seed yield of metribuzin tolerant mutants subject to 300 g/ha metribuzin in the field at Shenton Park in 2006.

Genotype	Seed yield (t/ha)	Visual damage score against metribuzin[A]
Tanjil-AZ33	4.23	0
Tanjil-AZ55	2.62	0
Tanjil	1.86	3
Mandelup	4.63	0
Lsd. (P<0.05)	1.19	

[A] Damage score against metribuzin with 0=no symptom, 3=most plants had scorch on first six leaves and 5=plant dead.

Seed yield in the absence of metribuzin

Seed yield of the two mutants Tanjil-AZ33 and Tanjil-AZ55 in the Stage 2 field evaluation (in the absence of metribuzin) was not significantly different (P <0.05) from that of Tanjil at three regional locations in Western Australia in 2006 (**Table 2**), although the actual seed yield varied from location to location. Mandelup had significantly higher yield than the two mutants at the sites of Merredin and Wongan Hills.

Anthracnose resistance

The visual scores of resistance to anthracnose of the mutants Tanjil-AZ 33 and Tanjil-AZ 55 were not significantly (P < 0.05) different from those of Tanjil in the disease nursery in 2006 (**Table 2**), suggesting a similarly high resistance to anthracnose. The mutants had slightly higher scores than cv Mandelup.

Table 2. Anthracnose resistance and seed yield (t/ha) of mutant lines in the absence of metribuzin at regional sites in 2006.

Variety	VarietyAnthracnose resistance score[A]	Badgingarra	Merredin	Wongan Hills
Mandelup	6.0	1.14	1.38	1.52
Tanjil	7.0	1.08	1.12	1.29
Tanjil-AZ-33	6.8	1.05	1.01	1.26
Tanjil-AZ-55	7.0	0.99	0.99	1.27
lsd (P = 0.05)	1.0	0.34	0.32	0.22

[A] Disease resistance was evaluated in disease nursery at Medina Research Station in 2006. 1=plants severely damaged, 9=plants immune.

Discussion

Two mutants induced from the metribuzin-sensitive Tanjil are highly tolerant to metribuzin, with a six-fold increase in tolerance to metribuzin compared to the original parent Tanjil as revealed by the dose response study, thus confirming that induced mutagenesis is a useful breeding tool to develop tolerance to herbicides. Mutation breeding has been successfully used in soybean for an increased tolerance to sulfonylurea herbicide [5]. The mutant Tanjil-AZ-33 has even greater tolerance to metribuzin than the tolerant cultivar Mandelup. Mandelup was released in 2004 as a high-yielding, early-flowering cultivar tolerant to metribuzin in the field at recommended rates. The degree of metribuzin tolerance in Mandelup is similar to other tolerant cultivars [3]. Tanjil-AZ-33 is the most herbicide tolerant genotype in the lupin germplasm collection. This, in fact, indicates that mutation created new tolerance. Mutants with tolerance better than Mandelup are valuable sources for the development of lupin cultivars with greater tolerance. Tanjil-AZ-33 has been used as a parent as a source of metribuzin tolerance in the breeding programme.

Cultivars with greater tolerance would lead to greater safety margin to this herbicide and could potentially lead to higher application rates to allow better weed control. When 300 g/ha metribuzin was applied to seedlings at the six-leaf stage, tolerant mutants had no symptoms of foliage damage whilst Tanjil seedlings were severely scorched. Consequently, seed yield of Tanjil-AZ-33 was twice that of Tanjil in presence of metribuzin, even though seed yield potential of the mutants is similar to Tanjil in absence of metribuzin as tested in the Stage 2 field trials across three locations. Seed yield of Tanjil-AZ-55 was lower than Tanjil-AZ-33, largely due to the observed damage caused by thrips during flowering. Mandelup yielded higher than Tanjil and the two mutants in the Stage 2 field trials. However, seed yield of Tanjil-AZ-33 was comparable to Mandelup at 300 g/ha metribuzin. It is expected that new cultivars combined with the high yielding background of Mandelup and high metribuzin tolerance of Tanjil-AZ-33 would be able to tolerate higher herbicide rates and coupled with a greater safety margin and hence higher yield.

The two mutants Tanjil-AZ-33 and Tanjil-AZ-55 were selected among several other tolerant mutants on the basis of their carrying the molecular marker of anthracnose resistance gene [4]. Presence of the specific molecular marker for Tanjil's resistance to anthracnose in all seedlings of the two mutants suggests that these mutants retained the anthracnose resistant gene [6]. We hoped that some mutants would retain the anthracnose resistance of Tanjil but also show an improvement in the tolerance to the herbicide. Anthracnose resistance screening in the disease nursery confirms that both mutants are as highly resistant to anthracnose as Tanjil.

In conclusion, induced mutagenesis has created two new mutants highly tolerant to metribuzin, but has not altered the characteristics of Tanjil in yielding capacity and anthracnose resistance. The induced mutant Tanjil-AZ-33 has replaced the original parent Tanjil as parental source for both anthracnose resistance and metribuzin tolerance in the lupin breeding programme. Mutation breeding will continue to provide genetic variation for the improvement of lupin required to adapt to the changing farming systems.

ACKNOWLEDGMENTS

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Results of Utilization of Chernobyl Radio Mutant in Breeding Programmes of *Triticum aestivum* L.

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Abstract

A large spectrum of mutations was observed as a result of winter wheat *T.aestivum* L. irradiation for two consecutive vegetative seasons (during 1986-87) in the fields close to the ruined reactor of the Chernobyl Nuclear Power Station. Mutants taken from the different generations (L147/91, BC 47 square head, dwarf 20104/89) were used for development of the varieties Lybid, Yasochka and Tsarivna to utilize traits from the mutants such as hardiness, drought tolerance, disease and lodging resistance and bread quality. These varieties were included in the State Variety Register of Ukraine, while another one, Lisova Pisnya is included in the list of perspective varieties.

Introduction

The genetic changes in winter wheat that occurred due to the ionizing radiation, which appeared as a result of Chernobyl Nuclear Power Plant accident, were investigated during 1988-2007 at the research station in Bila Tserkva. Irradiation received by plants was chronic. External gamma-irradiation, internal irradiation, irradiation by beta and alpha particles from incorporated radionuclide formed a cumulative incorporated doze. [1] 239 accessions of common wheat, which during two years in plantations in 1986 and in self-sowing 1987, grown near the Chernobyl Reactor were picked up by academician D.M. Grodzinsky, and professors P.K. Shkvarnikov and V.F. Batygin, who kindly provided them to our station for further investigation and analysis. The objective was to assess their possible utilization in breeding by selecting among them the mutants with attractive agronomic important traits to be subsequently introduced in breeding programmes. Another goal was to identify the remote consequences of the irradiation, which may later be used to forecast genetic changes for numerous populations remaining in the zone polluted by the radionuclide. [2]

Results and Discussion

The four varieties of common wheat, Bilotserkivska 47, Poliska 70, Myronivska 808 and Kyianka were planted at the fields of Chernobyl area before the accident. Even in M_2, a large spectrum of mutations was discovered. Because of genetic instability, the mutant diversity increased each year and, to date, the collection includes up to 2,000 mutants. [3] Plants with different kinds of abnormalities in structure appeared among mutants of different generations. We called them chimerical plants and they had no value for breeding. Moreover, the direct selection from mutants of all studied varieties did not yield positive results due to the high instability of all characteristics in many generations. This was particularly true for the segregation in plant height and morphology observed as well as for yield and gluten quality.

At the same time, several selected lines of mutants that showed some advantageous agronomic characteristics, were applied to the breeding programmes. The first one was Mutant 20168/89, where even in M_3 segregation was observed in the progeny of seeds collected from one ear: 54% of plants had square head ear with awns, a stem height 90-105cm and were resistant to brown rust, whereas 46% of plants closely resembled Myronivska 808 - lyutestsens sub-variety, with a stem height of 116cm and were highly susceptible to lodging and brown rust.

In M_4, a heterozygous ear mutant of Lyutestsens 147 (L147) was selected. The segregated population from this mutant (M_5) exhibited 89% awnless plants with a normal ear density, 10% of plants appeared to be square head form and 1% of plants were small chimeras with a height of 25cm. A separate segregation by height was also observed simultaneously, with variation between 75 and 110cm. Genetic instability of Mutant 20168/89 was also observed in later generations. (**Fig. 1**)

Figure 1 Splitting of genetically unstable mutant – 20168/89 in later generations (M6-M12): 1 – Lyutestsens with normal ear density L147/91, which became the female parent in the creation of Lybid and Yasochka; 2 – compactoid semi-awned with twisted ear stem; 3 – "transparent" ear without ear stem, with spikelets attached to each other; 4 – ear with deformed awns and sterile upper part; 5 – awnless squared head; 6 – well developed awned squared head with noticeable changes in the middle of the ear.

In M_6, the segregation of L147 continued by ear morphology and plant height. In this population the constant mutant L147/91 was selected, awnless and with high resistance to brown rust, septoria and *Fusarium* spp., because of which the plants have green leaves during all stages of ripening. This mutant has high gluten content in seeds – up to 41-48%, coupled with low quality of this gluten.

Further, mutant L147/91 with normal awnless ear was selected as a female component for crossing with the strong common wheat variety Novoukrainka Bilotserkivska. This was aimed at decreasing height and increasing thickness of the stem of cv. Novoukrainka Bilotserkivska while simultaneously improving its resistance to a number of diseases.

An F_2 population of 2,000 plants from those progenitors was studied and 105 among them were collected and sown. In the F_3 population from those plants, 23 families were selected for control nursery. Such lines were highly productive, winter-resistant (especially in winter conditions

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with snow) as well as lodging-resistant, and they had high gluten content of good quality. Therefore, in this crossing the low gluten quality derived from mutant L147 was inherited as a recessive trait and the level of gluten content as heterozygous.

Line № 728/98, which was selected for further investigation in control nursery, was registered in Ukraine in 2006 as variety Lybid` and recommended for cultivation in all zones of Ukraine. This variety is awnless, with an intermediate date of ripeness, high winter resistance (it successfully passed the 90-days of ice crust winter condition in 2003), drought tolerant, has resistance to brown rust, powdery mildew and septoria. It is a semi-dwarf, highly productive variety and belongs to the strong wheats. A maximum productivity of 9.6 t. per hectare was achieved by Bila Tserkva State Variety Testing Station (SVTS) of Kiev State Center for Plant Variety Expertise (CPVE) during the very dry 2007 season, which superseded the standard variety Podolianka by 0.9 t. per hectare.

Mutant L147/91 was also successfully used as female parent with variety Napivkarlyk 3 crossing, resulting in the creation of variety Yasochka. Unlike regular crossings with stable varieties, splitting by awn trait was recorded immediately in F_1: crossing of two awnless parental forms generated 1% of awned offspring. A wide variety of recombinants was also observed in further generations. There were 56 hybrid lines studied in F3 with 14 selected for the control nursery. A wide variability was obtained, both morphologically and for traits useful in breeding.

Thus, the variation in the control nursery for winter resistance ranged from three to four (on a five-point scale); brown rust resistance ranged from 2% to 65%, yield from 5.4 to 7.4 t. per hectare, stem height from 90 to 100cm, gluten content from filler with Gluten Deformation Index (GDI) of 113 to strong wheat with 15% of protein and GDI of 78. The difference in ripening season reached 10 days. The most productive line from this crossing, 199/02, was included after the state variety testing in the State Plant Variety Register for 2006 as Yasochka variety. Yasochka is awned, of mid-size height and mid-term ripening. It has a high drought tolerance, above average winter resistance and big kernels, having inherited resistance to brown rust, septoria, *Fusarium* spp. and lodging from mutant L147/91. Gluten quantity was also inherited from the mutant L147/91 with no connection to gluten quality. As a result, the variety was classified among strong wheats by gluten. A maximal yield of 8.5 t per hectare was achieved in 2004 in Vinnytsa CPVE and 8.4 t per hectare in Dnipropetrivsk CPVE. Yasochka is recommended for cultivation in the Steep-forest region.

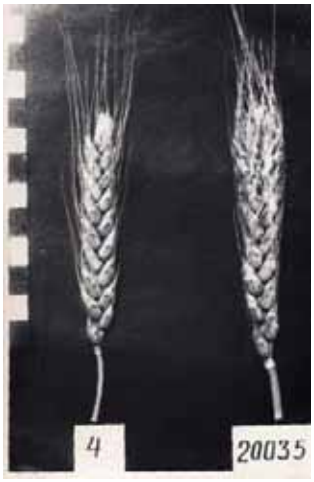

Figure 2 Mutants of Bilotserkivska 47 common winter wheat. 4 – parental variety Bilotserkivska 47, 20035 – mutant of Bilotserkivska 47 – female form in crossing leaded to development of varieties Tsarivna, Lisova Pisnya and Romantica.

In the collection of Chernobyl mutants the most numerous and least stable are a group of mutants of Bila Tserkva 47 – BC 47 squared head (BC 47 sqr). In M_3 they differ from the original variety by thicker upper half of the ear, meaning they possess a squared head ear. In 1989, there were 40 families of such mutants, with 25% of them awned and of even height. Others got split by height from 85 to 105cm, different morphological traits of the ear stem and leaves. In different generations different systemic mutations were noted. They possessed traits of other species: *T.spelta* (L), *T.compactum* (Host) and *T.vavilovi* (Tum. i Jakubz). [4] All of them, like the original genotypes, belong to hexaploid wheat. Some mutations had no noticeable morphological differences but had different quantitative traits such as productivity, bread quality, winter resistance, disease resistance etc., of practical application for breeding. For their identification, analysis of useful traits was conducted along with the study of morphological changes, whreby mutant BC 47 sqr. # 774/89 (**Fig. 2**) was selected.

Unlike many other mutants in this group (**Fig. 3**), BC47 had no significant deviations from normal ear structure with an exception of hardly noticeable square head and doubled spikelets on some parts of the ear. In addition, during nine years of testing (1991-1999), BC 47 sqr. has proven to be winter-resistant and of high bread quality. After multiple selections by pedigree method, BC 47 sqr. became more stable and was introduced for crossing with a steppe ecotype variety, Odesska 162, to improve winter resistance of the latter. After multiple selections from the progeny, three selected lines became new varieties: Tsarivna (included in the State Registry in 2008), and Lisova Pisnya and Romantica (included in 2009). Romantica variety is presently under state testing. All three varieties were found to be winter-resistant when tested in freezers, classified as belonging to the strong wheat group, and having high productivity. Maximal productivity of Tsarivna variety is 8.8 t. per hectare, while 9.0 t. per hectare was achieved with Lisova Pisnya in the Variety Study Centre in Kiev region in 2006.

Figure 3 Splitting of genetically unstable mutant of Bilotserkivsra 47 in M9: 9.- spikelets after threshing; 11.- T. spelta; 10,12- Spelta-Compactum; 14- spikelets are on a single side of ear stem.

Varieties obtained from crossing with radio mutants are characterized by high drought tolerance. In dry 2007 in Steppe region (Kirovograd Testing and Breeding Station), Tsarivna yielded 8.3 t per hectare and Lybid yielded 8.5 t. per hectare, which is higher than the standard variety Podolyanka by 0.9 and 1.1 t. per hectare, respectively. In the marshy woodlands zone at Borodianska Testing and Breeding Station, Lisova Pisnya delivered 6.2 t per hectare, outperforming Podolyanka by 2.8 t per hectare.

Another mutant, dwarf 20104/89, was found to be a prospective parent for future selection. In M3, it was heterozygous by many traits, the progeny obtained from one ear having a height ranging from 48 to 76cm, with compactum-type short ear. In the upper part of the ear many

spikelets had sterile flowers. In M_4 and M_5, the splitting continued with square headed, awned and awnless forms, tall and dwarf plants, early and late ripened plants. In M_6 a dwarf plant (60cm tall), with a strong stem and good ear density, but with low productivity was selected, and was introduced for breeding with the good bread-making quality variety Novoukrainka Bilotserkivska, which was susceptible to lodging. Transgression by productivity was obtained as a result. In the control nursery 24 numbers out of 63 surpassed the productivity of the original variety. The most productive lines achieved 7.7-7.8 t per hectare. They were resistant to lodging and to brown rust belonging to strong wheats. The best line, named Vidrada, was taken for the state testing. From dwarf mutant 20104/89 it inherited strong stem and quality of the strong wheat.

Thus, disease resistance (L 147/91), lodging resistance (20104/89), drought tolerance and winter resistance traits (BC 47 sqr.), plus gluten content and gluten quality (20104/89 and BC47 sqr.) were utilized for breeding. The use of stable (after multiple selections) lines of Chernobyl mutants as parental genotypes in crosses enhanced the genetic pool of winter wheat and helped to develop highly productive varieties with good bread-baking qualities and increased adaptive potential for hostile environments.

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Sunflower Mutants with Improved Growth and Metal Accumulation Traits Show a Potential for Soil Decontamination

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Abstract

Over the last two decades, the use of plants has been proposed as an alternative technique to remove toxic metals from contaminated soils. This technique, called phytoextraction, can use either hyperaccumulating species, able to accumulate and tolerate high amounts of metal, but producing low biomass, or high-yielding crops compensating moderate metal accumulation by a high biomass. Both types of plants can be considered for metal removal, but soil decontamination still takes quite a long time. Therefore, plants used for metal removal need to be improved.

This paper summarizes our previous and present work aimed at the improvement of sunflowers for phytoextraction by chemical mutagenesis. Improved yield and metal accumulation in sunflower mutants were already observed in the M_2 mutant generation, where three new sunflower phenotypes were found: mutants with a significantly enhanced biomass production and no changed metal accumulation; mutants with a slightly improved biomass production and an enhanced metal accumulation in shoots; and mutants with reduced metal uptake. The same alterations in growth and metal accumulation were observed in the following generation. The best M_3 sunflower mutants showed a three to five times higher cadmium, a four to five times higher zinc, and a three to five times higher lead extraction, as compared to the control inbred line. The stability of improved traits, yield and metal uptake, was confirmed also in the fourth generation, where mutant lines still provided a significantly enhanced metal extraction.

Metal translocation from root to shoot and distribution within the shoot (stem, leaves and flower) of mutant lines and control sunflowers grown on a metal contaminated soil was studied in detail in the fifth generation under greenhouse conditions. Sunflower mutant seedlings show a very good metal translocation capacity after three months of cultivation on contaminated soils; thus the metals were primarily accumulated by sunflower leaves.

Introduction

Soils contaminated with metals (such as cadmium, chromium, nickel, zinc, lead, etc.), arsenic, and various radionuclides are nowadays a major environmental and human health problem. Main sources of soil contamination are the metal smelting industry, residues from metalliferous mining, combustion of fossil fuel, sewage sludge, waste incineration, car exhausts as well as some pesticides and fertilisers used in agriculture. In the European Union, more than 16% of the total land area, an estimated 52 million hectares, is affected by some level of soil degradation [1]. In contrast to the organic contaminants, which can undergo biodegradation, heavy metals cannot be destroyed and remain in the environment. Moreover, they can enter the food chain via agricultural products or leach into drinking water. Therefore, there is a need for an effective and affordable technological solution for soil remediation.

Nowadays, phytoremediation is becoming very popular as a novel strategy to clean up polluted soils. This decontamination technique needs green plants and their associated micro-organisms, soil amendments and agronomic techniques to remove, contain or render harmless environmental contaminants [2, 3]. A great scientific and commercial interest now focuses on a phytoremediation strategy called phytoextraction. It is based on the ability of plants to take up, transport and concentrate metals from the soil into the above-ground parts of plants [3, 4]. Plants used for phytoextraction have to be finally harvested, then disposed or converted into valuable products. The main advantage of metal phytoextraction is *in situ* application without further disturbance of the site. Another advantage is a lower cost than conventional methods to decontaminate land. The possible recycling of metals and recovery of bioenergy could provide further economic advantages of phytoextraction. One of the possible limitations of this method is that its applicability is restricted to the upper soil layers and low or moderately contaminated soils [5]. The greatest disadvantage of metal phytoextraction is the need of a long cleaning up time. The phytoextraction process should preferably not exceed a few decades [6, 7]. The interest of many scientists is thus focused on a reduction of time needed for phytoextraction.

Based on long years of experimentation, two groups of plants are considered to be useful for metal phytoextraction: (1) hyperaccumulator species (e.g. *Thlaspi cearulescens* L., *Arabidopsis halleri* L.) which can accumulate and tolerate metals that are toxic to other organisms even at low dosage [8, 9], but produce low biomass; and (2) high biomass producing species, like Helianthus annuus L. [10, 11]. Although these plants are among the best candidates for phytoextraction, they are still not efficient enough to remove sufficient amounts of metals from the soil within 10 years. For a practical use of this green technology, it is necessary to enhance phytoextraction efficiency.

Phytoextraction efficiency can be enhanced either at the level of soil, using fertilizer and chelating agents to enhance metal bioavailability [12, 13] or at the plant level, improving insufficient metal uptake characteristics of high yielding crops or increasing the biomass of hyperaccumulators. The main attention of improvement of phytoremediation technology is focused on the achievement of high shoot metal concentration in high-yielding plants [14]. Genetic engineering as well as traditional breeding (classical mutation and *in vitro* breeding techniques) may help to improve the existing insufficient capacity of the metal phytoextraction by high-yielding crop species [15, 16, 17].

This paper gives an overview about the possible use of chemical mutagenesis to improve the capacity of sunflowers for metal uptake, accumulation and removal.

Mutagenesis as a tool to improve metal tolerance and metal accumulation by plants

Mutation techniques have contributed significantly to world-wide plant improvement, including yield, oil quality, disease, salt and pest resist-

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ance in crops. According to the FAO/IAEA Mutant Varieties Database, more than 1840 mutant varieties involving 164 plant species have been officially released up to December 1997 and some of them have made an outstanding impact on the productivity of a particular crop [18, 19]. In some countries mutant varieties of economically important crops, e.g., barley, durum, wheat and cotton occupy the majority of cultivated areas. Mutation techniques have also been successfully used for the induction of Al tolerance in wheat [20] and barley [21, 22]. Based on the fact that ionizing treatments and also certain chemical mutagens could induce a lot of useful alterations in the genome of crop plants, artificially induced mutations might lead as well towards crop improvement of metal accumulation.

Chemical mutagenesis has already been used to obtain new mutant variants with enhanced metal accumulation traits. Mutant seedlings of *Arabidopsis thaliana* L. accumulate a 7.5 times higher amount of manganese and 4.6 times more copper from the soil than the control [23]. Zinc accumulation is enhanced by a factor of 2.8 and magnesium by a factor 1.8 in the mutant variants. It was found that this recessive mutation shows a positive correlation with ferric-chelate reductase activity. Two cadmium-tolerant mutants, initially assessed by root growth, have been isolated from ethyl methanesulphonate (EMS) mutagenised *Arabidopsis* seeds. One mutant, *cdht1*, shows an LD50 of 200 μM Cd versus an LD_{50} of 110 μM Cd for the control plants. The mutants *cdht1* and *cdht4* accumulate 2.3 times less cadmium than control plants exposed to 150 μM $CdCl_2$ [24]. Induced mutations have also been used for rapid creation of variability in Al tolerance in barley. Thirteen mutants with increased levels of tolerance to Al have been selected in the M_3 generation after mutagenic treatment of four barley varieties with N-methyl-N-nitroso urea and sodium azide (NaN_3) [21]. An enhanced aluminium (Al) tolerance has also been observed in barley cell lines obtained through mutagenesis by EMS, sodium azide and Gamma-rays [22]. The chemical mutagen EMS has also been used recently to improve metal uptake and accumulation properties in *Helianthus annuus* L. [25].

Mutagenesis of sunflowers to improve metal uptake, accumulation and extraction capacity

Over the last 40 years, mutagenesis has played an important role to improve agronomic characteristics of *Helianthus annuus* L., one of the most important oil seed crops in the world. An increased variability in the fatty acid composition in oil of sunflower mutants, obtained from seeds mutagenized with EMS has been reported [26]. Chandrappa [27] has obtained new sunflower mutants with enhanced oil content and enhanced biomass production after mutagenesis with EMS or DES (diethyl sulphate). In another study [28], sunflower mutants of M_2 and M_3 generations with high linoleic acid content for diet food and mutants with high oleic acid content for special purposes like frying oils after EMS mutagenesis were developed and characterized.

New sunflower variants promising for phytoextraction have been developed by means of chemical mutagenesis by [25] and tested on a metal-contaminated field in Switzerland. Sunflower seeds (about 8,000) of inbred lines have been treated with 0.08 M EMS for 5 h. Mutagenised seeds have been directly sown out on a sewage sludge contaminated field with total metal concentrations of 0.9 Cd, 813 Zn and 492 Pb mg/kg dry weight [25].

Sunflower mutants of the M_1 and M_2 generations have been cultivated under free-land conditions to assess the effect of mutagenesis on yield, metal accumulation and extraction characteristics by sunflowers on metal-polluted soil. The mutant screening was done in the second generation, where 320 mutants were investigated. Results of cadmium, zinc and lead shoot concentrations in the tested mutant plants showed three phenotypes: 1) mutants with a significantly enhanced biomass, but no changed metal tissue concentration; 2) mutants producing a higher biomass with an enhanced metal concentration; and 3) mutants with a reduced metal concentration (exclusion) in the shoots, potentially interesting for improving food safety [25]. Biomass production and the ability of metal shoot accumulation in plants are two key factors for an efficient phytoextraction. Metal extraction (metal accumulation x dry weight) was thus the main criterion for sunflower mutant screening in the field experiments. In the M_2 generation the best sunflower mutant showed a strongly enhanced metal extraction, as compared to the control plants: Cd 7.5 x, Zn 9.2 x and Pb 8.2 x [25]. Ten individual M_2 lines with the significantly increased biomass and ten individual M_2 lines with improved biomass and an enhanced metal shoot accumulation were selected for a further study. Descendants of these mutant clones are termed M_3 and M_4 generation. To study the distribution and stability of the selected traits we analyzed individual descendants of these 20 lines and present here the overall results for the whole population without considering the individual mutant families.

The next two generations of sunflower mutants (M_3 and M_4) were grown on the metal-contaminated field to study the stability of improved yield and metal uptake traits. In the third and fourth generations, these sunflower mutants also produced a significantly higher biomass leading to an improved metal extraction than the control inbred line (**Fig. 1**). The best individual M_3 sunflower mutants of particular lines showed a three to five times higher cadmium, a four to five times higher zinc, and a three to five times higher lead extraction as compared to the non-mutagenized sunflowers. The most interesting M_3 sunflowers were self-pollinated and tested for their metal removal efficiency in the fourth generation. The improved growth and metal accumulation/extraction characteristics were assessed for each mutant clone individually to study the stability of the trait or the possible heterogeneity between sunflowers originating from the same mutant line. Results showed that the mutant lines kept the improved traits of yield and metal extraction. The following enhancement of metal extraction by mutants was found in the M_4 generation: cadmium thee to four times, zinc five to seven times, lead six to eight times and chromium five to seven times higher than control sunflowers [29].

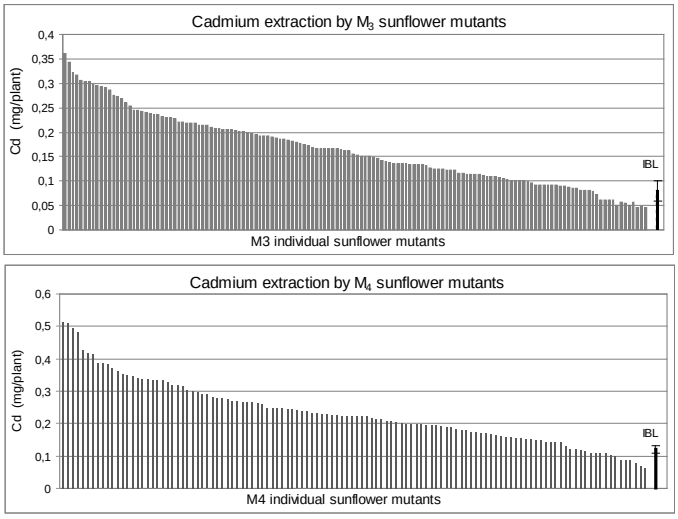

Figure 1 Cadmium extraction by individual sunflower mutant progenies of the third and fourth generation and inbred line (IBL).

We found that EMS mutagenesis mainly led to an enhanced shoot biomass and consequently an improved metal extraction. The capacity of metal accumulation in the shoot was also partially improved.

The phytoextraction potential of sunflower mutants and control sunflowers was calculated per hectare and year. Results obtained from three field experiments show that sunflower mutants can produce up to 20.9

t dry matter per ha and year and remove 10-11 kg Zn and 16-25 g Cd per ha and year from the metal contaminated field. In contrast, control sunflower IBL 04 produced only 4.1 t dry matter per ha and year, with the following metal removal: 4.8 g Cd, 2.2 kg Zn per ha and year [29].

Thus, sunflower mutants obtained during this study show a high potential for the removal of zinc and also of cadmium, as compared to the metal extraction efficiency by other sunflower cultivars, tobacco, maize or even by the hyperaccumulator *T.caerulescens*. For example, for zinc it was significantly higher than reported for other sunflower plants [30], where zinc removal is only 2 kg per ha and year.

As already mentioned, a sufficient metal shoot concentration and biomass production are key factors for the practical use of phytoextraction. Due to this fact, the next objectives of our research were aimed at the assessment of the metal translocation capacity of these new sunflower mutant lines. Descendants of M_2 lines with improved metal accumulation in shoots and biomass were grown in a greenhouse on a metal-contaminated soil with 10 mg/kg cadmium and 1110 mg/kg zinc in the M_5 generation. Cadmium and zinc concentrations in roots and shoots were measured after three months of growth on the polluted soil. Moreover, several growth parameters, such as shoot and root dry weight, were evaluated in sunflower lines cultivated on non-contaminated soil and polluted soil to assess the effects of cadmium and zinc on plant growth and productivity. We observed a growth reduction of both non-mutagenized inbred line and sunflower mutants on the metal contaminated soil, as compared to non-contaminated soil. But no other symptoms of cadmium and zinc toxicity were observed on the polluted soil. However, M_5 sunflower mutants still produced a higher shoot and root biomass on the metal contaminated soil compared to the inbred line. Leaves of the mutant line exhibited a 2.3 times higher cadmium and a 2.5 times higher zinc extraction than the inbred line; mutant roots with a 1.5 times higher dry weight showed a 1.6 times better cadmium and a three times better zinc extraction than the non-mutagenized line (**Fig. 2**).

Sunflowers of the mutant line also showed a better root development compared to the inbred line. This root enhancement can lead to improved access to water, minerals, as well as toxic metals. Therefore, plants with a well-developed root systems are potentially very useful for phytoremediation.

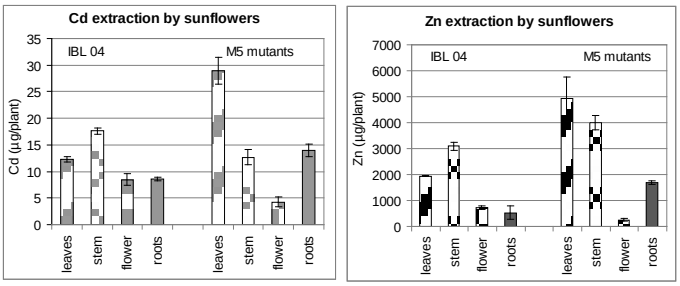

Figure 2 Cadmium and zinc extraction by leaves, stem, flower and roots of sunflowers tested on a metal-contaminated soil in the greenhouse.

The metal concentration was determined in the control inbred line IBL04 and sunflower mutants of the fifth generation after three months of growth on polluted soil.

Conclusions

Sunflower mutant lines obtained after chemical mutagenesis showed in four successive generations (M_2-M_5) an improved metal removal capacity. Due to the results obtained from field and greenhouse experiments, we can conclude that classical mutagenesis has a great potential to generate lines with enhanced metal extraction properties. It is thus still a valuable alternative to genetic transformation. Important advantages of this non-GMO approach for practical phytoextraction are the absence of restrictions for field tests and the direct use of new improved varieties. In addition, the possible subsequent molecular genetic analysis of the phytoextraction mutants may help to better understand mechanisms that govern metal accumulation in plants.

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Anjitha - A New Okra Variety through Induced Mutation in Interspecific Hybrids of *Abelmoschus* Spp.

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Abstract

Studies on interspecific hybrids of okra between *A.esculentus* (cultivated type) and *A.manihot* (wild type) revealed that no useful recombinants were obtained from the conventional breeding programme because of the strong linkage between yellow vein mosaic (YVM) resistance genes and the wild character of *A.manihot*. This study was aimed at breaking this undesirable linkage through gamma irradiation (100 200, 300 and 400 Gray) of F_1 seeds obtained by interspecific hybridization between *A.esculentus* var. Kiran and *A.manihot* and further evaluating and selecting high yielding YVM resistant types from the generations segregating until F_6M_6. The mutagenic effectiveness and efficiency increased with increasing doses of Gamma-rays. In the segregating generations, the irradiated treatments were late flowering and had more leaves, flowers and fruits per plant. Average fruit weight was maximum in 200Gy , while fruit yield was maximum in 400Gy due to larger number of fruits. A few high yielding disease-resistant plants resembling the cultivated plants were obtained in 300Gy which suggested that 300Gy could be the ideal irradiation dose in okra. Superior genotypes selected from F_6M_6 generation based on yield and YVM resistance were advanced to CYTs and farm trials. Cultivar AE18 outyielded the others and was released as "Anjitha" during 2006, for cultivation in the Thiruvananthapuram District of Kerala. Anjitha is a high yielding variety having the fruit characters and quality of the cultivated parent *A.esculentus* var. Kiran combined with the YVM-resistant character of the wild parent *A.manihot*.

Introduction

Okra or bhindi (*A.esculentus* (L.) Moench) is an important annual vegetable crop grown throughout India for its tender green fruits. Due to its high adaptability, it can be cultivated under a wide range of environmental conditions. However, the susceptibility of most okra cultivars to yellow vein mosaic (YVM) disease is a major problem limiting the growth and yield of the crop considerably, with yield losses ranging from 50 to 90% depending on the stage of crop growth at which infection occurs [1]. In India, YVM disease was first reported [2]. The virus, neither sap nor seed transmissible, is readily transmitted by grafting and also through whitefly (*Bemisia tabaci* Gen.) [3]. *A.manihot*, the semi-wild species is resistant to the YVM virus [4], while the cultivated species *A.esculentus* is usually susceptible. Therefore, *A.manihot* could be used as suitable donor of resistance to improve susceptible adapted varieties, but interspecific hybridization between Abelmoschus esculentus and A. manihot did not yield useful recombinants due to a strong linkage between disease resistance and the semi-wild characters of *A.manihot* in the F_2 generation [5]. Variability can be induced by subjecting hybrid seeds of okra to mutation and compared to the F_2, the proportion of recombinants was higher in the F_2M_2 population indicating the breakage of such undesirable linkage through irradiation [6]. This study was undertaken with a view of breaking the undesirable linkage through gamma irradiation of F_1 seeds obtained by interspecific hybridization between *A.esculentus* var. Kiran and *A.manihot*, so that useful recombinants with YVM disease resistance, high fruit yield and quality could be obtained.

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Materials and Methods

The study was carried out in the Department of Plant Breeding and Genetics, College of Agriculture, Vellayani wherein the parents, Kiran, a high-yielding locally adapted *A.esculentus* cultivar (P_1), and *A.manihot*, a YVM disease resistant semi-wild species (P_2), were crossed, and the F_1 hybrid seeds were irradiated using Gamma-rays at four doses viz. 100, 200, 300, 400Gy along with a control. F_1M_1, F_2M_2 up to F_6M_6 generations were studied and evaluated. Compact Family Block Design was adopted for F_2M_2 and F_3M_3 generations using seven treatments, five replications and 10 progeny rows of 10 plants each per treatment, while Randomised Block Design was adopted for the remaining experiments. Since most progenies from F_6M_6 had become stable, 13 progenies were selected and selfed and were subjected to three Comparative Yield Trials (CYT). The superior cultures from the CYTs were subjected to Farm Trials along with the standard variety Kiran and a local check variety of the farmer. The crops were raised under insecticide free condition and susceptible check Kilichundan was grown as border plants for all the experiments. Incidence of YVM disease was scored based on the rating scale [7].

Results and Discussion

F_1M_1 generation

Studies on F_1M_1 generation revealed that seed germination, survival of plants and plant height decreased with increased dose of Gamma-rays while pollen fertility increased in the irradiated hybrids. The undesirable changes resulting from chromosomal aberrations and toxicity are manifested as M_1 damage such as lethality, injury, sterility, and these are measured as reduction in germination, survival, plant growth and fertility, and increase in frequency of chromosomal aberrations and chlorophyll deficient chimeras. There was a progressive reduction in the mean values for percentage germination and survival in both laboratory and field conditions, and germination percentage was found to decrease with increase in level of irradiation. Such a decrease in germination at higher doses of Gamma-rays was also reported in brinjal [8]. The treated hybrids showed delayed germination compared to the control (**Table 1**). Similar results were reported in sorghum [9]. This may be due to the influence of mutagen on plant growth regulators which caused a delay in the initiation of germination. The reduction in the survival of plants is an index of post-germination mortality as a result of cytological and physiological disturbances due to radiation effect. The observations on internodal length, number of branches and plant height showed that the rate of growth was reduced by the mutagen. This reduction could be due to auxin destruction, and it may also be attributed to the influence of ionizing radiation leading to the genic loss due to chromosomal

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aberrations [10]. The irradiated hybrids showed an increased root to shoot ratio compared to the control. A progressive decrease in pollen sterility was observed with increase in radiation dose, that might be the result of normal chromosome pairing which was dependent on dose of gamma radiation and indicates the possibility of obtaining high fertile segregants in the succeeding generations of the irradiated population. The mutagenic effectiveness was found to increase with increase in dose of Gamma-rays. Mutagenic efficiency estimated on the basis of lethality and injury increased with increase in dose of Gamma-rays while on the basis of sterility, 300Gy treatment showed the highest efficiency. Number of fruits per plant increased with increase in dose of Gamma-rays while weight of fruits per plant was more in the irradiated treatments compared to both the parents. Fruit length decreased while girth increased in the irradiated treatments compared to the cultivated parent. YVM incidence was low in the irradiated treatments, similar to the semi-wild parent.

F_2M_2 and F_3M_3 generations

In the F_2M_2 and F_3M_3 generations, the irradiated treatments were found to be late-flowering compared to the un-irradiated treatment and the cultivated parent. Irradiation was found to increase the number of leaves per plant, flowers per plant, fruits per plant, pollen sterility and plant height and decrease in YVM disease incidence compared to the cultivated parent (**Table 2**). A preponderance of low yielding YVM resistant plants similar to the donor parents among the F_2 and F_2M_2 populations was observed, indicating the presence of a strong genetic mechanism preventing free recombination [6]. Gamma radiation created considerable genetic variability in interspecific *A.esculentus* x *A.manihot* F_1 hybrids and they observed that higher doses (above 250Gy) should be used to create wider variability in the interspecific hybrids and also reported that, compared to F_2, the proportion of recombinants was higher in the F_2M_2 population indicating the breakage of undesirable linkages through irradiation [11]. However, a few high-yielding YVM disease-resistant plants resembling the cultivated parent were observed in 300Gy in the present study. The fruit yield per plant was more in irradiated treatments due to the larger number of fruits. The same trend noticed in the F_1M_1 generation was observed in F_2M_2 and F_3M_3 generations with respect to fruit characters and yield.

F_4M_4 , F_5M_5 and F_6M_6 generations

In the F_4M_4 , F_5M_5 and F_6M_6 generations, out of the 50 families studied, nine recorded less number of days to 50% flowering than the cultivated parent. In F_4M_4, occurrence of segregants with higher mean values for number of flowers and fruits per plant than both the parental means was noticed. Average fruit weight exhibited a reduction in mean value, whereas fruit yield per plant increased as a result of increase in number of fruits per plant. This is in conformity with the findings that irradiation induced delayed flowering and produced increased number of flowers, fruits and weight of fruits per plant and YVM resistant plants in irradiated treatments [12]. Duration of the plants were longer than the cultivated parent while plant height of the progenies exceeded both the parents. Incidence of YVM was lower in the F_4M_4, F_5M_5 and F_6M_6 generations compared to the cultivated parent. By F_5M_5 generation, number of fruits per plant, yield of fruits per plant, length and girth of fruit increased in all the families compared to both the parents indicating good response to selection in the previous generation. The crude fiber content of the fruits was maximum in the wild parent. All the F_5M_5 and F_6M_6 families had fruits with crude fiber content equal to or less than than the cultivated parent. Only a narrow range of variability was noticed for crude fiber content of fruits by [13]. The majority of the

Table 1. Effect of Gamma-rays on different characters in F_1M_1.

Hybrid/ parent	Gamma-rays (GY)	Germination%	Survival%	Internode length (cm)	Branches/ plant	Plant ht (cm)	Root/shoot ratio	Pollen fertility	No. of fruits/plant	Wt.of fruits / plant (g)	Fruit length (cm)	Fruit Girth (cm)	YVM incidence
P1xP2	0	74.2(59.4)	100	10.8	25.5	155.8	0.78	15.2(22.9)	15.0	265.2	11.9	8.1	1.0 (1.0)
P1xP2	100	70.4(57.0)	95.4	10.0	27.5	141.9	1.08	18.2(25.2)	15.6	306.4	10.4	7.4	1.0 (1.0)
P1xP2	200	69.1(55.6)	90.8	10.3	24.9	135.9	1.11	22.0(27.9)	15.9	289.6	10.2	7.6	1.0 (1.0)
P1xP2	300	63.4(52.7)	89.7	9.4	21.9	127.1	1.18	24.7(29.8)	16.0	270.8	9.3	7.7	1.0 (1.0)
P1xP2	400	54.6(47.6)	84.0	8.7	29.7	120.7	1.27	29.0(32.5)	16.4	287.0	11.3	6.8	1.0 (1.0)
P1	0	79.1(62.8)	100	7.9	11.8	96.1	0.81	90.5(72.0)	10.4	202.2	14.8	6.0	3.3 (1.8)
P2	0	82.2(65.0)	100	6.8	18.1	108.0	0.83	94.0(75.8)	6.6	153.7	11.1	8.2	1.0 (1.0)
CD (0.05)		7.07	1.43	1.02	7.87	14.7	0.12	1.23	3.8	71.38	1.00	0.44	0.03

Transformed values are given in parentheses

Table 2. Effect of Gamma-rays on different characters in F_2M_2 and F_3M_3 generations (mean of 2 generations).

Treatment	N°. of leaves	N°. of flowers	Pollen sterility%	N°. of fruits	Wt. of fruits / plant(g)	Fruit length (cm)	Fruit girth (cm)	Plant height (cm)	Plant duration (days)	YVM incidence
0Gy	18.2	12.0	27.8	10.5	130.2	10.7	5.7	145.0	136.2	1.2 (1.1)
100GY	49.1	39.6	37.9	33.7	264.1	8.6	5.1	148.5	142.8	1.5 (1.2)
200Gy	50.4	40.6	31.2	35.1	313.4	9.4	5.3	152.3	154.5	1.6 (1.3)
300Gy	30.5	22.9	29.0	19.8	236.5	11.6	5.5	139.8	141.5	2.0 (1.4)
400Gy	45.8	22.5	30.5	32.1	314.6	12.0	6.1	134.4	154.4	2.3 (1.5)
P1	19.1	14.0	3.8	13.2	185.9	14.6	5.4	112.6	126.0	2.4 (1.6)
P2	22.0	15.1	26.4	14.1	248.0	14.5	8.3	121.5	163.0	1.2 (1.1)
SE	1.39	1.35	0.93	1.32	15.93	0.27	0.12	3.30	1.01	0.03

families exhibited increases in the mean values for the economically important characters and combined high yield with resistance to YVM disease. The best lines of the families in F_6M_6 generation were selected and advanced to Comparative Yield Trials.

Comparative Yield Trials

Comparative Yield Trials were conducted using 13 promising genotypes selected from the F_6M_6 generation for three seasons to get consecutive results. The pooled mean data with regard to yield and yield attributes and incidence of YVM disease incidence (**Table 3**) showed that culture AE 18 had the maximum number of fruits and highest yield, followed by AE 25 and AE 17. AE 18 had long fruits, the longest being for AE 8. Culture AE 18 had shorter duration compared to the check variety Kiran and the three cultures AE 18, 25 and 17 had no YVM disease incidence, while variety Kiran had a very high incidence of the disease (89.8%). Considering the superiority of AE18 in terms of yield, duration and YVM disease resistance, it was recommended for farm trials in Thiruvananthapuram district.

Table 3. Pooled mean data of three Comparative Yield Trials.

Cul.No	Fruits / plant	Length of fruit (cm)	Yield tons/ha	Duration(days)	Incidence of YVM (%)
AE 1	11.8	15.9	10.4	95.0	1.2
AE 4	13.1	19.2	10.6	95.1	0.0
AE 7	13.5	16.0	10.8	94.4	0.0
AE 8	11.5	22.8	9.0	95.8	0.9
AE 11	13.1	15.8	10.4	94.4	0.0
AE 12	13.3	18.9	10.7	93.8	0.0
AE 16	13.8	19.1	11.8	94.3	3.6
AE 17	14.9	18.8	13.5	96.0	0.0
AE 18	16.6	19.3	14.6	94.3	0.0
AE 19	12.9	17.7	11.0	93.8	0.0
AE 20	13.7	14.9	11.3	95.7	0.0
AE 24	13.5	17.3	11.1	94.8	0.0
AE 25	15.6	18.1	14.3	95.1	0.0
Kiran	9.6	14.7	6.6	97.3	89.8
CD (0.05)	2.84	2.73	2.80	2.10	7.18

Table 4. Farm trial with okra culture AE 18.

Sl No	Locations	Fruit yield tons / ha		YVM Incidence (%)		Length of fruit (cm)	
		AE 18	Kiran (std check)	AE 18	Kiran (std check)	AE 18	Kiran (std check)
1.	Nedumangad	14.9	8.1	2.5	40	15.0	13.0
2.	Pothencode	13.1	5.9	1.0	100	16.0	14.5
3.	Kariyavattam	11.7	9.6	0	78.0	18.5	13.5
4.	Anad	13.7	8.5	1.0	33.0	16.0	13.0
5.	Neyyattinkara	11.3	5.3	0	40.0	16.5	11.0
6.	Kattaikonam	13.3	8.9	0	30.0	17.0	11.0
7.	Kazhakuttam	10.0	3.4	0	90.0	16.0	14.0
8.	Kalliyoor	19.1	7.2	0	95.0	18.5	10.5
9.	Thannimoodu	14.7	5.8	0	75.0	16.0	13.5
10.	Karakulam	17.5	8.3	0	100	16.0	15.3
11.	Venganoor	8.8	3.2	1.7	95.0	18.0	11.0
12.	COA Vellayani	17.3	4.1	0	98.0	19.5	14.0
	Mean	13.78	6.52	0.51	72.83	16.9	14.0
	CD (0.05)	2.099		16.415		1.05	

Farm Trials

Farm trials were conducted with culture AE 18 and the standard check variety Kiran in farmer fields in 12 locations in Thiruvananthapuram district during the summer of 2004. Culture AE 18 was found to be significantly superior to the check variety Kiran with regard to mean fruit yield, fruit length and YVM disease incidence (**Table 4**). Hence it was released as a new variety 'Anjitha' for cultivation in the Thiruvananthapuram district of Kerala by the XXIII State Seed Sub Committee during 2006.

Table 5. Description of the variety Anjitha and the parents P_1 and P_2.

Sl.No.	Character	*A.esculentus* var Kiran (P_1)	*A.manihot* (male parent) (P_2)	Anjitha
	Nature of variety/ species	Cultivated variety	Wild species	New variety
1	Days to first flowering	42	55	39
2.	No. of fruits per plant	9.6	11.5	16.6
3.	Length of fruit (cm)	14.7	8.5	19.3
4.	Girth of fruit (cm)	5.3	8.1	6.0
5.	No. of ridges per fruit	5.0	7.6	5.0
6.	Fruit yield tons /ha	8.9	9.6	14.6
7.	Height of the plant (cm)	103	80.2	135
8	Duration	97.3	159	94.3
9.	YVM incidence	susceptible	resistant	resistant
10.	Shoot &fruit borer incidence (%)	22.5	3.0	4.0
11.	Crude Fiber (g/100g)	1.7	2.6	1.5

Summary

Anjitha (AE18) is an early flowering type with a larger number of long fruits having five ridges per fruit developed through induced mutation (300Gy dose) in interspecific hybrids of *Abelmoschus* spp (**Table 5**). The plant has high fruit yield and good fruit quality with less fiber content. It is resistant to YVM disease and tolerant to fruit and shoot borer attack. Anjitha has the fruit characters and quality of the cultivated parent *A.esculentus* var. Kiran combined with the YVM-resistant character of the wild parent *A.manihot*.

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Genetic Improvement of Chickpea (*Cicer arietinum* L.) Using Induced Mutations

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Abstract

The main target of chickpea breeding programmes has been to develop high yielding cultivars. In an attempt to induce genetic variability for improvement of locally popular chickpea cultivar Vijay (Phule G-81-1-1), we employed three well known mutagens, sodium azide (SA), ethyl methane sulphonate (EMS) and gamma radiation (GR). The objective was to provide genetic variability in the yield contributing traits that can be exploited for a genetic improvement of chickpea. Seeds of Chickpea cultivar Vijay were treated with three different concentrations / doses of SA (2, 3 and 4 mM), EMS (8, 12 and 16 mM) and gamma radiations (400, 500 and 600 Gy). In M_1 generation no dominant mutations were observed, many different mutants were screened and isolated in M_2 generation such as chlorophyll mutations (alnina, chlorina and xantha); leaf mutations (gigas, compact and curly); pod mutations (small, roundish, gigas and narrow elongated); seed mutations (green, dark brown, rough seed coat); flower mutations (white flower and open); morphological mutations (early, sterile, tall and gigas). True breeding mutant lines in M_3 generation differed considerably in their quantitative traits from the parent cultivar. The early mutant lines matured 10 days earlier than the parent variety. The range in plant height was expanded from 0.02 to 14.91cm. Gigas mutant lines obtained after 400 Gy gamma irradiation were the tallest (44.44cm), with a 2-3 fold increase in pod and seed size over the control. Mutagenic treatments also caused changes in seed size and seed coat. Considerable genotypic variation was observed with regards to the number of seeds and pods per plant. Small leaf mutants showed double the number of seeds and pods per plant. As a result of mutagenic treatments, genetic variation was induced in mutants with respect to different quantitative characters. Induced mutant lines showed both positive and negative increase in the quantitative traits. Variation was also observed for crude protein, globulin and albumin content of mutants.

Introduction

Chickpea (*Cicer arietinum* L.) is a widely cultivated and important food grain legume in the Indian sub-continent. It is a major source of protein for both humans and livestock. In spite of its high economic importance, its yield did not witness much appreciation during the past decade [1]. It has been argued that one of the reasons for failure to achieve a breakthrough in productivity in chickpea is the lack of genetic variability [2]. The improvement of chickpea using conventional breeding approaches has been hampered due to lack of sufficient genetic variability. Therefore, there is an urgent need to develop new plant types for different situations. A common and efficient tool to create new desirable genetic variability in chickpea is mutagenesis [3]. Although studies on induced mutations have been undertaken in the past in some legumes, limited reports are available on chickpea [4]. In the present investigation, an attempt has been made for genetic improvement of the locally adapted cultivar of chickpea, Vijay, through induction of mutations employing potent mutagens like gamma radiation, sodium azide and ethyl methane sulphonate.

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Materials and Methods

Seeds of Chickpea (*Cicer arietinum* L.) cultivar Vijay (Phule G-81-1-1), were obtained from the Mahatma Phule Agriculture University, Rahuri, India. Healthy seeds containing 10-12% water were treated separately with chemical (SA and EMS) and physical (gamma radiation) mutagens. For chemical mutagen treatments, seeds were presoaked in distilled water for 6 hours and then subjected to 2, 3 and 4 mM SA and 8, 12 and 16 mM EMS, for 12 hours at 25±2°C. The treated seeds were thoroughly washed under running tap water for an hour to terminate the reaction of the chemical. For physical mutagen treatment, dry seeds were irradiated with 400, 500 and 600 Gy from a ^{60}Co source available in the Department of Biophysics, Government Institute of Science, Aurangabad (M.S., India). Each treatment was carried out for 250 seeds.

All treated seeds along with control were sown in the field at a spacing of 15cm within rows and 45cm between rows to raise the M_1 generation during the 2002 growing season. All M_1 plants were harvested separately to raise M_2 generation. Screening and evaluation of M_2 generation was performed during the 2003 growing season, using a randomized block design (RBD) with 3 replicates at experiment field of Shri Anand College, Pathardi. In M_3 generation, mutant seeds were planted in RBD, with 3 replications. Data were collected for 6 agronomic traits (plant height, plant spread, number of pods per plant, number of seeds per plant, yield per plant and 100 seed weight). Total protein, globulin and albumin were estimated following the method of Lowry, *et al.* [5]. The nitrate reductase activity from leaf samples of chickpea at flowering stage was performed as described earlier by Sawhney, *et al.* [6]. The analysis and comparison of proteins were carried out by the SDS-PAGE following the method of Laemmli [7]. Data was analyzed using Dry software programme. The genotypic and phenotypic coefficients of variation were estimated following the method of Burton and De Vane [8]. The heritability and genetic advance were calculated following the methods suggested by Hanson, *et al.* [9] and Johnson, *et al.* [10], respectively.

Results and Discussion

Spectrum and frequency of mutations

M_2 generation was comprised of 189 families with a total plant population of 4898 surviving plants at harvest. The morphological mutants isolated mainly showed an altered leaf structure, plant shape, seed size, seed colour, seed structure and days of the maturity (**Plate 1**). A high frequency and spectrum of viable mutations was observed in the M_2 generation of chickpea cultivar Vijay with all three mutagens, which were completely absent in the control, and increased in a concentration/dose dependent manner of the mutagen employed (**Table 1**).

A high frequency of viable mutations was observed with gamma

Table 1. Spectrum and frequency of induced mutations in M_2 progeny of chickpea cultivar Vijay

Treatment Conc./Dose	M_2 population	M_1 families	Spectrum of mutations				Total frequency of mutations
			Leaf mutants	Plant type mutants	Pod/ seed mutants	Sterile mutants	
2 mM SA	431	21	00	0.92	0.23	00	1.16
3 mM SA	582	21	00	1.03	0.51	00	1.54
4 mM SA	615	21	0.16	1.47	0.65	00	2.28
Mean			**0.05**	**1.14**	**0.46**	**00**	**1.66**
8 mM EMS	525	21	1.33	1.71	0.57	00	3.62
12 mM EMS	541	21	1.08	1.80	0.73	0.36	3.97
16 mM EMS	550	21	2.40	1.66	1.10	0.18	5.34
Mean			**1.60**	**1.72**	**0.80**	**0.18**	**4.31**
400 Gy GR	596	21	0.17	1.51	0.67	00	2.34
500 Gy GR	539	21	0.74	4.82	0.37	0.18	6.12
600 Gy GR	519	21	0.38	4.62	1.54	0.38	6.93
Mean			**0.43**	**3.65**	**0.86**	**0.19**	**5.13**
Total	**4898**	189					

Table 2. Mean performance for quantitative traits among selected M_3 mutant lines of chickpea

Mutant/ control	Mutagen and dose	Plant height (cm)	Plant Spread (cm)	Number of Pods plant-1	Number of seeds plant-1	Seed yield plant-1 (g)	100 seed weight (g)
Control	-	29.53	25.00	36.33	37.00	8.23	22.99
Small leaf	12 mM EMS	32.74	30.94	72.00	76.33	11.54	15.13
White flower	8 mM EMS	32.40	28.77	26.33	27.33	5.31	19.47
Narrow leaf	400 Gy GR	33.77	22.31	45.33	47.66	7.02	14.87
Rough seed	3 mM SA	37.50	22.66	39.66	45.33	9.33	19.64
Early	500 Gy GR	29.55	20.44	42.33	48.33	9.46	19.58
Gigas	400 Gy GR	44.44	24.66	15.33	15.33	5.77	37.78
Compact	4 mM SA	31.41	27.66	35.66	37.01	6.06	16.46
Green seed	500 Gy GR	34.08	30.55	24.00	25.33	4.68	18.65
Bold seeded	500 Gy GR	32.63	26.11	29.66	31.33	6.75	31.79
C V		6.423	8.333	24.48	23.81	20.12	10.17
SE +		1.236	1.240	5.12	5.31	0.844	1.19
CD(p=0.05)		3.671	3.684	15.22	15.78	2.507	3.556
CD(p=0.01)		5.035	5.053	20.88	21.65	3.438	4.876

radiation followed by EMS and SA treatments. The frequency of viable mutations ranged from 1.16 to 6.93. Gamma radiation induced a wider spectrum of viable mutations. At 500 Gy of gamma radiation, mutation frequency was highest as seen in the mutagenized population for plant type, 16 mM EMS treatment induced a high frequency of leaf morphological mutations (2.40). On the other hand, SA treatments showed the least spectrum and lowest frequency of viable mutations. Kharkwal [11] attributed the differences in frequency and spectrum of viable mutations induced by various mutagens to genetic differences in the cultivars, while Konzak, *et al.*, [12] have reported that even as small as a single gene difference could bring about significant changes not only in the spectrum but also the frequency of recoverable mutations.

In this research with Vijay chickpea, we observed that the spectrum and frequency of induced viable mutations increased with increasing concentrations/doses of SA, EMS and gamma radiation. This could be due to a differential mode of action of the mutagens on different base sequences in various genes.

Quantitative traits

Nine mutants were compared for mean values of quantitative traits with parental cultivar Vijay in the M_3 generation. Both positive and negative mutation occurred as compared to the parental cultivar. The plant height ranged from 29.55 to 44.44cm in M_3 generation as compared to 29.53cm in the parent. Overall, mean height increase ranged from 33.56% in Gigas mutant to 21.26% in round seed mutant as compared to the control.

Most mutants showed both a positive and a negative plant spread. A significant reduction in plant spread was observed in the early mutant. Conversely, small leaf and green seed mutants showed a significant increase in plant spread over the control. The maximum number of pods per plant compared to the control was observed for the small leaf mutant followed by the narrow leaf mutant. Among all mutant lines, the highest number of seed per plant was observed in small leaf mutant (76.33) compared to control (37). The small leaf mutant showed a significantly higher yield per plant (11.54 gm) over the parental cultivar (8.23 gm), whereas it was reduced in the white flower mutant and gigas mutant in M_3 generation. Narrow leaf, small leaf and compact mutants had lower 100 seed weight. Gigas and bold seeded mutants showed significantly higher 100 seed weight, which was attributed to the increased cotyledonary cell volume whilst retaining a similar cell number per unit area [13].

In all the mutants, days to maturity ranged from 89.66 to 110 days. The early maturing mutant was significantly earlier (at least 10 days) in flowering and maturity compared to the parental cultivar (Table 3). Rough

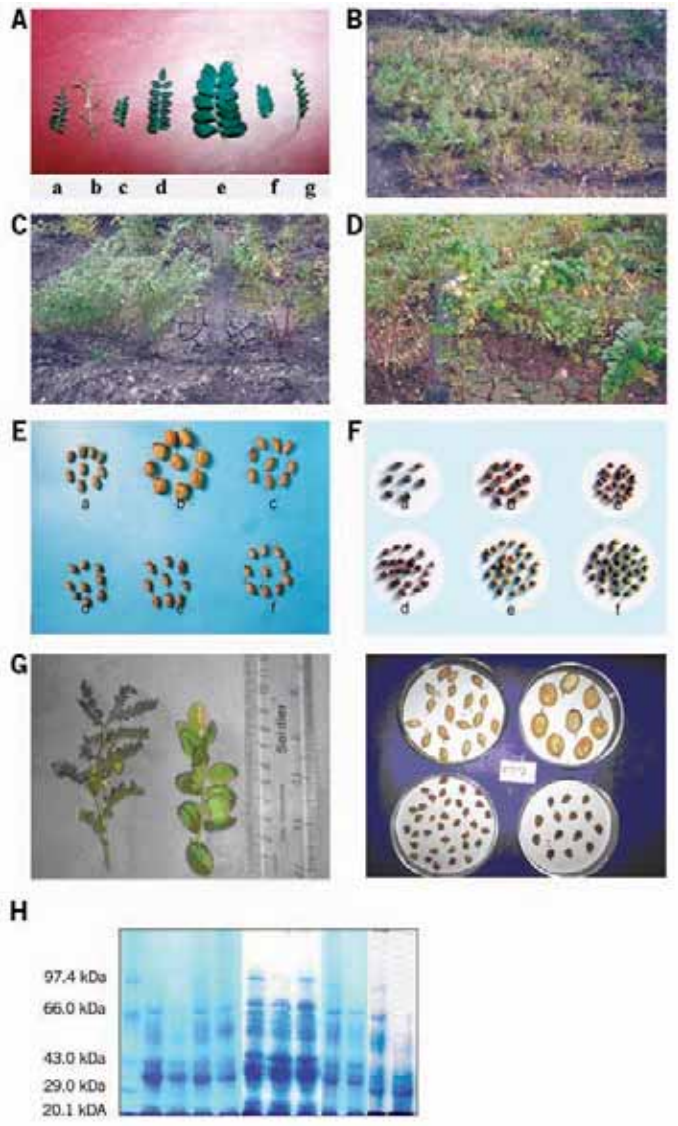

Figure 1 (A) Leaf mutation-a. narrow, b. tiny, c. small, d. parent (control), e. gigas, f. compact and g.curly leaf, **(B)** Early mutant and parent, **(C)** Parent (right) and compact mutant (left), **(D)** Parent (left) and gigas mutant (right), **(E)** Pod- a. parent, b. gigas, c. long, d. small roundish, e. narrow elongated and f. small, **(F)** Seed- a. parent, b. bold, c. blacked spotted, d. green, e. rough seed coat and f. brown, **(G)** Parent (left) leaves, pods, seeds and gigas mutant (right) leaf, pods and seeds, **(H)** SDS-PAGE protein profile of chickpea in induced mutants of Vijay in M_3 generation.

seed and bold seeded mutants showed similar maturity as the parent. Early mutant matured earlier than the parent and thus will cope better with the late season and moisture stress that is usually encountered in the chickpea growing areas in the state of Maharashtra. Early maturing mutants can be of great importance in the areas with short rainfall also. Early mutants have been reported earlier in chickpea [14] and pigeonpea [15]. These findings showed that the mutations induced have generated a variability for quantitative traits that offers a wide scope for genetic improvement of chickpea in forthcoming breeding programmes.

Estimation of protein content and nitrate reductase activity

Seed protein content in the parent cultivar was 256.66 mg g^{-1} while it ranged between 228.73 to 284.2 mg g-1in mutants (**Table 3**). Bold seeded, compact and gigas mutants showed a significantly higher protein content. The bold seeded, compact and small leaf mutants showed significantly higher globulin and albumin contents among mutants in the M_3 generation. The highest nitrate reductase activity was observed in gigas mutant followed by compact mutant. The lowest value was observed in green seed mutant in the leaves examined. Nitrate reductase activity showed a positive correlation with protein content. Therefore, the results indicate that nitrate reductase could be used as a tool to correlate with protein content and overall productivity of mutants in early stage. Our result for nitrate reductase activity is in agreement with those obtained by Aparna, *et al.* [16].

Table 3. Mean performance of selected M_3 mutant lines for maturity, protein, globulin, albumin and nitrate reductase activity of chickpea

Mutant/ control	Days to maturity	Protein (mg g –1)	Globulin (mg g –1)	Albumin (mg g –1)	N R (µ moles / g.fr.wt.)
Control	99.66	256.66	163.30	55.1	2.06
Small leaf	106.33	261.06	181.33	60.16	2.16
White flower	98.66	257.56	170.76	56.36	2.10
Narrow leaf	99.33	235.86	130.6	56.43	1.76
Rough seed	99.66	265.43	171.5	77.23	2.27
Early	89.66	261.73	175.63	79.03	2.16
Gigas	110.00	274.50	171.66	61.96	2.64
Compact	102.66	274.76	176.76	82.33	2.72
Green seed	106.66	228.73	151.63	74.33	1.62
Bold seeded	99.66	284.20	187.81	73.21	3.10
C V	1.638	1.41	1.082	1.60	0.19
SE +	0.960	2.08	1.099	0.70	0.08
CD(p=0.05)	2.853	6.18	3.265	2.078	0.25
CD(p=0.01)	3.913	8.48	4.479	2.851	0.34

Heritability and variability components for quantitative traits among the mutants

Data in **Table 4** indicates that a consistently greater PVC was observed than the GCV in different quantitative traits among the induced mutants. Comparison among traits indicated that the number of seeds per plant recorded the greatest PCV (43.36%) followed by number of pods per plant and 100 seed weight. Plant height and plant spread had the lowest PCV among the mutants. Because of the enhanced reproductive growth in terms of the number of seeds per plant, diversion of the photosynthates towards vegetative growth probably was minimized resulting in the lowest PCV and GCV for plant height. High PCV and GCV values for number of pods per plant and number of seeds per plant indicated further scope of yield improvement through selection of the donor for breeding in chickpea.

Table 4. Heritability and variability for quantitative traits among the mutants in M_3 generation

Quantitative traits	PCV (%)	GCV (%)	H_2 (%)	GA
Plant height (cm)	13.62	12.80	88.32	8.34
Plant spread (cm)	16.77	13.87	73.66	6.43
Number of Pods (plant-1)	42.94	41.84	94.94	30.79
Number of seeds (plant-1)	43.36	42.72	97.07	33.85
Seed yield (plant-1g)	30.97	28.34	87.71	9.97
100 seed weight (g)	34.92	34.20	95.74	14.87

High heritability coupled with high genetic advance was observed for quantitative traits like number of seeds per plant and number of pods

per plant (Table 4), may be due to additive genes. On the contrary, both heritability and genetic advance were less for plant spread. Badigannavar and Murty [17] reported a high heritability associated with a high genetic advance for plant height, pod yield and seed yield in gamma rays induced mutants of M_8 generation. We are of the opinion that selection based on heritability and genetic advance for number of seeds per plant and number of pods per plant may be effective induced mutations for improvement of chickpea.

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Gamma-Ray Induced Mutations in Soybean [*Glycine max* (L.) Merill] for Yield-Contributing Traits

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Abstract

Attempts were made to induce genetic variability in yield contributing traits in soybean [*Glycine max* (L) Merrill] employing gamma radiation. Germplasm of a locally adapted cultivar of soybean, MACS-450 was irradiated with different doses (10, 20, 30, and 40 kR) of Gamma-rays and sown in the field during the kharif season of 2006. M_2 progeny was raised from the M_1 seeds, and was screened for yield contributing traits. The M_2 progeny raised from 30kR dose of gamma radiation exhibited several induced mutations for yield contributing traits. Important among them was a High-yielding mutant, of which about 10 mutant plants were obtained in the M_2 progeny. These High-yielding mutants were all uniformly tall and showed a two-fold increase in plant height. They produced double the number of pods per plant and thrice the yield per plant as compared to control. No change in pod length and number of seeds per pod were observed between the control and High-yielding mutant plants, except for the 100 seed weight, which was almost 1.5 times higher compared to the control. These mutants seem to be very promising in increasing the yield of soybean.

Introduction

Soybean is an important oil seed crop, cultivated in 64.50 Lakh hectares in India, as per estimates of 2003 [1]. Soybean provides a balanced diet to the poor to make up the deficiencies of proteins, fat, vitamins, minerals and salts and provide a nutritious diet, within the reach of the poorest in the country [2]. In spite of its nutritional importance, the yield of soybean is very low [3]. Mutation breeding is one of the plant breeding techniques used for creating genetic variability in yield contributing traits and to improve the yield of crop plants [4]. In the present investigation, attempts were made to improve the yield of the existing, locally adapted soybean cultivar MACS-450, by improving its yield-contributing traits through mutation breeding.

Materials and Methods

Experimental plant material used in the present investigation was soybean (*Glycine max* (L) Merrill) cultivar, MACS-450. The main features of the cultivar are its semi-determinate growth habit, medium maturity and high-yield potential. Germplasm of soybean (MACS-450) were obtained from Agarkar Research Institute, Pune. They were irradiated with different doses (10, 20, 30, and 40 Kr) of Gamma-rays at the Department of Biophysics Government Institute of Science, Auarangabad (Maharashtra State). Each treatment included 300 seeds, out of which 50 seeds were used for planting along with the control. Both irradiated and control (non-irradiated) seeds were sown in the experimental fields following randomized block design (RBD) with three replications, at a spacing of 15cm within rows and 45cm between rows, to raise the M_1 generation, during the *kharif* season of 2006. All the surviving M_1 plants were selfed and harvested individually to give the M_2 generation population along with controls during *kharif* season of 2006-2007. Necessary cultural practices were adopted to produce a healthy crop. The M_2 progeny was raised following randomized block design with three replications.

Each treatment comprised of 20-21 M_1 plant progenies and each M_2 progeny row consisted of 10 to 25 plants in three replications. The cultural operations and application of FYM were done as per the standard schedule. Treated, as well as control plant progenies were carefully screened from the day of emergence, in all generations for the yield-contributing traits viz., plant height, number of branches per plant, number of nodes per plant, pods per plant, number of seeds per pod, pod length, 100 seeds weight, seeds per plant and yield per plant.

Figure 1 Field view (A) of dried specimens of control and of High-yielding mutants, (B) of soybean.

Results and Discussion

The M_2 progeny from the 30Kr dose of gamma radiation, exhibited several induced mutations for yield-contributing traits. Statistical analysis of data clearly indicated significant variations in yield-contributing traits of the mutant as compared to the control. The mutants were taller (98.48cm) than the control (49.10cm), and had double the number of nodes. No difference in number of branches was found between the control and mutants. The mutants produced double the number of pods and of seeds per plant as compared to the control (**Fig. 2** and **Table 1**). The yield per plant in the mutants was almost three fold as compared to the control (**Table 1**). The difference in yield per plant observed between the control and mutant is statistically significant (**Table 1**). This mutant was named High-Yielding Mutant (**Fig. 1 A and B**). No change in pod length and number of seeds per pod was observed between the control and High-yielding mutants. The data recorded on M_2 generation are presented in **Table 1**.

The positive correlation between seed yield and number of pods observed in the present investigation is in agreement with results obtained by Anand and Torrie [5] and Lal and Haque [6]. A positive

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and significant correlation between the fertile branches and pods, fertile branches and seed yield and 100 seed weight and seed yield were also observed in M_2 the mutants. The positive and significant correlation between various quantitative characters of rice and soybean was reported earlier by Venkateswarulu, *et al.* [7] and Malhotra, *et al.* [8]. A negative and significant relationship between seed yield and 100 seed weight was also reported in chickpea by Baradjanegara, *et al.* [9]. Seed weight per plant was found to be positively associated with plant height [10]. The significance of each correlation was tested using t-test. The mutants seem to be very promising in increasing the yield of soybean. Such a high-yielding mutant, which yields almost three times as much as the existing locally adapted high-yielding variety (MACS 450) has not been reported so far. The present study confirms that Gamma-rays are highly effective in inducing polygenic variability for the yield contributing traits in soybean. A similar increase in yield parameters with Gamma-rays has been reported by Saric, *et al.* [11], Micke [12] and Sparrow [13]. These authors have attributed the increase in plant height to an increase in the number of growing points due to irradiation. Ionizing radiations are also known to induce a host of physiological changes in addition to genetic effects [14,15]. Such stimulatory effects were attributed to an increased enzymatic activity arising from a depletion of an inhibitor or an effect on the enzyme itself [14, 16].

Table 1. Yield-contributing traits of control and high yielding mutant at M_2 generation.

S.No	Parameter	Control	High-yielding Mutant
1	Plant height (cm)	49.10	98.48*
2	Number of branches per plant	09.40	09.90
3	Number of nodes per plant	13.20	24.10*
4	Pods per plant	97.40	244.40*
5	Number of seeds per pod	02.86	02.87
6	Pod length (cm)	5.01	5.39
7	100 seeds weight (gm)	12.985	17.857
8	Seeds per plant	278.6	702.3*
9	Yield per plant (gm)	36.134	125.447*

* Significant at P=0.001

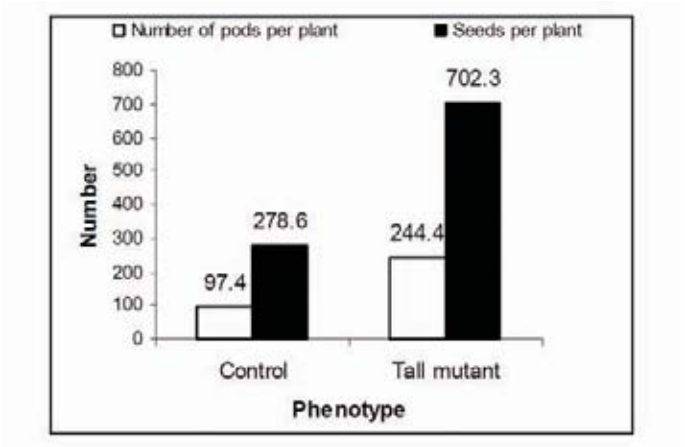

Figure 2 Number of pods and seeds per plant of control and High-yielding mutant of soybean.

These results on the induction of useful genetic variability for a number of economically important yield contributing traits, which can contribute to the development of high-yielding genotypes having an improved plant type, clearly indicate the vital role of mutation breeding in crop improvement. Systematic and serious efforts in pursuing the methodology of mutation breeding have already been successfully demonstrated in development and release of large number of improved high-yielding varieties in several crops [17].

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Induced Mutagenesis in Mungbean (*Vigna radiata* (L.) Wilczek)

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Abstract

A wide range of viable morphological and physiological mutants were observed in M_2 and M_3 progenies of mungbean (*Vigna radiata* (L.) Wilczek) cultivars (Vaibhav and Kopargaon-1) raised from seeds treated with different concentrations of sodium azide, ethyl methane sulfonate and different doses of gamma radiation. The most striking type of mutants obtained in the M_3 progeny included plant habit, leaf structure, flower type, pod type, seed type, early-maturing and high-yielding and Lhb mutants. Some of these mutants are new and are being reported for the first time in this crop. The true breeding mutant lines of M_3 generation were compared with their parent cultivar (control) to assess whether the induced genetic variability was statistically significant. These mutants can be better fitted in new cropping patterns, with improved agronomic management and good yielding ability, or can be used in the genetic improvement of mungbean crop. Chemical mutagens were more efficient than physical ones in inducing viable and total number of mutations. Along with simple viable mutations, multiple mutagenic effects on two or more characters were also found in all the mutagenic treatments. Differences in the mutation frequency and spectrum depends on the interaction of three factors such as mutagen, plant genotype, and physiological state of the organism at the moment of treatment. The Kopargaon-1 cultivar was more resistant towards mutagenic treatment than Vaibhav cultivar. All mutants were analyzed for their protein, albumin and globulin contents by Lowry's method and for protein banding patterns employing SDS Polyacrylamide Gel Electrophoresis. Mungbean mutants with high as well as low protein contents ranging from 29.3% to 14.75% vis-à-vis 22.2% in the control were isolated. Results showed that early flowering mutant and Lhb mutant differed between each other as well as with other mutants and controls in their protein-banding pattern. Our results indicated that mutational breeding was effective and useful for induction of agronomically important mutants in mungbean.

Introduction

Induced mutations have been used to enhance genetic variability, which was utilized not only to increase crop productivity but also for basic studies in various crops [1]. Induced mutagenesis has played an important role in improvement of mungbean, and as many as 25 cultivars have been developed so far through induced mutagenesis [2]. In order to induce variability and utilize useful mutations for efficient plant breeding, the systematic study of induced viable morphological mutations in M_1 and M_2 generation is the most dependable index [3].

Material and Methods

The experimental plant material used in the present investigation were two local varieties of mungbean (*Vigna radiata* (L.) Wilczek); Vaibhav and Kopargaon-1. These two cultivars were treated with different concentrations of two chemical mutagens; ethyl methane sulphonate (EMS) and sodium azide (SA) and one physical mutagen, gamma radiation, to induce mutations in the M_2 and M_3 progenies of the selected plant materials. Stock solutions EMS (1.0M) and sodium azide (1.0M) were prepared in phosphate buffer (pH. 3.5). From these stocks, working solutions of 0.01, 0.02, 0.03, and 0.04 M concentrations each of EMS and SA were prepared. The seeds were surface sterilized with 0.1% mercuric chloride solution for about one minute, washed thoroughly and soaked in distilled water for eight hours. Pre-soaked seeds were subjected to treatment with various test concentrations of EMS and SA for 12 hours at room temperature. The source of gamma radiation used in this study was ^{60}Co. Dry seeds of Vaibhav and Kopargaon-1 (about 700 each) were irradiated with 30, 40 and 50 kR doses of gamma radiation. Treated as the control, seeds were sown in the experimental fields at a spacing of 30 x 20cm apart on the same day.

Each M_1 plant was harvested individually and M_2 progeny was raised in separate rows. The treated and control populations of M_2 generation were carefully screened for viable mutations and spectrum of mutation was counted in M_2 and is presented in **Tables 1 and 2**, respectively. Total proteins, globulin and albumin were estimated in three replications following the method of Lowry, *et al.* [4] and Polyacrylamide Gel Eletrophoresis was carried out following the method of Laemmli [5].

Results and Discussion

Frequency and spectrum of viable mutations

Mutations affecting gross morphological changes in leaf morphology, plant habit, flowers, pods, days to first flower and maturity, high yield, the same as mutations affecting seed color, size and shape, were scored as viable mutations. These mutants were characterized and named on the basis of specific characters constantly observed in them throughout the course of the investigation. Effect of mutagens on the frequency and spectrum of different types of viable mutations in both cultivars of mungbean in M_2 generation is presented in Tables 1 and 2. Various types of viable mutants observed in M_2 generation are described below.

Plant type mutations

Four classes of viable plant type mutations were observed in the M_2 progeny of mungbean cultivars Vaibhav and Kopargaon-1: tall, dwarf, compact mutations and spreading mutant.

i. Tall mutant: Plants were exceptionally tall (75 to 96cm. vs. 49-51cm in the control) and vigorous. Tall mutants could be observed in both varieties after treatment with different concentrations of SA and EMS. They appeared with a frequency of 30.21% in Vaibhav and 6.18% in Kopargaon-1.

ii. Dwarf mutant: Plants were dwarf, ranging from 32 to 35cm in height vs. 49-51cm in the control, and they remained dwarf throughout the growth period. They showed profuse branching at the base, normal flowers and pods. Dwarf mutants were induced by all concentrations of SA, EMS and all doses of gamma radiation in both varieties.

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They appeared with a frequency of 34.14% in Vaibhav and 22.08% in Kopargaon-1.

iii. Compact mutant: These mutants were dwarf due to compactness of branches. The branching was more at the base, giving rise to dense, interwoven secondary branches, which ultimately made the mutant compact. This type of mutant was induced by 50 kR dose of gamma radiation with a frequency of 1.64% and they were recorded only in Vaibhav variety.

iv. Spreading mutant: This type of mutants showed spreading or semi-spreading habit. They were induced by 40 kR dose of gamma radiation with a lowest frequency of 0.70%. Pods of spreading mutants were small (3.5 to 4cm) and bore faint colored grains. They were found only in Vaibhav variety.

Leaf mutations

A broad spectrum of leaf mutations with remarkable variation in shape, size, number and arrangement of leaflets were observed with various mutagenic treatments.

i. Broad leaf: The leaflets were larger in size with broad lamina. The plants of ranged from 60 to 86cm high and had pollen sterility up to 22%. Frequency of this type was 13.5% in variety Vaibhav and 7.33% in Kopargaon-1, and were induced by 0.02 M and 0.03 M of sodium azide and EMS.

ii. Leathery leaves: The leaflets were thick, leathery and showed waxy coating on the surface. Plants attained a height up to 55-58cm and the flowers were normal and had a pollen sterility up to 15%. These mutations appeared in Vaibhav with a frequency 0.93 and in Kopargaon-1 with a frequency 3.69. These were induced by 0.04 M EMS, and all concentrations of gamma radiations.

iii. Irregular leaves: These mutants were characterized by the presence of leaves with notched leaflets and irregular shape of the lamina. In most leaves, the notch appeared in the middle of the leaflets. Plants were of medium height but were weak in nature. The pods were small and possessed smaller seeds. Rate of seed germination was very low and plant had up to 73% pollen sterility. These mutants appeared in Vaibhav and Kopargaon-1 with a high frequency of 18.58% and 14.15%, respectively.

iv. Dissected leaves: The lamina of the leaves was dissected in a specific pattern (**Fig. 1**). The pods were small and seeds were faint green in color. They appeared in both varieties with a frequency of 2.12% in Vaibhav and 0.74% in Kopargaon-1, with 40 kR dose of gamma radiation.

v. Tetrafoliate leaves: This type of mutants produced leaves with four leaflets. All four leaflets were different in shape and the leaves showed different arrangements. The plants produced normal flowers, bore medium pods and had pollen sterility up to 15%. The plant height was 60-65cm. They appeared in Vaibhav and Kopargaon-1, with a frequency 6.78% and 5.11%, respectively.

Flower mutations

Four different types of flower mutations viz., large flower size, small flower mutations, flower color and abnormal (stamen) flower mutations were observed in the M_2 progeny of mutagen administered mungbean cultivars. All the doses of gamma radiations were effective in inducing flower mutations. Flower mutations were recorded only in the variety Vaibhav.

Pod mutations

Five types of pod mutations were observed in the M_2 progeny following mutagenesis as follows:

i. Long Pod Mutant: The pods of these mutant plants are long (9.8 to 10.1cm) as compared to those of the control (6.9 to 7.9cm), and contained medium-size green seeds. This type of long pod mutations was observed in M_2 progeny subjected to treatment with different concentrations of SA and EMS. They were recorded in Vaibhav variety with a frequency of 19.14% and in Kopargaon-1 with 21.15%. EMS induced higher frequencies of mutations in both varieties.

ii. Curved pod: This type of mutation was characterized by the presence of medium to small sized pods, which were curved and possessed small seeds. This type of mutation was recorded with a frequency of 12.12% in Vaibhav and 15.34% in Kopargaon-1, as a result of treatment with different concentrations SA and EMS and all the doses of gamma radiation.

iii. Hairy pod: This mutation was characterized by thick, dense and hairy pods (**Fig. 2**). The pods turned black at maturity and contained black seeds. This type of mutation was recorded only in Vaibhav variety, with a frequency 2.34% as a result of treatment with a 50 kR dose of gamma radiation.

iv. Flat pod: These pods contained small seeds. The mutants were late in maturity (91 days). They were induced by 0.03 and 0.04M concentrations of SA and EMS and all the doses of gamma radiation with a frequency 8.96% only in Kopargaon-1 variety.

v. Pod Color: This type of mutant pods had a pod wall of different colors, ranging from dark green to brown and black. Such mutations in pod color were recorded in Vaibhav variety only. EMS (0.02M) and gamma radiations (40 kR) were effective in inducing pod color mutations with a frequency of 3.14% and 1.83%, respectively.

High-yielding mutants

High-yielding mutants were isolated in M_2 generation of both varieties. They showed high yield contributing characters viz. number of pods, 100 seed weight and yield per plant. These mutants were induced only by lower concentrations of chemical mutagens in Vaibhav and Kopargaon-1 varieties with the frequencies 3.83% and 2.98%, respectively.

Mutations affecting seed color, size and shape

A large number of seed mutations were isolated in M_2 generation. Many of these mutations were associated with other characteristics such as dwarfness, various types of leaf morphological modifications, plant type mutations, etc. Mutations of seed shape and size included small, bold, small with rough seed coat, bold with rough seed coat, wrinkled seeds, elongated seeds, etc. The seed color mutations included black, faint brown, dark green and reddish brown (**Fig. 3**).

Several workers have reported induction of viable mutations employing physical and chemical mutagens in mungbean [6, 7]. In our studies, EMS has emerged as a more effective mutagen than sodium azide and gamma radiation, in terms of mutation spectrum. Comparison of the spectrum of viable mutation had shown that particular mutagens induced specific mutations in a relatively large number, which was produced rarely by other mutagens. The variation in mutation frequency, within and between treatments noted in the present study may be due to the number of genes involved in the mutational process. A 50 kR dose of gamma radiation induced a novel mutant that showed multiple morphological mutations like large flowers with dark yellow petals, dense, thick hairy pods and black colored seeds. It was named Lhb mutant (large flower, hairy pod and black seed mutant). Such a mutant had not been reported earlier in mungbean. These Lhb mutants were also recovered in M_3 populations of mungbean. Lhb mutants also showed multiple mutagenic effects on various other traits. The Lhb mutant raised through gamma radiation-induced mutagenesis in the present research seems to be promising in at least for two characters i.e., semi-dwarf habit and presence of thick dense hairs on the pods. The semi-dwarf habit of the plant enables it to be lodging resistant. The thick dense hairs on the pod help in protecting it from insects and caterpillar predators and may be an economically promising and important character. It can be used or incorporated in breeding programmes that are aimed at genetic improvement of mungbean. According to Patil [8], multiple mutations are either due to mutation of a pleotropic gene, mutation of gene clus-

ters, or to a loss of chromosomal segments. Leaf, flower, pod and seed mutations obtained in the present investigation might have arisen as a result of mutations in the genes that control the ontogeny of these organs through their gene products and altered biosynthetic pathways. Among the stable mutants only high-yielding mutants showed high levels of proteins, amino acids, albumins and globulins followed by tall and dwarf mutants (**Table 3**). The large seeded mutants may be utilized in breeding programmes as donors. The early maturing mutant exhibited the presence of 12 polypeptide bands while the Lhb mutant showed the presence of nine polypeptide bands (**Fig. 4**). These bands differed from those of control and other mutants in position and molecular weight. This difference in banding pattern of these two mutants (early-maturing and Lhb mutants) can be used as molecular markers to identify them from other mutants and controls. These mutants are promising and can be used in breeding programmes of mungbean aimed at genetic improvement of protein, albumin and globulin contents in the mungbean genotypes.

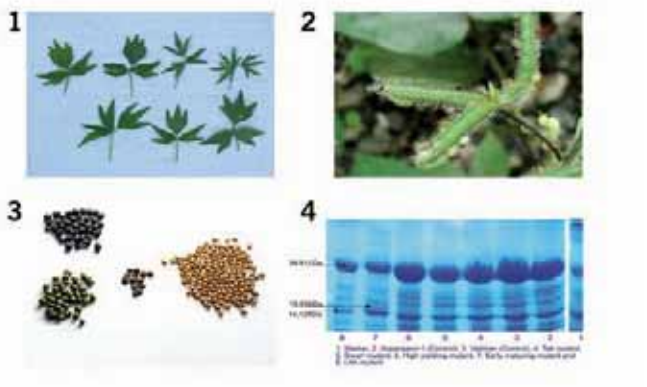

Figure 1-4 1. Dissected mutant leaves; 2. Hairy pod mutant; 3. Seed mutants; 4. Electrophoregram of mungbean mutants.

Table 1. Frequency of induced viable mutations (in%) in M_2 progeny of mungbean cv. Vaibhav.

Treatment	Total mutation Frequency (%)	Plant type mutations	Leaf mutations	Flower mutations	Pod mutations	Seed mutations	Maturity type mutations
			SA				
0.01M	2.22	0.44	0.22	-	0.44	0.66	0.44
0.02 M	3.01	0.46	0.69	0.23	0.46	0.69	0.46
0.03 M	3.85	0.45	0.90	0.22	0.68	1.13	0.45
0.04 M	2.74	0.45	0.68	0.22	0.45	0.45	0.45
			EMS				
0.01M	2.91	0.44	0.44	-	0.67	0.89	0.44
0.02 M	4.64	0.46	1.16	0.23	0.69	1.16	0.92
0.03 M	4.90	0.44	1.32	0.66	0.89	1.11	0.44
0.04 M	4.35	0.45	1.37	0.45	0.68	0.91	0.45
			Gamma				
30 kR	2.55	0.23	0.69	0.23	0.46	0.69	0.23
40 kR	3.52	0.23	0.47	0.69	0.69	0.69	0.47
50kR	4.21	0.46	0.70	0.93	0.93	0.70	0.46

Table 2. Frequency of induced viable mutations (in%) in M_2 progeny of mungbean cv. Kopargaon-1.

Treatment	Total mutation Frequency (%)	Plant type mutations	Leaf mutations	Flower mutations	Pod mutations	Seed mutations	Maturity type mutations
			SA				
0.01M	1.13	0.45	0.22	0.45	-	-	0.44
0.02 M	2.51	0.45	0.68	0.68	-	068	0.46
0.03 M	2.92	0.22	0.67	0.9	0.67	0.45	0.45
0.04 M	2.79	0.23	0.69	0.69	0.69	0.46	0.45
			EMS				
0.01M	1.60	0.45	0.45	0.45	0.22	-	0.44
0.02 M	3.71	0.46	0.16	0.46	0.69	0.92	0.92
0.03 M	3.29	0.23	1.41	0.70	0.70	0.23	0.44
0.04 M	2.95	0.22	1.36	0.68	0.68	-	0.45
			Gamma				
30 kR	2.15	0.23	0.71	0.71	-	0.47	0.23
40 kR	3.19	0.24	1.23	0.73	0.49	0.49	0.47
50kR	2.5	0.25	0.5	0.75	0.5	0.5	0.46

Table 3. Variation in the protein, globulin and albumin contents of mungbean mutants.

Mutants	Protein Mean (%)	Globulin Mean (%)	Albumin Mean (%)	Mutants	Protein Mean (%)	Globulin Mean (%)	Albumin Mean (%)
	Vaibhav				Kopargaon-1		
Control	22.2	12.7	10.2	Control	21.36	12.76	10.2
Tall mutant	23.3	13.3	10	Tall mutant	21.5	12.26	8.5
Dwarf mutant	24.7	13.1	9.2	Dwarf mutant	22.1	11.46	9.23
High yielding	29.3	16.1	13.7	High yielding	26.46	14.43	12.76
Dissected leaf	19.5	10.4	7.2				
Early maturing	23.4	11.7	9.8	Early maturing	21.26	12.7	9.56
Late maturing	17.5	9.3	5.7				
Variegated leaf	14.7	7.3	5.1				
Lhb mutant	18.9	10.5	6.4				
S.E.	0.35	0.37	0.26	S.E.	0.65	0.16	0.42
C.D. at 5%	1.04	1.12	0.78	C.D. at 5%	2.13	0.54	1.38
C.D. at 1%	1.43	1.54	1.07	C.D. at 1%	3.1	0.79	2

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Genetic Enhancement of Groundnut (*Arachis hypogaea* L.) for High Oil Content through Gamma-Ray Mutagenesis

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Abstract

Breeding for increased oil content (OC) is important in groundnut since 70% of the Indian groundnuts are utilized for oil purpose. To induce mutations for higher OC, seeds of TAG 24 were irradiated with 150, 250 and 350Gy Gamma-rays. OC in M_3 seeds from M_2 plants estimated by Nuclear Magnetic Resonance Spectrometer, ranged from 36.39% to 52.85% as compared to 43.38% to 50.83% in the parent. In the M_2, 60 plants had superior OC as well as seed yield, 46 plants had superior OC and 62 plants had superior oil yield. Based on OC and seed yield, 107 plants were advanced. Progeny mean OC in M_4 seeds indicated 14 progenies bred true by recording 1.5 - 4.9% higher OC than parent. Of these, 11 progenies also recorded superior seed yields of 3.0 - 86.0% and oil yields of 6.2 - 92.4%. Further in the M_5 generation, four mutants scored significantly higher progeny mean OC, seed yield and oil yield with 2.4 - 5.8%, 46.6 - 67.8% and 54.4 - 71.2% superiority, respectively. True breeding behavior of high oil mutants was confirmed by progeny evaluation in M6 generation. All the mutants had significantly superior OC with three mutants having greater seed and oil yields. Genetic improvement for OC was brought about by Gamma-ray mutagenesis of TAG 24, wherein seven mutants exhibited consistently superior OC of 4.3 - 6.1% based on pooled mean from M_3 to M_6 generations, in addition to an improved seed yield and oil yield. Thus, induced mutagenesis was successful in bringing about genetic improvement for a complex trait like oil content in groundnut.

Introduction

Groundnut (peanut) is an important food, feed and oilseed crop and contributes 27.7% to Indian and 8.5% to world's oilseed production [1]. Among vegetable oils, groundnut oil stands in second position in India and fifth in the world, contributing 21% and 4% respectively to total oil availability. Around 46% of global groundnuts are crushed for oil purpose while in India the rate is at 70%. Per capita consumption of groundnut oil in India (1.568 kg) is almost double that in the rest of the world (0.778Kg). Frequent groundnut consumption promotes cardiovascular health by lowering serum LDL-cholesterol levels and reduces the risk of development of type II diabetes [2].

Groundnuts are valued for their high quality oil, rich in monounsaturated fatty acids like oleic acid. Most of the oil is found in the cotyledons, which form 72.4% of the seed. Groundnut oil is considered an excellent cooking medium in Indian culinary, as it does not impair the flavour of herbs and spices by contributing its own. Besides, it can be stored at room temperature for 18 months without deterioration [3]. Though modifications for oil quantity in induced mutants of *Brassica* spp. [4-5], *Gossypium* spp. [6], *Arachis hypogaea* [7-8], *Glycine max* [9] and *Helianthus annuus* [4,10] were reported, there were no concentrated studies for breeding for improved oil content using induced mutagenesis. Here, we report breeding for oil content in cultivated groundnut using Gamma-ray induced mutagenesis.

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Materials and Methods

For the induced mutagenesis, a popular groundnut variety, TAG 24 was irradiated with 150, 250 and 350Gy Gamma-rays (500 seeds each) and sown in the field along with 100 untreated seeds [11]. M_1 generation was raised and all the plants were harvested individually dose-wise. Seed oil content (OC) was estimated by Nuclear Magnetic Resonance (NMR) Spectrometer (Oxford MQA 6005 Model, Oxford Instruments UK Ltd., Oxan, UK). Seed sample size was standardized initially by analyzing OC of single, two, three and more sound mature seeds until they reached the mark on glass tube of NMR. Sound mature M_3 seeds from 2781 M2 plants (1872 plants in 150; 511 in 250 and 398 in 350 GY) were used for estimating OC by NMR. Mutant populations were advanced as plant to row progeny in M_4, M_5 and M_6 generations and OC was estimated progeny-wise in each generation. Progeny means for OC of mutants were compared with parent using t-test in each generation.

Results and Discussion

Since 70% of the Indian groundnuts are utilized for oil purpose, breeding for increased oil content is an utmost important objective in groundnut breeding programmes. Increased oil yield is achieved by increased seed yield and/or increasing oil content. Based on results of oil estimation by NMR for two seasons for two genotypes, TAG 24 and TG 18A, it was found that oil content (OC) was stable from a sample size of 10g to 18g. Accordingly, 10-18g of sound mature seeds was analyzed for the OC.

In order to achieve increased oil content, induced mutagenesis of TAG 24 was carried out using Gamma-rays. It was found that mean oil percentage in the entire 150, 250 and 350Gy treated and parental populations was between 45% and 47%. There was a spectrum of genetic variability for oil content in the M_2 population (M_3 seeds). The widest range of oil content of 36.39 - 52.85% was observed in the 150Gy treatment, compared to 43.38 - 50.83% in TAG 24, followed by 350 and 250Gy (**Table 1**). The highest was 52.85% oil, noted from plants obtained by 150Gy treatment. In this M_2 population, 60 plants had superior oil content (50.40 - 52.72%), as well as seed yield (19.3 – 44.7g plant-1), 46 plants had superior oil content (48.52 – 50.36%) and 62 plants had superior oil yield (9.5 – 22.1g plant-1) compared to TAG 24 (oil content: 46.47%; seed yield: 18.3g plant-1; oil yield: 8.5g plant-1). Based on seed yield and OC, a total of 107 plants were advanced to the next generation.

Of the 107 progenies grown, 14 progenies bred true for high oil content by recording 1.5 - 4.9% increase in mean oil content of M_4 seeds over parent. Among these, 11 progenies also recorded superior seed yields of 3.0 - 86.0% and oil yields of 6.2 - 92.4% compared to TAG 24. TGOM 142 recorded the highest mean oil content (51.55%) and TGOM 61 recorded the highest seed yield (43.9 g plant-1) and oil yield (22.1g plant-1). Further, 10 plants (TGOM 167 to TGOM 176) with oil content 50.00 - 56.18% were selected and harvested separately.

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In an evaluation in M_5 generation, TGOM 2, TGOM 60, TGOM 61 and TGOM 168 scored significantly higher progeny mean oil content with a superiority of 2.4 - 5.8% (**Fig. 1**). Additionally, TGOM 61, TGOM 119, TGOM 168 and TGOM 171 recorded significantly greater seed and oil yields with 46.6 - 67.8% and 54.4 - 71.2% increase over parental mean, respectively (**Fig. 2, 3**). Further, TGOM 61 had the highest oil content, while TGOM 119 had the highest seed and oil yields.

Table 1. Frequency distribution of number of M2 plants in oil content classes

Oil%	TAG 24	150GY	250GY	350GY
36.00 - 36.99		3		
37.00 - 37.99		2		
38.00 - 38.99		10	5	4
39.00 - 39.99		6	5	2
40.00 - 40.99		25	12	15
41.00 - 41.99		44	4	23
42.00 - 42.99		103	19	37
43.00 - 43.99	3	221	38	45
44.00 - 44.99	6	366	63	62
45.00 - 45.99	6	391	112	62
46.00 - 46.99	5	282	133	65
47.00 - 47.99	3	149	67	37
48.00 - 48.99	2	87	26	26
49.00 - 49.99	2	76	22	10
50.00 - 50.99	3	63	5	7
51.00 - 51.99		32		2
52.00 - 52.99		11		1
Total plants	30	1872	511	398
Mean	46.47	45.58	45.73	45.1
Range	43.38 - 50.83	36.39 - 52.85	38.54 - 50.46	38.24 - 52.47
SD	2.09	2.38	2.08	2.43
S Em±	0.382	0.055	0.092	0.122

True breeding behavior of high-oil mutants was confirmed by studying their performance in M6 generation. All seven mutants had significantly superior oil content with the highest oil percentage in TGOM 119 (**Fig. 1**). Among these, TGOM 60, TGOM 61 and TGOM 119 also recorded greater seed and oil yields with 27.3 - 35.3% and 29.8 – 48.4% superiority, respectively (**Fig. 2, 3**). However, oil content in TGOM 167 was higher than TAG 24 while its seed and oil yields were inferior. Genetic improvement for oil content was brought about by induced mutagenesis of TAG 24, wherein mutants TGOM 2, TGOM 60, TGOM 61, TGOM 119, TGOM 167, TGOM 168 and TGOM 171 exhibited consistently superior oil content in M_3 to M_6 generations. These mutants had an improved mean oil content of 4.3 - 6.1% compared to the parent. Additionally, except TGOM 167, these mutants also registered an improved seed yield and oil yield of 13.3 - 42.1% and 18.6 - 50.2%, respectively. TGOM 171 was obtained with 250Gy treatment while rest of the mutants with 150Gy.

Thus, induced mutagenesis was successful in bringing about genetic improvement for a complex trait like oil content. In the literature, it was reported that oil content varied from 36.0 to 60.3% in cultivated groundnut and from 42.2 to 63.2% in wild species based on the genotype, seed maturity, environment, post-harvest treatment, insect or disease incidence, time of planting and harvesting, planting density, plant nutrition, and irrigation. In inter-mutant groundnut crosses, an improvement for oil content was observed due to a combination of favourable genes resulting in increased genetic variance in mutated backgrounds [12]. Through pollen mutagenesis using ethyl methane sulfonate (EMS), Jiang and Ming [13] produced maize mutants with high oil contents. Oil content in developing seeds increased due to an increase in the number of oil bodies without changing in size [14]. Wang, *et al.* [15] found that lipase activity was proportional to the oil content in high oil and low oil maize lines. Increased seed oil content may also result from partitioning of increased amounts of photosynthates into the embryo and decreased amount into hull. In safflower, the oil content was raised from 20% to 48% due to reduced hull content [16]. In *Arabidopsis* mutants, the high oil phenotype is caused by the disruption of the GLABRA 2 gene which encodes a homeobox protein required for normal trichome and root hair development [17].

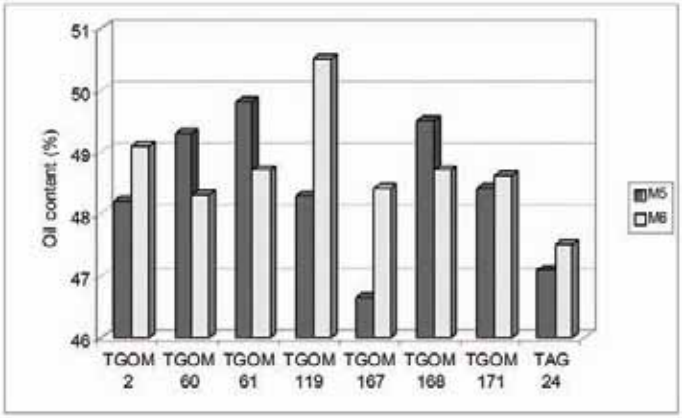

Figure 1 Mean progeny oil content in induced mutants in M_5 and M_6 generations.

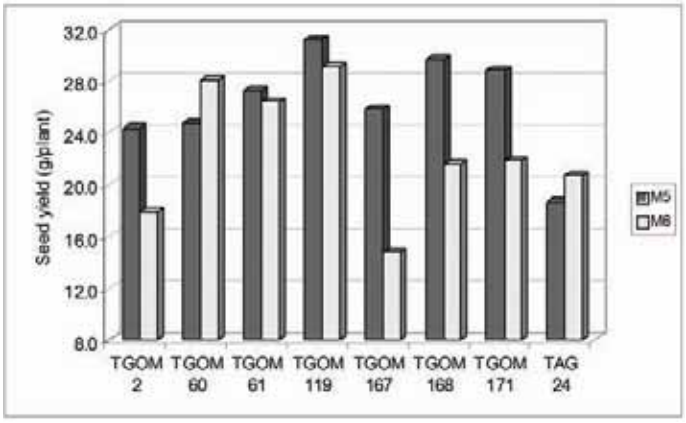

Figure 2 Mean progeny seed yield in induced mutants in M_5 and M_6 generations.

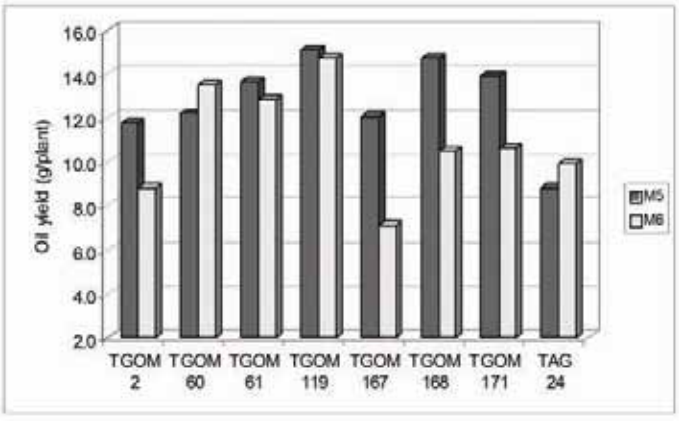

Figure 3 Mean progeny oil yield in induced mutants in M_5 and M_6 generations.

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Development and Utilization of Genetic Variability through Induced Mutagenesis in Sunflower (*Helianthus annuus* L.)

S J Jambhulkar* & A S Shitre

Abstract

Studies on mutation breeding at Bhabha Atomic Research Center (BARC), Mumbai, India, were initiated with the objective of isolating black seed coat mutant from zebra stripped seed coat variety 'Surya' whose yield potential is equivalent to hybrids. Besides black seed coat mutants, a large number of morphological mutants were isolated. Prominent among them are a fasciation stem mutation and an extreme dwarf mutant, each controlled by a single recessive gene. One of the dwarf mutants had a 90cm plant height with sturdy stem. Furthermore, variations in number, shape, and size of ray florets were also isolated. In seed coat color black, white, and brown colored mutations were isolated. Seed and oil yields of one of the black seed coat mutant genotype TAS82 were superior over three checks. Also, TAS82 was relatively tolerant to sunflower necrosis disease (SND) and tolerant to low rainfall conditions. Based on these superior characters, it was identified for release in the state of Maharashtra and notified by the government of India in 2007. Morphological and biochemical mutations isolated in other laboratories along with genetic control are also mentioned here.

Introduction

Sunflower is one of the youngest cultivated crop plants. Wild sunflower is native to North America. In India, it was introduced in the 1960s. The cultivated area is around 1.8-2 mha and it ranks fourth after groundnut, rapeseed-mustard, and soybean. Present yield potential of 10-12q/ha has been exploited through the development of hybrids using cytoplasmic male sterility. However, changing nutritional requirements and environmental vagaries have imposed new trust areas for sunflower improvement programmes. Genetic improvement through induced mutagenesis in sunflower could pave the way to develop desirable varieties/hybrids with higher seed and oil yields, better nutrition, and tolerance to biotic and abiotic stresses. Mutation breeding has been successful for induction of desirable variability and its utilization in developing high-yielding varieties [1, 2, 3]. Compared to self-pollinated crops, mutation breeding in sunflower has been limited. However, efforts to enhance the spectrum of variability for morphological, biochemical, yield and its attributes have been carried out [4, 5, 6, 7]. Mutation breeding in sunflower is briefly overviewed in this article.

Morphological mutations

Plant height, stem, leaf, and head (inflorescence) are the important sunflower morphological characters. Inflorescence consists of ray and disc florets. Mutations in various morphological characters are presented in **Table 1**. Most of these mutations are controlled by single recessive genes.

Work carried out at BARC

Mutation studies were initiated in 1991 with the objective of isolating black seed coat mutants from the zebra-striped seed coat variety 'Surya' whose yield potential is equivalent to hybrids. Besides 7 black seed coat mutants, a large number of morphological mutants were isolated [7]. Prominent among them are a fasciation mutant [16] with 125 leaves against 30-35 in the parent and an extreme dwarf mutant [12] with a plant height of 11cm against 180cm of the parent. Single recessive genes control each of these mutations. Various morphological mutations are controlled by either a recessive or dominant or additive gene [14] and could be exploited in sunflower improvement programmes. Dwarf and semi-dwarf mutants avert lodging and stalk breakage and they have prominence in breeding [24]. One of the dwarf mutants of 'Surya', which grows to 90cm with a sturdy stem, is being exploited to develop dwarf hybrid/varieties.

Table 1. Various morphological mutations and their inheritance.

Character	Mutant	Genetic control	References
Chlorophyll	chlorophyll defi- cientXantha, virescent Yellow leaf veins		[8],[9] [7]
Plant height	Reduced plant height	Recessive Additive & dominance	[10] [11]
	Extreme dwarf (11cm)	Single recessive	[12]
Leaf	Basilicum leaf	Single dominant	[13]
	Upright leaf	Single recessive	[14]
	Leaf curling	Single recessive	
	Wrinkled & involuted	-	[7]
	Spoon-like leaf roll	Single recessive	[4]
	More leaf number	Quantitative	[4]
Leaf petiole	Erectum	Single recessive	[4]
	Short petiole	Two complementary dominant	[4]
		Two cumulative dominant	[15]
Leaf color	Dark green color	Two complementary dominant	[4]
Stem	Zigzag stem	-	[7]
Stem and leaf	fasciation	Single recessive	[16]
	stem fascination	Single recessive	[17]
Inflorescenece	Chrysanthemoides (*chry*)	Semi-dominant w modifiers	[18]
(Ray & Disc	Tubular ray flower	-	[19]
florets)	Reduced, zigzag, short	-	[4]
	Broad, thin & more	Single recessive	[20,[21]
	ray florets	-	[7]
	Reduced ray florets	Two unlinked pairs of alleles	[4]
	Various shades	-	[4]
	Color shades	-	[21], [22]
Cotyledon	Tricotyledon	-	[23]

In seed coat color black, white, and brown seed coat mutations were isolated. Black seed coat mutants were exploited to develop high yielding varieties as preferred in the market. Sib-mating of seven black seed coat mutants resulted in the development of high yielding genotypes. Seed yield of one of the mutant genotypes TAS 82 (1348 Kg/ha) was superior

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by 17.42%, 12.05% and 53.53% over checks PKVSF9 (1148Kg/ha), Surya (1203 Kg/ha) and Morden (878 Kg/ha), respectively. Oil yield of TAS 82 (528kg/ha) was also superior by 32%, 20% and 78% over check varieties, PKVSF9 (397kg/ha), Surya (440kg/ha) and Morden (296Kg/ha), respectively. Other morphological characters were similar to 'Surya'. Besides, TAS 82 was relatively tolerant to sunflower necrosis disease (SND) and yielded better in low rainfall conditions. Based on these superior characters it was identified for release in the state of Maharashtra and notified by Ministry of Agriculture, Government of India in 2007. Induced mutations for yield and yield components and their use in breeding were carried out since 1973 [6]. In the USSR, 'Pervenets' is the only high yielding variety developed using DMS [33].

Mutations for fatty acids

Edible oil constitutes an important component in human diet and acts as main energy source. In sunflower oil oleic (30%) and linoleic (60%) acids contribute more than 80% of the total fatty acids. From a nutritional point of view, increased oleic acid (70%) and decreased linoleic acids (20%) are desirable. Various mutations for fatty acid content have been isolated [1],[5] and are listed in **Table 2**.

Table 2. Mutations for various fatty acids.

Fatty acids	Parent	Mutagen	Mutant	References
Palmitic acid	Zarya RHA274	Gamma-rays NMU/EMS	275HP (25.1%) *fap1* low	[25] [26], [27]
	BDS 2-691	X-rays	CAS5 (25.2%)	[28], [29]
	BSD-2-423	X-rays	CAS5, CAS12 increased	[30], [31]
Stearic acid	HA821	NMU/ EM	Low Stearic	[27]
	RHA 274	NMU/ EMS	M430 (2%) Low	[26]
	RHA 274 LS-2	NMU/ EMS	Low Stearic	[27]
	RDF 1-532	EMS/ NaN_3 Sodium Azide	CAS3,CAS4,CAS8 incr. CAS14	[28] [32]
Oleic acid	V1M1K-8931	DMS	Pervenets (79.3%)	[33]
	HA382 (18.9%)	EMS/ NMU	M4229 (86.2%)	[26]
	BSD-2-423	X-rays	CAS12 reduced	[30]

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Genetic Improvement of Soybean Variety VLS-2 through Induced Mutations

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Abstract

The narrow genetic base of cultivated varieties in soybean is of global concern. Mutations, spontaneous or induced, are an important source of genetic variability. Seeds of soybean cultivar VLS -2 were irradiated with 250Gy Gamma-rays. A large number of mutants with altered morphological characters like plant height, flower color, sterility, leaf shape, number of pods per plant, seed color, early or late maturity were identified and characterized. Significantly higher oil content was observed in the mutant M-387 (22.7%) and dwarf mutant M-126 (20.7%) as compared to the parent VLS-2 (19.7%). A modified rapid and reliable micro titration plate technique was developed and used for screening trypsin inhibitor (TI) content in the seeds of 7480 M_2 plants. The TI content in the M_5 generation ranged from 13.5 TIU/mg seed meal to 22.9 TIU/mg seed meal. Significantly lower level of TI was observed in the mutants M-104 (13.5 TIU/mg seed meal), M-213 (14.0 TIU/mg seed meal) and M-291 (15.7 TIU/mg seed meal) as compared to the parent VLS-2 (22.4 TIU/mg seed meal). In the M_5 generation, 24 mutant lines were evaluated for various quantitative characters. Analysis of variance showed highly significant variations among mutant lines for yield per plant. Mutant M-17 had the yield per plant of 13.1gm, significantly higher than the parent VLS-2 (8.3 gm). This mutant also exhibited more branches, more pods and higher harvest index. Genetic diversity study indicated that mutant M-17 had maximum dissimilarity value of 24% from the parent. These identified mutants can be utilised in the breeding programme for developing elite varieties of soybean.

Introduction

Soybean [*Glycine max* (L.) Merrill] is an economically important leguminous crop for oil, feed, and soy food products and since 2005 it has occupied first position among the oilseed crops grown in India; it shares nearly 37% of the total oilseeds produced and is cultivated over an estimated area of 8.87 million hectares with a production of around 9.46 million tons. Considering the economic importance of the soybean, crop genetic research on soybean has increased and with the help of conventional plant breeding procedures, dramatic improvements have been accomplished in Indian soybean cultivars. So far more than 93 soybean varieties have been released in India for commercial cultivation. However, the productivity remains only 1t/ha in India as compared to world average of 2.2t/ha. In India, along with various other factors, narrow genetic base of the released soybean varieties is responsible for low average yields [1].

Soybean is found to be associated with a number of anti-nutritional factors that exert a negative impact on the nutritional quality of the protein [2]. Protease inhibitor is one of the important anti-nutritional factors that exert a negative effect by causing pancreatic hypertrophy, hyperplasia which ultimately results in the inhibition of growth. The presence of protease inhibitors is a limitation for the use of soybeans in human and animal feeding. Soybean needs to undergo heat treatment in order to reduce protease inhibitors; however, thermal treatment does not eliminate the inhibitor completely and also reduces its economic viability as a food supplement [3]. Hence, development of cultivars with low trypsin inhibitors (TI) will help to improve nutritional quality of soybean for export and domestic use.

Improvement in yield is normally attained through exploitation of the genetically diverse genotypes in breeding programmes. Mutations, spontaneous or induced, are an important source for inducing genetic variability. Improvement in either a single or a few economic traits and quality characters can be achieved with the help of induced mutations within a short time span. In India, six soybean varieties (Birsa soy 1, VLS-1, NRC-2, NRC-12, MACS-450 and TAMS 98-21) have been developed using induced mutations. Therefore, attempts were made to induce genetic variability for morphological characters, yield attributes, and oil and trypsin inhibitor content in the soybean cultivar VLS-2.

Materials and Methods

Plant material and mutation studies

One thousand seeds of the soybean variety VLS-2 were exposed to 250Gy Gamma-rays in a gamma cell GC 220 with ^{60}Co source installed at BARC. The treated seeds along with the parental control were sown in the experimental field at Trombay to raise the M_1 generation. The data on germination was recorded at three to 12 days after sowing. In all, 748 M_1 plants were harvested individually and the seeds obtained were used to raise the M_2 population in plant to row progenies. In M_2 generation, 7,480 plants were carefully screened for morphological mutations. Plants appearing different from the control for one or more morphological traits were harvested separately. The frequency of the mutants was calculated on the basis of number of mutants identified versus total plant population. In the M_5 generation, 24 mutants for different traits were evaluated for various quantitative characters in comparison to the parental cultivar VLS-2.

Determination of oil and protein content

Oil content of seed samples was determined by solvent extraction using Soxhlet apparatus [Soxtec system – HT (1043)]. The nitrogen content of the seed was determined by the micro-Kjeldahl method [4] and the amount of total protein was calculated from percent nitrogen content using a conversion factor of 6.25.

Analysis of trypsin inhibitor content

One hundred milligrams of ground seed meal/ powder was mixed with 1 ml of 2.5mM HCl and shaken for 30 minutes. The suspension was centrifuged at 25,000g for 10 minutes at 4°C. The supernatant was collected and used for analyzing the TI as described by Page, *et al.* [5]. The TI activity assay was carried out in micro titer plates in duplicate. The first two wells had 10µl of trypsin, 80µl Tris-HCl buffer pH 8, 5µl seed extract,

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25µl of 30% acetic acid (to inhibit the trypsin) and 125µl of BAPNA. The last two wells of the row had 10µl trypsin, 85µl Tris-HCl buffer pH 8, 5µl (Tris-HCl buffer, 2.5mM HCl in the ratio of 1:1) and 125µl of 1mM BAPNA. The first two wells of the row were considered to be blank and the last two wells were considered as control (100% trypsin). For measuring the TI activity of the sample, two adjacent wells of the micro titer plate were used. The wells had 10µl trypsin, 85µl Tris-HCl buffer, 5µl seed extract and 125µl of 1mM BAPNA. After 30 minutes, the reaction was stopped by adding 25µl of 30% (v/v) acetic acid. The absorbance of each well was measured at 405nm in an ELISA reader. One TIU is defined as an increase of 0.01 absorbance unit at 405nm under the above described conditions. One TIU corresponds to one unit of trypsin inhibited.

Statistical methods

Significance of various observations was tested using standard statistical methods. Analyses of variance were calculated as described by Panse and Sukhatme [6]. Statistical analysis of the morphological characters of mutants was conducted using the programme Numerical Taxonomy Analysis System, version 2.00 [7].

Table 1. Frequency and spectrum of mutants in M_2 generation in the cultivar VLS-2

Character of the mutant	No. of plants selected in M_2	Frequency%
Chlorophyll		
Albina	7	0.09
Xantha	35	0.47
Chlorina	69	0.92
Leaf size and shape		
Round	7	0.09
Narrow	7	0.09
Small	38	0.02
Sterile	2	0.05
Plant characters		
Dwarf	80	1.07
Good bearing	32	0.43
High oil	26	0.34
High Protein	1	0.01
Low Trypsin inhibitor	3	0.04

Results and Discussion

Studies in M_1 and M_2 generations

Studies on effectiveness and efficiency based on undesired effects like plant damage, sterility or lethality of the physical and chemical mutagens have been carried out in soybean by several workers. Various parameters like germination percentage, reduction in plant height and plant survival in M_1 generation have been extensively used in soybean to measure the mutagenic effect [8]. In our studies, the germination rate observed in the M_1 generation was 74.8%. In the M_2 generation, chlorophyll and viable mutants affecting morphological and physiological characters were identified and selected. The frequency and the spectrum of the mutants in the M_2 generation are given in **Table 1**. Chlorophyll mutations occur with high frequency following mutagenic treatments of seeds. They are regarded as the test mutations assuming that their frequency is proportional to the rate of viable mutations. Various chlorophyll mutations like albino, xantha, chlorina, virescent, maculata and striata have been observed in soybean [9]. The various chlorophyll mutations observed in the present investigation were albino, xantha, virescent and chlorina. The morphological mutants included those affecting plant height, sterility, leaf shape and number of pods per plant. All 7,480 M_2 plants of the cultivar VLS-2 were screened for oil content using the NMR technique. In the parent, oil content was 19.9% while in the selections it ranged from 13.9% to 24.61%. Twenty-six selections with higher oil content ranging between 23% to 24%, identified and mutation frequency was 0.35% (**Table 1**). Trypsin inhibitor activity was estimated in the seeds of 7,480 M_2 plants of the cultivar VLS-2 by microtitration plate technique. These studies have indicated considerable variation regarding the TI level and it ranged from 28.5 TIU/mg seed meal to 13.7 TIU/mg seed meal. All the selections showing low TI as compared to parent VLS-2 (21.8 TIU/mg seed meal) were identified (Constantin, *et al.*) [10], observed decrease in survival, plant height and seed yield with increase in dose rate of mutagen and found 200 to 300Gy of gamma radiation to be useful in inducing genetic variability in soybean. In another study 100 to 300Gy dose of Gamma-rays was found very effective for inducing genetic variability [11]. In the present study also 250Gy Gamma-rays dose was found effective for inducing genetic variability in the cultivar VLS-2.

Evaluation of mutants of VLS 2

Twenty-four mutants in the M_5 generation were evaluated for various quantitative characters in comparison to the parental cultivar VLS-2. Mean values for important morphological characters are shown in **Table 2**. The mean plant height of the parent cultivar VLS-2 was 22.5cm. The plant height in the mutants ranged from 12.0cm to 21.5cm (**Fig. 1**) (**Table 2**). Extreme dwarf mutant M-494 (**Fig. 2**) showed plant height of 12.0cm. Multi-branch mutant M-17 showed significantly higher number of pods and high harvest index as compared to the parent VLS-2. Analysis of variance showed highly significant variation among the mutants for yield per plant. Yield per plant ranged from 2.8gm to 13.1gm as compared to the parent VLS-2 (8.3gm). Only one mutant M-17 showed high yield per plant 13.1gm (**Table 2**). Induced mutations for quantitative traits [12], leaf and floral modifications [9, 13 and 14] have been reported.

High oil content was observed in the mutant M-387 (22.7%) and dwarf mutant M-126 (20.7%) as compared to the parent VLS-2 (19.7%) (**Table 1**). Mutants with higher oil content have been reported by other workers as well [15]. Protein content in the mutants ranged from 36.7% to 41.7%. Dwarf mutant M-60 demonstrated highest seed protein (41.7%) as compared to the parent VLS-2 (39.7%).

A simple, reliable method of screening a large number of plants for identifying biochemical mutants is a pre-requisite for an efficient breeding programme. In the present study, a rapid and reliable microtiter plate technique was developed and used for assaying TI activity. Three mutants namely M-213 (13.7 TIU/mg seed meal), M-104 (15.4 TIU/mg seed meal) and M-291 (15.9 TIU/mg seed meal) showed lower levels of TI content as compared to parent VLS-2 (21.8 TIU/mg seed meal) (**Table 2**). One of the mutants M-225 (28.5 TIU/mg seed meal) showed higher TI content as compared to the parent. Low TI mutants identified using this technique can be used in the cross-breeding programme for developing low TI lines.

Genetic diversity studies of soybean mutants

Induced mutants affecting quantitative characters have been studied in soybean [12]. The coefficient of variation was found to be higher in mutants of soybean induced by Gamma-rays [16]. In the present study, six major clusters were observed based on dissimilarity values between mutants of VLS-2 (**Fig. 3**). A maximum of eight mutants was grouped in Cluster III followed by seven mutants in Cluster I. Cluster II, Cluster IV and Cluster V had three mutants each. Only one mutant M-17 was grouped in cluster VI and had maximum dissimilarity value of 24% from th e parent. Based on the present study the mutant M-17 was found distinct and diverse and can be utilized in the breeding programme for developing better varieties of soybean.

Table 2. Morphological and biochemical characters of VLS-2 mutants in M_5 generation

M-16	31	95	16.1	20.4	14.7	17.9	40.0	16.8	38.5	6.9
M-17	32	95	17.9	36.6	16.0	19.0	39.7	20.3	48.2	13.1
M-22	31	95	12.5	21.1	12.0	18.8	39.1	20.0	31.6	4.1
M-26	31	96	17.0	27.8	15.4	18.5	40.2	19.8	34.9	9.0
M-40	31	95	16.5	19.4	16.5	17.7	40.5	17.6	33.5	4.7
M-51	31	96	19.2	22.9	15.0	18.5	40.7	17.9	38.9	6.2
M-60	31	95	13.0	8.5	14.8	16.9	41.7	18.3	31.6	3.3
M-104	31	95	17.4	24.8	15.6	19.4	38.9	13.5	42.6	8.4
M-126	31	96	15.7	17.4	15.7	20.7	37.7	16.3	31.6	4.6
M-170	31	95	18.8	14.7	15.4	18.4	39.9	19.4	33.5	5.0
M-180	32	95	14.0	28.3	15.8	18.0	40.0	22.3	41.6	7.4
M-213	31	95	18.3	17.1	15.2	17.7	40.0	14.0	35.9	4.6
M-225	32	96	14.8	20.1	15.9	18.4	38.6	20.9	41.5	7.4
M-226	31	97	13.3	7.9	14.3	19.2	37.7	18.4	31.5	5.5
M-229	31	95	15.0	15.0	16.4	19.4	36.7	21.7	29.7	3.6
M-231	32	96	15.8	14.8	15.5	17.9	40.5	20.6	21.3	3.6
M-235	31	95	17.0	18.3	16.4	17.7	40.4	22.9	42.3	4.8
M-285	31	96	17.0	9.4	14.2	18.9	39.7	21.4	22.8	2.8
M-291	31	96	17.3	15.4	15.4	18.9	38.9	15.7	35.0	3.9
M-387	31	96	17.5	26.0	16.1	22.7	37.1	18.2	47.1	7.2
M-450	31	96	21.5	19.0	15.2	18.7	40.0	17.3	36.0	6.5
M-468	31	96	12.5	15.2	15.6	19.3	36.9	20.4	46.3	8.7
M-494	31	96	12.0	25.3	14.2	18.5	39.6	20.0	44.4	7.2
M-624	32	96	21.5	27.9	16.5	18.3	37.3	21.8	41.0	7.9
VLS-2	31	95	22.5	24.9	15.6	19.7	39.7	22.4	39.8	8.3
SE			0.4	1.3	0.3	0.3	0.5	1.5	1.6	0.8
CD 5%			1.3	3.9	1.0	0.8	1.5	1.6	4.5	2.4
CD 1%			1.8	5.3	1.4	1.1	2.0	2.1	6.1	3.2
CV%			3.8	9.5	3.3	2.14	1.85	4.05	5.9	18.5

Figure 1 Comparison of dwarf mutants

Figure 2 Extreme dwarf mutant

In the breeding programme, hybridization provides unlimited possibilities of generating new combinations of characters, which can be selected in the segregating population. In contrast, with induced mutations it is possible to improve a single trait without causing extensive disruption in the genome. The use of mutation techniques for crop improvement over the past few decades has shown that it is an effective plant breeding method to improve yield, quality and resistance to biotic and abiotic stresses. Thus induced mutations can be widely accepted as a supplementary approach in the crop improvement programme, thus speeding up the breeding programme considerably. The results also indicated that a dose of 250Gy Gamma-rays is useful to induce broad genetic variability in soybean. The other mutants identified may be a useful genetic stock for applied and basic research.

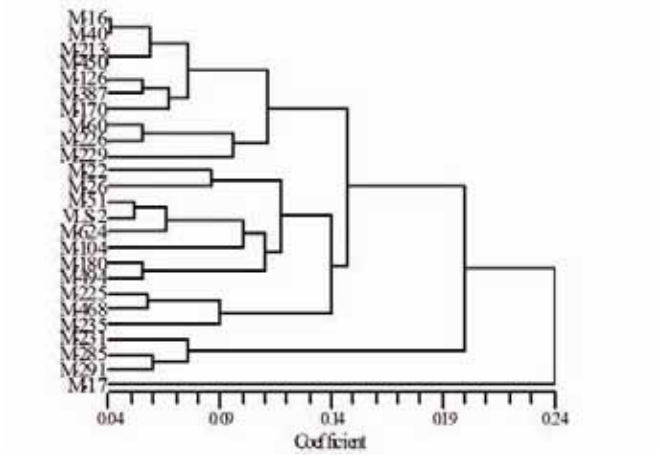

Figure 3 UPGMA dendrogram obtained from dissimilarity index values.

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