Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

Plant Breeding Unit

Activities Report 2006

FAO/IAEA Agriculture & Biotechnology Laboratory
Agency's Laboratories, Seibersdorf
International Atomic Energy Agency
Austria

Executive Summary

Under the auspices of the Sustainable Intensification of Crop Production Systems sub-Programme of the Joint FAO/IAEA Programme on Nuclear Techniques for Food and Agriculture, the Plant Breeding Unit (PBU) of the Agriculture & Biotechnology Laboratory of the Agency's Laboratories, in conjunction with the Plant Breeding and Genetics Section of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, implement activities that relate to enhancement of capacity in Member States to develop superior crop varieties using induced mutagenesis. Within this mandate the Plant Breeding Unit focuses primarily on research and development (R&D) activities, the provision of services, and human capacity-building.

The R&D activities aim at the development and adaptation of technologies that enhance efficiency in the induction, detection and deployment of induced mutants in crop improvement, and driven by the needs and circumstances of developing Member States (MSs) of both the Agency and the FAO. The strategy of the Unit continues to be the use of a matrix of three primary crops with differing biological systems and production constraints (banana, rice and cassava). Additionally, the activities address global or regional breeding objectives for these and other crops.

A major part of the Unit's activities during 2006 related to the establishment of a high throughput reverse genetics platform, TILLING, for the detection of mutation events. Three crops, *Musa, Oryza* and *Manihot* were used for these activities. For *Musa*, shoot tips of the banana cultivar 'Grandnain' were treated with different EMS concentrations to determine the optimum concentration of mutagen for TILLING. Bulk mutagenesis was performed on a total of 4000 shoot tips using four different concentrations of EMS. To select the most suitable genomic regions to screen for induced mutations, a wide range of databases were searched to develop sequences for 14 primer pairs that target genes involved in desired traits in Musa. Candidate primers were synthesized from these sequences. Validation of the primers for use in TILLING was performed by traditional PCR amplification followed by analysis of the yield and molecular weight of the amplicon by agarose gel analysis.

For rice, a total of 153 varieties, representing a wide range of geographic regions were surveyed for the identification of naturally occurring allelic variants in agronomically important genes involved in drought tolerance. To validate the TILLING methodology, a population mutagenized with the chemical mutagen EMS is being developed.

For cassava seventeen elite cassava accessions from IITA, Ibadan, Nigeria were selected for use in validating methodologies for Ecotilling, a high throughput polymorphism discovery method for the detection of spontaneous point mutations, SNPs. Primer pairs from regions of the cassava Granule Bound Starch Synthase and Starch Branching Enzyme genes were used in PCRs to amplify the target genomic regions of the cassava genotypes to validate the fidelity of these primers for use in TILLING.

Through the use of *in vitro* techniques a dwarf variants from a salt tolerant but tall uncultivated

relative of rice were developed and segregation for height was observed in subsequent generations when allelisms test were performed.

Collaborative activities with strategically located and mandated international and national agricultural R&D Programmes constitute the mainstay of our activities especially with regard to validation of technologies in target environments. Our collaborative activity aimed at adding value to elite African cassava varieties.

The Unit continues to support the activities of MSs through the provision of services that include the irradiation of plant propagules to generate mutants using cobalt 60 source; molecular genetic fingerprinting of induced mutants; and ploidy determination using flow cytometry.

Training, when properly conducted, is an activity that provides instant gratification for the trainer and trainee. The areas covered included the induction and detection of mutants; *in vitro* techniques in crop improvement; molecular genetic markers; molecular cytogenetics; high throughput reverse genetics; and population genetics and data management.

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1. Introduction

1.1. Sub-programme and Unit objectives

The Plant Breeding Unit (PBU) is one of the 5 component Units of the FAO/IAEA Agriculture & Biotechnology Laboratory (ABL), Agency's Laboratories, Vienna and Seibersdorf. The Unit works with the Plant Breeding and Genetics Section (PBG) of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in implementing the crop improvement components of the activities of the Sustainable Intensification of Crop Production Systems (SICPS) sub-Programme of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture. The PBU has responsibility for the laboratory components of these activities that include research and development (R&D) activities, and the provision of related services and training. These complement the activities of the PBG which include the overall sub-Programme management; technical backstopping of crop improvement activities in Member States (MSs); and coordination of collaborative research activities. Jointly with the PBG, the activities of both entities target the holistic enhancement of capacity in MSs for the use of induced crop mutagenesis to develop superior crop varieties.

In recent years, the activities of PBU have been driven by a strategic paradigm shift towards an ever increasing reliance on novel biotechnologies to mitigate some of the critical bottlenecks to the efficient use of induced mutagenesis to enhance crop germplasm and the uptake by plant breeders of the ensuing mutants into breeding programmes. The inherent unpredictability mutation induction has per force necessitated the generation of large mutant populations with the attendant consequence of increased costs in terms of time, space, labour and money for growing out and evaluating these populations. This is a discouraging factor especially when viewed in the context of the low percentages of useful mutants that are realized in the end. The potential of novel biological technologies to ameliorate these drawbacks have been amply demonstrated, making it now possible to rapidly regenerate whole plants from single cells and small tissues, to maintain 'miniatures' of such plants under slow growth conditions in aseptic growth media, and to transplant them to the field at will. The use of such *in vitro* techniques in rapidly producing starting materials (cells or tissues) for mutation induction, in multiplying putative mutants, and in dissociating chimeras mutated and wildtype sectors harboured in the same plant have contributed enormously to increasing the efficiency in induced mutations.

One of the major advantages of molecular biology, another relatively new biotechnology, is in the neutrality of its suite of tools. In simple terms, a molecular genetic marker, akin to landmarks and road signs, can be used to find out the allelic form of gene, for instance. The results of such an investigation are not dependent on environmental factors or indeed even on the interaction of that allele with another gene or allele as morphological markers are wont to be, thereby opening up immense potentials for its applications. Additionally, molecular strategies can be used for querying the genetic make up of the individual once deoxyribonucleic acid (DNA) can be extracted from the individual, obviating therefore the need to grow out the experimental materials in the field. Time, space, labour and ultimately, money, are saved in this way. Additionally, more reliable results that are not amenable to environmental influences are obtained.

Another modulating factor for the Unit's strategies and hence activities has been the global trend of crop improvement's R&D priorities for developing MSs. While, in addition to high yield and resistance to a plethora of biotic and abiotic stresses, which have long been at the forefront of plant breeder's goals, enhanced income generation within the resource-poor farmer's limited options (land, crop types, climate, agricultural inputs, farming systems, markets, etc.) is becoming increasingly important in identifying R&D themes. The Unit's approach over the years has been to identify crops and their respective limiting factors to productivity in such manner that technologies developed

and adapted through our activities could be relatively easily translated to addressing the specific own country issues being addressed by individual plant breeders in developing MSs.

Our work on bananas, rice and cassava are therefore predicated upon above considerations. Their seeming disparate biological systems and breeding objectives engender the desired diversity necessary for capturing the widest spectrum of needs in our MSs. Cultivated edible bananas and plantains are parthenocarpic, asexually propagated plants whose seedless fruits are major food security and cash crops in the tropics. The most critical constraint to their productivity is a fungal plantation disease, banana leaf spot disease, the so called black sigatoka disease with causative agent, *Mycosphaerella fijiensis.* On the other hand, rice is a seed propagated crop. A major production constraint for this crop, especially in South East Asia is its susceptibility to salts found in the irrigated paddy fields. Enhancement of the levels of bioavailable micronutrients is another breeding objective for this major global staple and cash crop. Cassava is a euphorbiaceous shrub with starchy storage roots that constitute the mainstay of the caloric intake in the diets of hundreds of millions of people in the tropics. On account of this high contents of starch, its potentials as a cash crop has been recognized and is already being exploited in varied applications in pharmaceutical, textile, paper, adhesive, foods and feeds industries. A bourgeoning area that will revolutionize cassava agriculture is bioenergy. Potentially, the cheapest source of starch, given the right starch configurations – amylose vs. amylopectin contents, for instance – this crop could very easily replace cornstarch and compete with sugarcane in industrial applications.

Through an R&D platform that employs induced mutagenesis in concert with *in vitro* techniques and molecular genetics, the Unit is addressing above select identified production constraint to these three crops (rice, banana and cassava). The ultimate aim for these crops and the identified traits is to, for each crop – trait complex, build a seamless pipeline of a continuum of dovetailing interventions from the selection of initiating materials for induced mutations and inducing such materials to mutate; through their multiplication and evaluation; to the eventual integration into a breeding scheme; and the subsequent field trialling and release as an improved variety.

A number of validated *in vitro* techniques are available both from Agency-supported activities and information in the public domain. These include cell suspension cultures; somatic embryogenesis (including friable embryogenic callus) regeneration systems; doubled haploidy and other forms of rapid regeneration mechanisms. Our activities do not target further technology development nor adaptation in these areas but rather are focussed on their routine applications and their eventual use in concert with molecular techniques, the latter being an area that is currently receiving a substantial investment of the sub-Programme's efforts. To highlight the vital importance of *in vitro* techniques however in induced mutations, we shall be presenting the findings on the induction of mutation on a rice variety using a doubled haploidy strategy in this report.

Molecular genetic strategies that facilitate the tracing of the inheritance of alleles in progenies and for germplasm characterization in general are also available and hold promise for integration into crop improvement programmes that involve induced mutagenesis. In tandem with the renaissance of induced mutations – mostly for genomics studies – have been the development of a number of reverse genetic methodologies that permit the targeted querying of specific genes for mutation events. One such strategy, Targeting Induced Local Lesions in Genomes (TILLING), amenable to high throughput platforms, allows for the detection of single nucleotide polymorphisms in genes. During the reporting period, a significant portion of the Unit's activities had targeted the establishment of proofs of concept for the use of TILLING in banana, rice and cassava improvement. The status of these endeavours will be presented by crops in the following R&D sections of this Activity Report.

The Unit also invests substantial resources in the provision of training (group and individual) to scientists from developing MSs. A summary of these activities for 2006 will also be highlighted in this report. Another Unit-based mechanism for supporting induced mutagenesis activities in MSs is through the provision of services. These include irradiation of plant propagules; determination of ploidy levels; and molecular genetic fingerprinting of promising mutants. This report also includes a summary of such services to MSs for 2006.

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The following were staff members of the Unit (01 January to 31 December 2006):

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2. Research and Development Activities

Through an R&D platform that employs induced mutagenesis in concert with *in vitro* techniques and molecular genetics, the Unit is addressing critical production constraints to rice, banana and cassava in addition to Member States' specific issues addressed through Fellowship training activities. For rice, the aim is to develop variants that are tolerant to abiotic stresses such as salinity and drought while for banana, the interest is in developing induced banana mutants that are tolerant to black sigatoka disease. For cassava, added value is the main improvement objective, to develop cassava variants with modified starch characteristics to make them suitable for varied industries and bioethanol production. The enhancement of the nutritive value through the reduction of anti-nutritional factors as well as enhancement of micronutrient contents also receives substantial attention. The ultimate aim for these crops and the identified traits is to, for each crop – trait complex, build a seamless pipeline of a continuum of dovetailing interventions from the selection of initiating materials for induced mutations and inducing such materials to mutate; through their multiplication and evaluation; to the eventual integration into a breeding scheme; and the subsequent field trialling and release as an improved variety.

For the year under review, the main focus of our R&D interventions has been to establish a proof of concept for the use of a high throughput reverse genetics methodology for the identification of mutation events in target genes. This has involved the generation of appropriate populations as well as the initiation of laboratory assays. The status of these activities for rice, cassava and banana are presented in the following sections.

High throughput reverse genetics strategy for identification of mutation events

The drudgery associated with producing, handling and assaying the requisite large populations of mutant stocks for crop improvement remains a major drawback to the routine application of induced mutagenesis in forward genetics. This could also be extrapolated to include the applications relating to genomics and reverse genetics strategies. As large populations must always be used since the outcomes of mutation induction are unpredictable, the expensive, laborious and time consuming natures of this technology remain impediments to the routine large-scale adoption of induced mutagenesis.

Of recent, there has been a surge in the quantity of publicly available genomics resources as result of the relative ease for obtaining and disseminating genome sequences. A major contribution to the coupling of these available genomics resources to the development, identification and deployment of induced mutants has been the demonstration of the efficacy of a high throughput reverse genetics platform, TILLING (McCallum et al., 2000; 2000a). This strategy, through the provision of allelic series of point mutations in genes of interest allows for a rapid low-cost discovery of induced point mutations in mutated populations. Simply, TILLING entails the pooling of DNA and the use of appropriate polymerase chain reaction (PCR) primers to identify point mutations in genes of interest whose target regions are being amplified. A mismatch-specific endonuclease is used to detect natural or induced DNA polymorphisms in PCR. (McCallum et al., 2000a; Till et al., 2003; Till et al., 2004; Colbert et al., 2001; Henikoff and Comai, 2003; Gilchrist and Haughn, 2005; Greene et al., 2003). The so-called EcoTILLING variant of this strategy involves the detection of spontaneous point mutations, say in ecotypes, as against induced mutant populations. Simply put therefore, the TILLING assay searches the genomes of mutagenized organisms for mutations in a chosen gene, typically single base-pair substitutions. The assays involve principally the use of dual fluorescent labelling of site-specific amplicons from the samples followed by digestion of the amplicons with single strand specific endonuclease, CEL1. This endonulclease selectively cleaves mismatches positions in heteroduplex molecules. On account of the labelling, the location(s) of the mismatch(es) light up and could therefore be identified and further studies through cloning and sequencing.

The advantages of TILLING over other reverse genetic strategies include its versatility (applicable to virtually any organism); adaptability to high throughput; independence of genome size, reproductive system or generation time (Gilchrist and Haughn, 2005). TILLING, though primarily conceived as a functional genomics method – for gene function elucidation -, has also been demonstrated to hold promise for the genetic improvement of agricultural crops via induced mutagenesis (Henikoff et al., 2004). This has been successfully demonstrated for maize (Till et al., 2004); and for wheat it has been used in identifying over 200 alleles of the waxy starch genes (Slade et al., 2004). Soybean genetic improvement is also being enhanced through the application of the TILLING technique by the United States Department of Agriculture (USDA) (http://www.ars.usda.gov/is/AR/archive/jul05/genes0705.htm).

We present in the following sections the status of the proofs of concept of this promising novel technique to the ongoing induced mutation activities in the Unit. These relate to abiotic stresses in rice; biotic stresses and quality traits in banana; and to quality enhancement in cassava. For rice and cassava, the ease for carrying out these proofs of concept assays has to differing degrees been greater on account of the relatively more available genomics information than for the relatively less investigated Musa genome. This accounts for the varying degrees of details in the presentations. While we have strived to develop the Musa resources from the scratch, we have been able to make use of available information for rice and cassava. The results from our prior activities relating to the determination of optimal conditions for mutation induction were expectedly also fed into these activities. Brief summaries of these are also highlighted in the development of the mutagenic populations. In time, and with the establishment of these conditions over a range of genotypes and experimental conditions, mutations would be induced without the need for prior establishment of radiosensitivity parameters, for instance.

In our studies, we have used a chemical mutagen, ethyl methanesulfonate (EMS), the mutagen that has been widely reported for TILLING assays. The assumption has been that EMS provides more point mutations than physical mutagenic agents though there are evidences to suggest that this is not necessarily the case. The next phases of our investigations will involve scaling up to the use of physical agents for producing mutagenic populations. For determining the appropriate TILLING regions of the genome, a web-based platform, CODDLE [which stands for Choosing codons to Optimize Discovery of Deleterious LEsions], was used. CODDLE identifies the region(s) of a gene and of its coding sequence where the anticipated point mutations are most likely to result in deleterious effects on the gene's function. This is of course important for being able to relate the mutation to a phenotype. This platform enables the user to predict:

- nonsense changes and splice junction changes, i.e. changes which should truncate the protein and destabilize the RNA
- missense changes which should alter function of the gene product - those in conserved amino acid blocks in the coding sequence.

2.1. Towards the establishment of a TILLING platform for Musa

This report describes the status of activities for using a model genotype of *Musa acuminata* for adapting published TILLING protocols for routine detection of single nucleotide mismatches in this vegetatively propagated crop.

Producing the mutagenised population

Producing a mutagenized population suitable for TILLING requires careful consideration of several factors, including the genetic structure of the target population, the choice of mutagen and methods of application to the target material. Mutagens that induce point mutations with high efficiency are desirable. To this group belongs the chemical mutagen, EMS which is reported to cause a high density of point mutations, the so called single nucleotide polymorphisms (SNPs). Also, the 'intensity' of mutagenesis that is applied to the target genome is an important component of a TILLING strategy.

Mutagenesis is usually applied in a manner that produces some level of lethality in the treated stage. It is hypothesized that physiological differences underlie the enhanced sensitivity of certain species to lethality caused by alkylating agents. Several data suggest a possible role for polyploidy, but not genome size, in conferring tolerance to high mutation density. Summarising, one of the most crucial requirements for mutation induction is the selection of efficient doses of mutagenic agent for mutating the starting materials.

Reports of previous work involving *in vitro* mutagenesis in the Musa genome suggest a wide variation in responses to EMS treatment conditions without a clear consensus on modalities. Additionally, there is no information on the use of EMS to produce mutagenic populations suitable for TILLING assays. What seems to be clear from the previous reports is that longer periods of exposure to EMS at low concentrations, which produce the least amount of damages, would be preferred for producing high density point mutations and hence higher mutagenic efficiency. Based on these conclusions therefore,

incubation time and EMS concentrations were evaluated the optimal production of mutagenic populations. 300 plantlets were used for the determination of the optimum EMS concentration for producing mutagenic populations from the Grand Naine genotype. To determine the efficient dose of EMS, *in vitro* tissues were exposed to 4 EMS concentrations, 0.25%, 0.5%, 1%, and 1.5% for two different incubation times, 2 hours and 4 hours (Photo 1,2).

For each treatment 25 ex-plants were soaked in EMS solution. To facilitate absorption of the mutagen, 2% DMSO (dimethyl sulfoxide) was used as a permeable agent. After the treatment plantlets were rinsed several times in distilled water and transferred into liquid growth media, S-27 (media composition: 4.4g MS SIGMA Cat.Nr: M-6899; 40g Sucrose; 10ml Cystein; 20ml BAP; 1ml Thiamine; pH: 5.8). The plantlets were sub-cultured to a fresh S-27 media after 24 hours and left to grow for 30 days. After this growth period, the fresh weights of the plantlets were recorded. Figure 1 shows the responses of Grand Naine plantlets incubated for 2 hours in different concentrations of EMS. Figure 2 shows the pictures of plantlets from explants of the same genotype that were exposed to the same EMS concentrations but incubated for 4 hours. The fresh weights of these samples after 30 days are shown in Table 1. All the explants incubated for 2 hours germinated irrespective of EMS concentration indicating that lethality was not achieved by any of these concentrations. For the 4-hour incubation, it was observed that 1.5% EMS was lethal. The data were used to determine the optimal EMS concentration for mutation induction by plotting the percentage reductions in weight against EMS concentrations for the 2 different incubation periods (Figure 1). This is referred to as 50% lethal dose (LD_{50}).

LD_{50} calculations:
2h incubation time y = -30.39x + 101.92
LD_{50} (2h) = 2.04 %
4h incubation time y= -72.498x + 109.01
LD_{50} (4h) = 0.81 %

Photo 1. Grand Naine plantlets exposed to 4 concentrations of EMS and incubated for 2 hours, and controls – water, DMSO and untreated – after 30 days of growth in S-27 liquid media.

Photo 2. Grand Naine plantlets exposed to 4 concentrations of EMS and incubated for 4 hours, and controls – water, DMSO and untreated – after 30 days of growth in S-27 liquid media.

Table 1. Average fresh weights of 30-day old Grand Naine *in vitro* plantlets exposed to varying concentrations of EMS for 2- and 4-hour incubation periods.

	2h incubation time		4h incubation time	
	*Fresh weight average (g)	%	*Fresh weight average (g)	%
0 TIME	1.60	100	1.60	100
0.25% EMS	1.46	91	1.63	102
0.5% EMS	1.46	91	1.25	78
1% EMS	1.21	76	0.40	25
1.5% EMS	0.84	53	0.07	4

*Average of 25 plantlets weighed separately under sterile conditions.

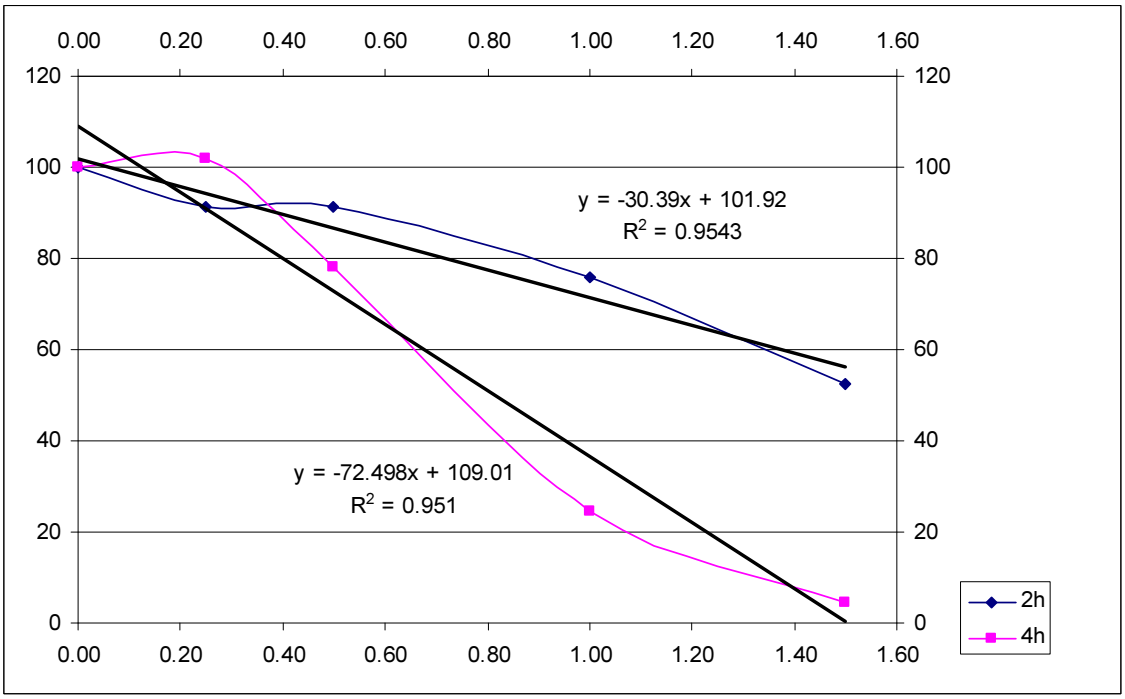


Figure 1. Percentage reductions in fresh weight of *in vitro* plantlets of *Musa acuminate* plotted against EMS concentrations for 2 incubation durations, 2 and 4 hours

This pilot experiment for the determination of the LD_{50} indicated that the lethal EMS concentration for ex-plants exposed to the mutagen for 4 hours was 1.5%. The inference from the graph (Figure 1) was that the damage due to exposure of the propagule to EMS for 2 hours was slower in comparison to the 4 hour-incubation. It was therefore assumed that lethality could only be achieved within a 2-hour incubation period with a higher EMS concentration. For TILLING purposes where the interest is achieving subtle genome alterations, ideally SNPs, the mutagenic treatments should therefore not achieve lethality. The practise is to select a range of treatments around the threshold of the lethal dose. We adopted the strategy of selecting treatment conditions characterized by short incubation periods and higher concentration and decreased concentrations and increased incubation times (with the LD_{50} as the baseline).

To generate the mutated populations for TILLING purposes therefore, bulk EMS treatments were

carried out with a total of 4000 plantlets which were induced to mutate using 4 different treatment combinations (1000 plantlets per treatment):

a) 3 hours incubation time in 1%EMS (Photo 3)
b) 6 hours incubation time in 0.5%EMS
c) 24 hours incubation time in 0.125%EMS
d) 48 hours incubation time in 0.06%EMS

This strategy would additionally achieve the development of structured mutated populations that would permit the linking of phenotypic changes to the mutagenic treatments.

These had a purpose to differentiate a mutated population and also to follow the different changes which may appear in the genetical structure of mutated plants during different times and different concentrations.

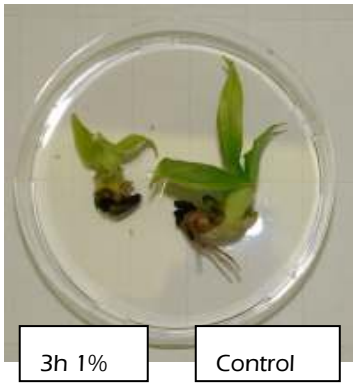


Photo 3. Response (after 30 days in liquid growth media) of *in vitro* banana plantlet incubated in 1% EMS for 3 hours and the control.

After mutagenic treatment, the plantlets were sub-cultured several times in fresh liquid media in order to eliminate the accumulating of phenolic compounds. Survival rates were scored and computed and are shown in Table 2 and Figure 2. The 24-hour incubation period with 0.125% EMS gave the lowest survival rate of 52% while the highest, 87% survival rate, was obtained under the 48-hour incubation time with 0.063% EMS.

Table 2. Survival rates of 4000 *in vitro* banana plantlets exposed to different EMS concentrations for varying durations of incubation.

Bulk Treatment Conditions *(1000 plantlets per treatment)*	***Survivals (%)**
Control	100%
3h 1% EMS	85%
6h 0.5% EMS	80%
24h 0.125% EMS	52%
48h 0.063% EMS	87%

*Average for 1000 plantlets per treatment condition.

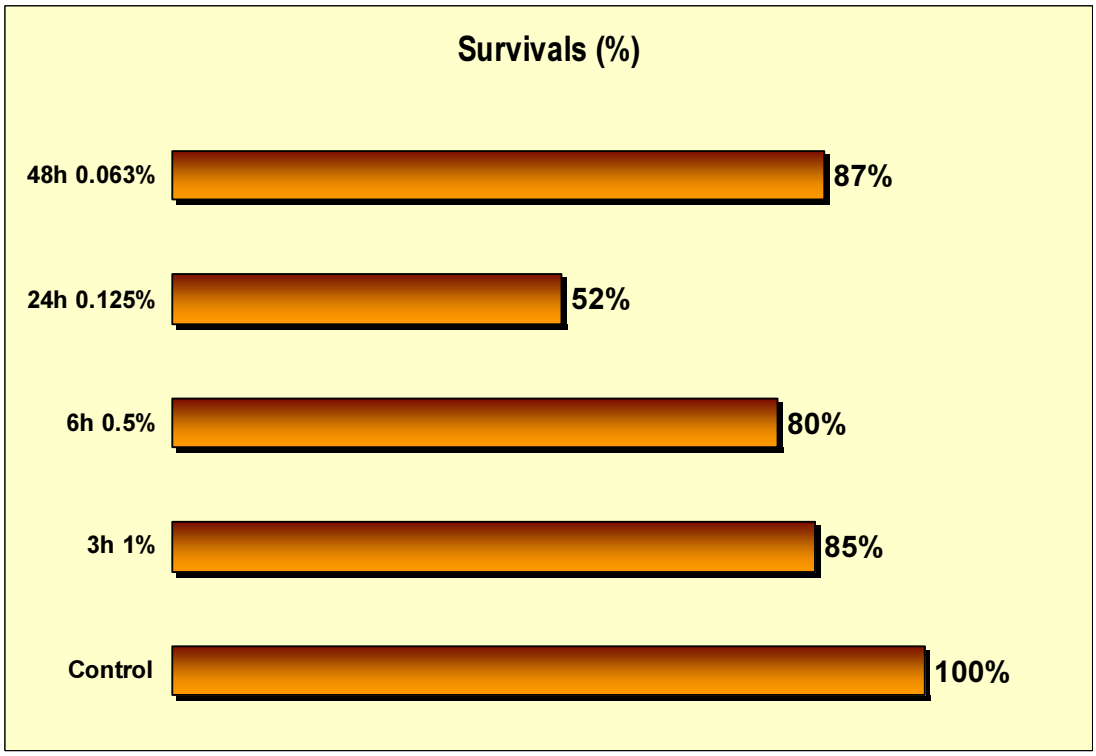


Figure 2. Survival rates of banana plantlets by EMS treatment condition (concentration and duration of incubation combination).

From above treatments, 3004 lines of mutagenised triploid Grand Naine are available for TILLING assays. For this, leaf tissue were collected for pooling DNA. A second cycle of leaf tissue collection is also planned and every single, mutated banana plantlet is being maintained as a new potential line.

Before using this large population, a smaller population of approximately 300 individuals will be used in a pilot study for standardising the TILLING protocols. The first assays will involve the diploid plants from International Transit Centre (ITC) collection to be followed by pilot experiments using triploid genotypes and finalised with the use of 250 EMS treated individuals. The pilot TILLING experiments will involve 14 target genes.

Selection of regions of target genes for TILLING assays

We adopted an *in silico* strategy for identifying suitable genomics resources for the TILLING pilot assays for the Musa genome. This involved a thorough search of the NCBI database. The sequences of 14 genes that met the criteria of being at least 1000 bp in length and that had been annotated were selected for these assays. These 14 genes were distributed on 2 Musa BAC clones, MA4_25J11 and MA4_106O17 that had been sequenced by TIGR (The Institute for Genomic Research; http://www.tigr.org). A schematic representation of these BAC clones showing their gene contents are presented in Figures 3 and 4 respectively.

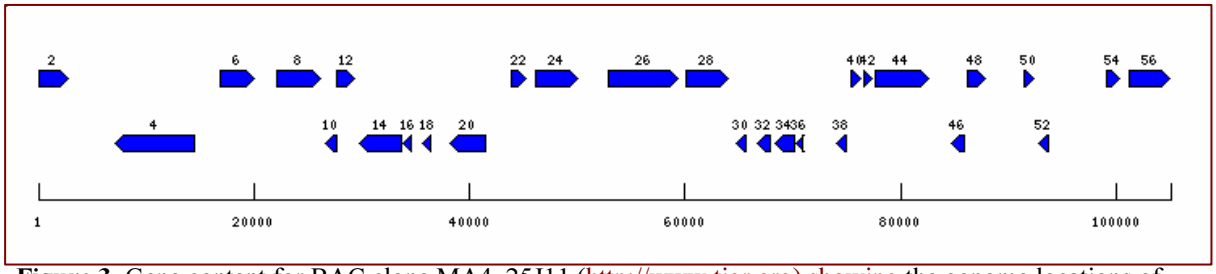


Figure 3. Gene content for BAC clone MA4_25J11 (http://www.tigr.org).showing the genome locations of respective genes.

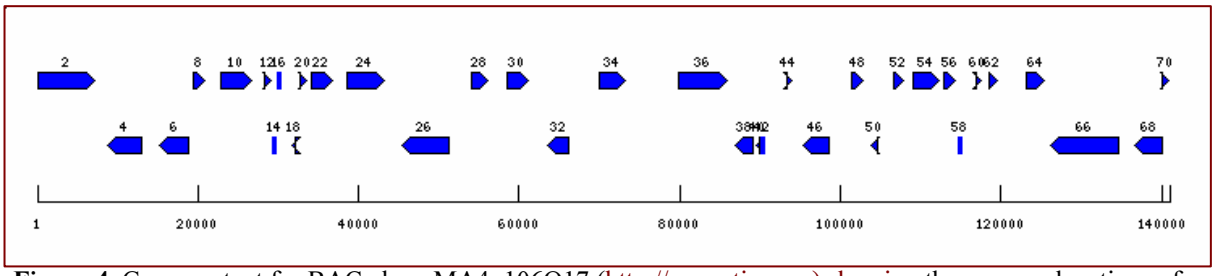


Figure 4. Gene content for BAC clone MA4_106O17 (http://www.tigr.org).showing the genome locations of respective genes.

These selected genes and their gene products as reported in NCBI are given in Tables 3 and 4.

Table 3. Summary of information on the genes selected from Musa BAC clone MA4_25J11.

Number on the Fig 6 graph	Locus	5' coordinate	3' coordinate	Gene product name
2	MA4_25J11.2	2	2747	AMP-dependent synthetase and ligase family protein, 5' partial
4	MA4_25J11.4	14463	7126	FtsJ-like methyltransferase family protein
8	MA4_25J11.8	22147	26245	GTPase family protein
12	MA4_25J11.12	27722	29314	(1-4)-beta-mannan endohydrolase (mannan endo-1,4-beta-mannosidase), putative
50	MA4_25J11.50	91461	92405	6-phosphogluconate dehydrogenase NAD-binding domain-containing protein

Table 4. Summary of information on the genes selected from Musa BAC clone MA4_106O17 .

Number on the Fig 7 graph	Locus	5' coordinate	3' coordinate	Gene product name
2	MA4_106O17.2	1	7153	soluble glycogen [starch] synthase, chloroplast, putative, 5' partial
4	MA4_106O17.4	13066	8765	glycosyl transferase family 31 protein
6	MA4_106O17.6	18876	15292	aminomethyltransferase, mitochondrial (glycine cleavage system T protein), putative
14	MA4_106O17.14	29742	29398	plant lipid transfer/seed storage/trypsin-alpha amylase inhibitor (LTP) family protein
24	MA4_106O17.24	38511	43138	transcriptional factor B3 family protein
30	MA4_106O17.30	58459	61100	nonphototropic hypocotyl 3 (NPH3) family protein
32	MA4_106O17.32	66200	63537	zinc finger (C3HC4 type RING finger) family protein
38	MA4_106O17.38	89185	86976	no apical meristem (NAM) protein, putative
46	MA4_106O17.46	98567	95379	heat shock protein DnaJ N-terminal domain-containing protein

Primer Selection: The requirements for a "good" TILLING region (where appropriate primers should be selected) are critical. In many cases the analyzed sequences didn't meet the requirements. The following are the recommended important criteria for the selection of genomic regions for designing and subsequently synthesizing PCR primers for use in TILLING:

- amplicon size which should be approximately 1.5 kb amplicon;
- approximately 5% truncation; and
- 48% missense

The interactive web-based platform CODDLE (Choosing codons to Optimize Discovery of Deleterious Lesions - http://www.proweb.org/input/) was used to identify the target regions of the genes where mutations would lead to identifiable phenotypic changes. These were the regions of selected genes in which G/C to A/T transitions are most likely to result in deleterious effects.

Figure 5, for instance, is a schematic representation of the predicted mismatches in *FtsJ-like methyltransferase* gene, following EMS treatment of the tissue.

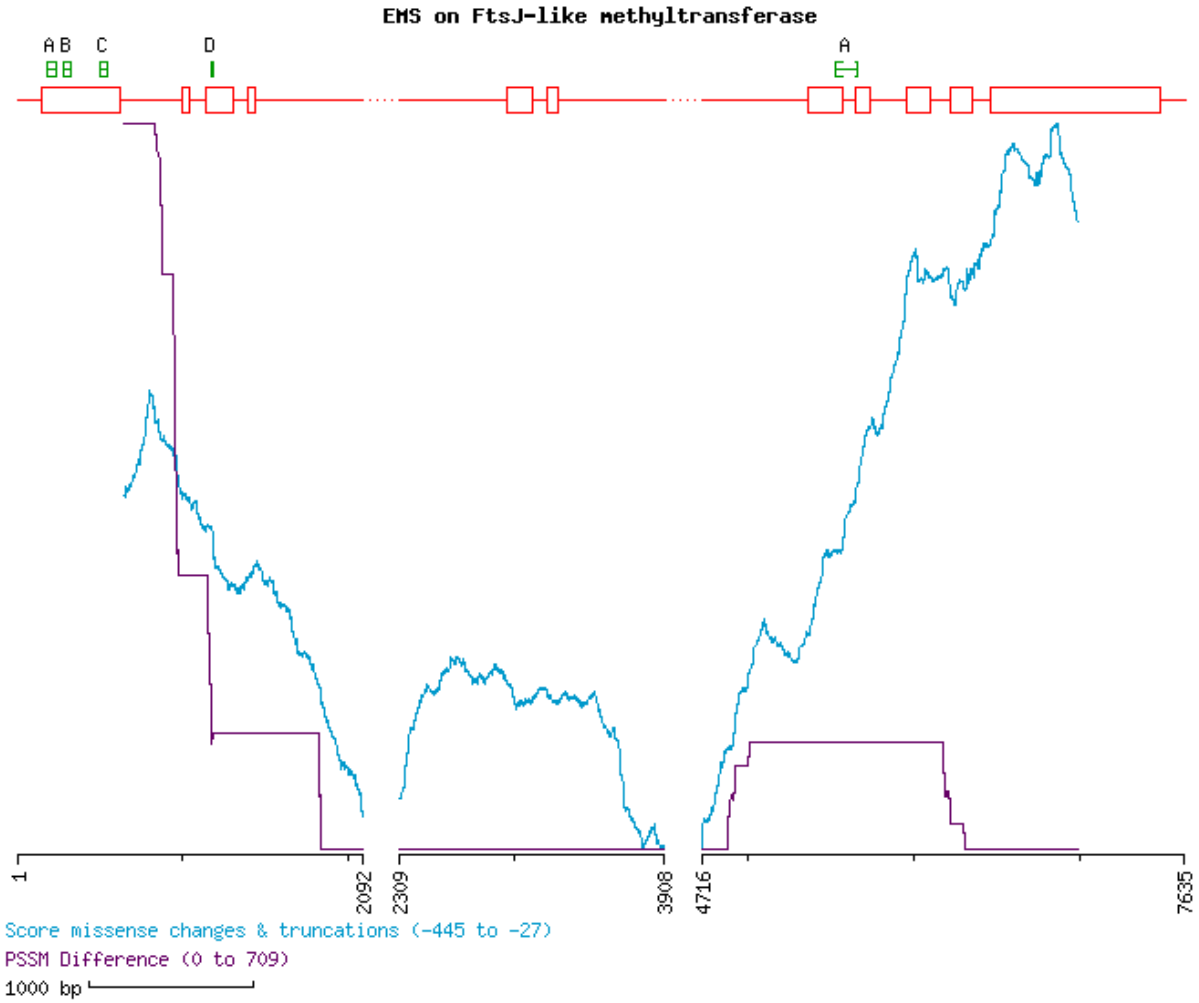


Fig 5. Output of CODDLE program showing the predicted effect of EMS mutagenesis on the *FtsJ-like methyltransferase* gene. The graphical output shows the gene model (red boxes and lines), protein homology (green boxes) and the score of the gene (purple and blue lines). The purple line indicates the score for predicted deleterious missense changes, and the blue line is the score for the total number of non-silent changes (http://tilling.fhcrc.org:9366/tillingdemo/computational_tools.shtml).

Primer Selection: The primer design software, Primer3 (http://frodo.wi.mit.edu/) was used for picking the sequences of the PCR primers. Based on CODDLE outputs, 14 regions were selected for meeting the above minimum requirements. Primers were therefore designed for these regions of the Musa genome targeting possible mismatches in selected genes that would result from exposure of the tissue to EMS. Figure 6 is a schematic representation of above described model for the gene *FtsJ-like methyltransferase*. These regions span a length of 1479 bp, from sequence position 6000 to 7478 on the genomic sequence which has been selected for primer design and synthesis. This region is 43% ideal, as compared to an ideal target containing only GC.

Based on the CODDLE prediction, polymorphisms in this gene should be detected in following portions:

- 4.9 % Truncation Changes (4.2 % Nonsense and 0.7 % Splice Junction Changes);
- 55.1 % Missense Changes; and
- 60 % Nonsilent Changes

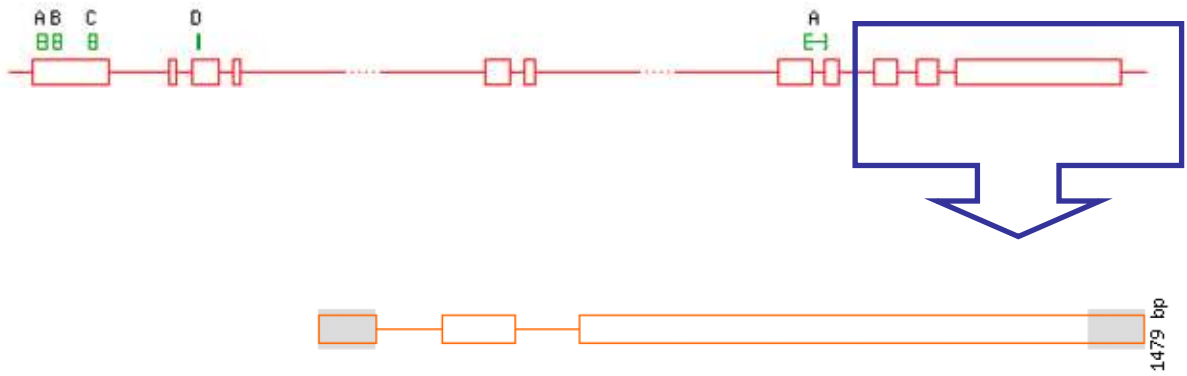


Figure 6. Schematic representation of the *FtsJ-like methyltransferase* gene highlighting (in blue box) the target region selected for primer design based on above prediction by CODDLE.

14 primer pairs targeting genes involved in several traits in Musa were synthesized (Figure 7). The validation of these primers for use in TILLING has been performed by using them as PCR primers and visualizing the amplicons on agarose gels after electrophoresis. Examples are shown in Figure 11. Based on the PCR product sizes, all the primers amplified the target regions of the genome.

Figure 7. Primer pairs (1 to 14) synthesized for use in TILLING assays for the Musa genome. The size of the amplicons for each primer pair is indicated in the row, Amplified Region.

1) TILL-FtsJmt *(ABF69946.1-FtsJ-like methyltransferase family protein)*

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	6000	27	70.275	51.852	6.00	1.00	ATACAGCAAGGGTGATGCAGCAGACAG
Right Primer	7478	27	69.373	44.444	4.00	0.00	ATTTGGCCTTTATTCTTGCGTCCCTTC
Amplified Region		*1479*			5.00	3.00	

2) TILL-AMPstlg (ABF69945.1-AMP-dependent synthetase and ligase family)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	1402	27	69.914	51.852	6.00	0.00	GGCATGTGTACAGCGTAGAACGGAAGA
Right Primer	2894	27	69.936	40.741	6.00	2.00	TTCCTTGAATTGTCGTTGGGCTCAAAT
Amplified Region		*1493*			4.00	1.00	

3) TILL- GTP *(GTP-ase)*

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	175	27	70.079	51.852	5.00	2.00	TATGTAGCTGTGCTAGGCAGGCCCAAT
Right Primer	1635	27	69.897	40.741	4.00	2.00	ATGCCAAAAACATTCCAATAGCCATGC
Amplified Region		*1461*			5.00	3.00	

4) TILL-betaMHD (beta mannan hydrolase)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	202	27	69.687	51.852	6.00	1.00	GTTGAGGCTGCAGGAGGAGAGTTCATT
Right Primer	1700	27	70.244	48.148	4.00	0.00	TTCTCTCTCATGGCCTTTGCCTTCTTG
Amplified Region		*1499*			6.00	3.00	

5) TILL-PHGdh (6-phosphogluconate dehydrogenase NAD-binding domain-containing)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	284	27	70.279	55.556	8.00	2.00	AACTCTCGGACCCGACTCGAGTTTAGC
Right Primer	1624	27	70.004	55.556	4.00	1.00	GAAGGTGTTCGAGAGCGAGCCTGTTAG
Amplified Region		*1341*			7.00	1.00	

6) TILL-starchST (soluble glycogen [starch] synthase, chloroplast)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	4372	28	67.112	39.286	6.00	3.00	TGAAAATGGTCATAAGCTGTTTGAGACG
Right Primer	5867	27	69.570	44.444	5.00	1.00	CTATTTCCATGATGTGGCTTCGGATGA
Amplified Region		*1496*			5.00	1.00	

7) TILL-glycTR (glycosyl transferase family 31)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	2554	27	69.689	48.148	7.00	3.00	AGTGCCACTTCTGGTGGAATTCTGGAT
Right Primer	4013	27	67.965	40.741	8.00	2.00	TTCATGTGATTAGATGAGGCGAAATGC
Amplified Region		*1460*			5.00	2.00	

8) TILL-amthTR (aminomethyltransferase)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	2255	27	70.274	44.444	5.00	2.00	CGGCATCCAAGTTTCTCATGCCTTTTA
Right Primer	3762	27	69.289	44.444	6.00	3.00	CAACTCGAGCAAAAAGCATCTCACGAT
Amplified Region		*1508*			7.00	2.00	

9) TILL-lptr (plant lipid transfer/seed storage)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	602	27	69.882	55.556	6.00	0.00	GCTCTGACCTCAGTCCCATCACCTCTT
Right Primer	1346	27	68.661	40.741	6.00	3.00	TTCTTGCTCGATCATGGTGACATTCAT
Amplified Region		*745*			5.00	0.00	

10) TILL-B3trsp (transcriptional factor B3 family protein)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	2306	27	70.986	51.852	7.00	1.00	ATCAGTGATCTTGACCCTGTCCGTTGG
Right Primer	3807	27	70.118	51.852	5.00	3.00	TGACACCAGTACCAAGACTTCGGATGC
Amplified Region		*1502*			5.00	1.00	

11) TILL-NPH3 (nonphototropic hypocotyl 3 (NPH3))

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	1058	27	69.958	48.148	7.00	0.00	TCGAACCTGCTGCCAAGTTCTGTTATG
Right Primer	2555	27	70.247	51.852	5.00	3.00	GTCCATGCTCACCTTCAAGACCTGGTT
Amplified Region		*1498*			4.00	1.00	

12) TILL-zincFNG (zinc finger (C3HC4 type RING finger) family)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	1456	29	68.945	37.931	7.00	1.00	TGGATTTAGCTTGTCAATGGAAATGAGGA
Right Primer	2880	27	69.095	44.444	6.00	2.00	ACACAAAACTGGGGCTAATGTTGTCCA
Amplified Region		*1425*			5.00	2.00	

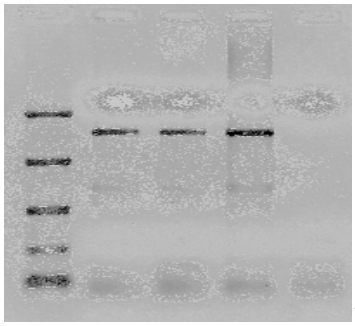
13) TILL-NAM (no apical meristem (NAM) protein)

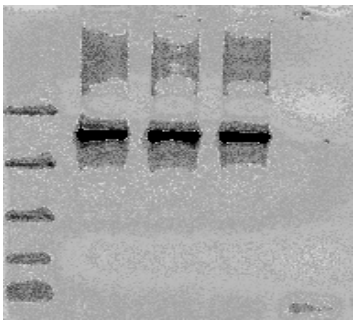
Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	970	27	67.481	37.037	5.00	0.00	TCTTTCTCAACCCCCAAAAAGAAAACA
Right Primer	2466	27	70.644	51.852	3.00	0.00	TCCTTCCTTGTGTGGGGACATCGTTAG
Amplified Region		*1497*			7.00	2.00	

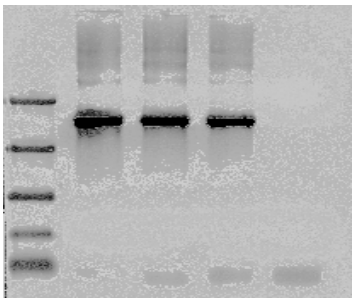
14) TILL-DnaJ (heat shock protein DnaJ)

Oligo	Start	Length	Tm	% GC	Any	3'	Sequence
Left Primer	1553	27	69.863	51.852	4.00	2.00	AGGAGAAGTCAGGGACCAGAACCGAAT
Right Primer	3025	27	70.160	48.148	4.00	2.00	TATAAACCGCCCAAATCTCACCACAGC
Amplified Region		*1473*			4.00	0.00	

Fig 8. Pictures of 1% agarose gels after electrophoresis of the PCR amplified products of some regions of some of the genes to be used for TILLING assays in the Musa genome. These regions were amplified in the Musa genotypes, Calcutta 4, Grand Naine and a mix of both Calcutta 4 and Grand Naine. A: amplicons of a region of ABF69946.1-FtsJ-like methyltransferase family protein; B: amplicons of a region of soluble glycogen [starch] synthase, chloroplast; C: amplicons of a region of aminomethyltransferase.

A)  *ABF69946.1-FtsJ-like methyltransferase family protein*

B)  *soluble glycogen [starch] synthase, chloroplast*

C)  *aminomethyltransferase*

APPENDICES

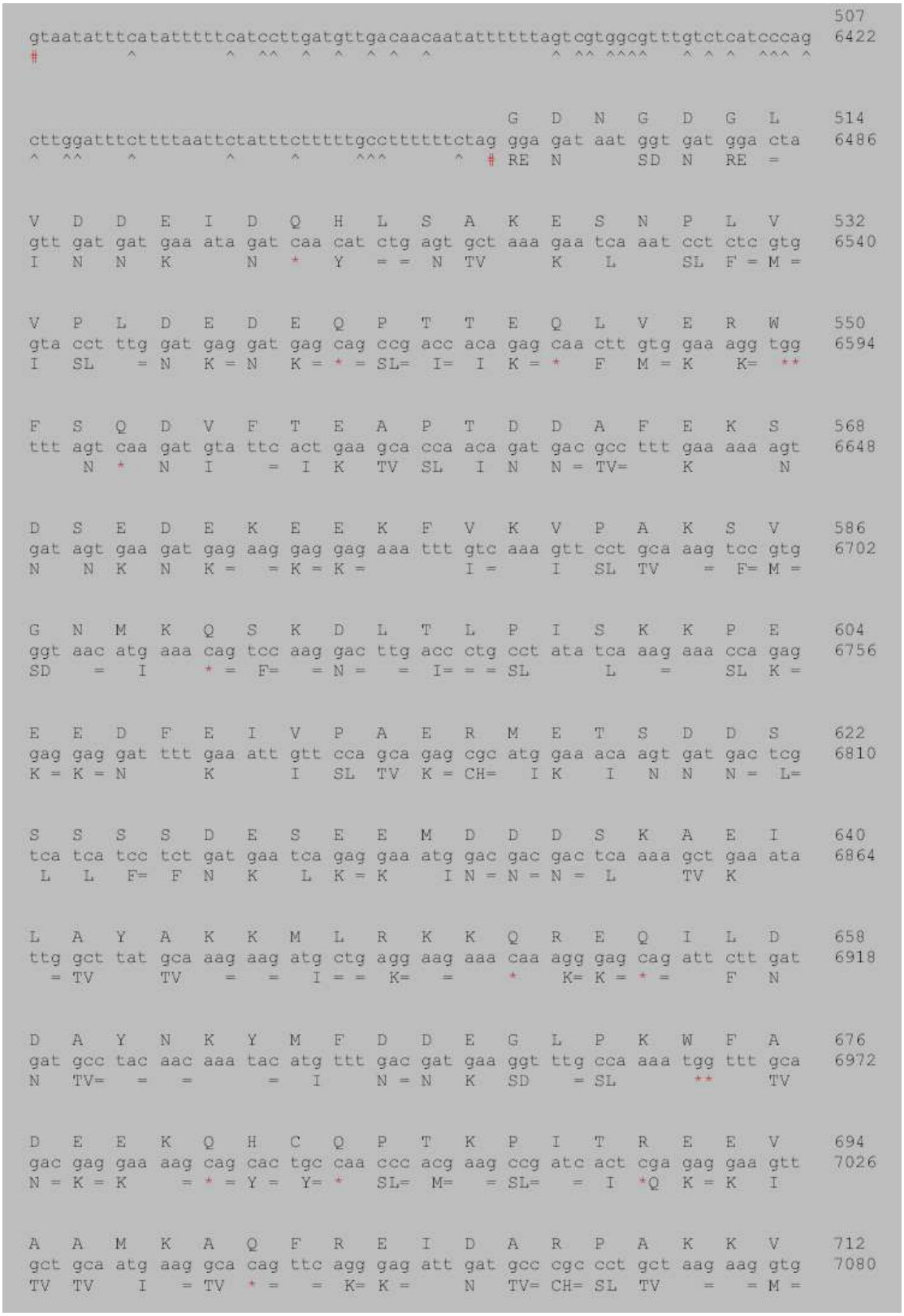


Fig 9. Part of *FtsJ-like methyltransferase* gene selected for TILLING. The possible changes are indicated.

Conclusions and future perspectives

The use of TILLING as a tool for detecting SNPs in the induced mutagenic populations of Musa seems promising going by the successful recovery of the expected amplification products from the selected primers. The next stages will involve the following sequential steps:

- Validation of the TILLING protocol involving the use of endonucleases to selectively cleave mismatches in these target regions using single plant samples;
- Validating the TILLING protocol using pooled samples;
- Design of adequate population structures, field trialing, biochemical and and other assays to relate the mutation events to phenotypic expressions.

2.2. Towards the establishment of a TILLING platform for Rice

Rice population

A total of 153 rice varieties were surveyed for the identification of naturally occurring allelic variants at agronomically important genes using a reverse genetic approach, EcoTILLING. These rice varieties represented a wide range of geographic samples from Iran (Middle East), Myanmar (Asia) Hungary (Europe) and from Sierra Leone (Africa). Additionally, a mutagenic population of an Iranian basmati rice variety, Fajer, has also been developed and will be used for the identification of induced mutations in the genome.

Genomic regions surveyed

Eight different drought tolerance genes have been identified for use in amplifying the target genomic regions in these 153 rice accessions with the aim of identifying single nucleotide polymorphisms or haplotypes. The PCR primer pairs targeting identified regions of these drought tolerance genes were designed using the web-based primer picking software, CODDLE (http://www.proweb.org/input/) the primer pairs were synthesized by VBC-GENOMICS(oligo@vbc-genomic.com or www.vbc-genomic.com.) Table1 shows the primer information including the expected amplicon sizes.

Identification of naturally occurring allelic variants – EcoTILLING

In addition to the 153 rice accessions, Nipponbare, a well-studied rice genotype whose sequence information is easily available, was included in the assays as reference and internal control. The initial assays have involved the use of the primer pairs for the gene, Extensin, a cell wall protein involved in growth expansion. The appropriate genome locations were amplified as confirmed by the product sizes (Figure 1).

Table 1. DNA sequences and other properties of the primer pairs designed from rice (Oryza) drought candidate genes for use in EcoTILLING analyses.

Gene	Bioprocess	Functional Evidence	STP primer name	Sequence	Amplicon
Pp2a4	protein phosphatase	Up-regulated under drought on Syngenta array	pp2a4bL pp2a4bR	TGGCCTGTAGAATTTTAACTTGGTGTTT GG CAACCTCCAGGATGGATGCCATGT	971
DREB1	drought responsive element binding protein 1	Transformation of homologue results in enhanced survival under stress; cM 137 locus is closest hit to 16.1 cM locus	osdreb1L osdreb1R	CATCGTGGCGCAACATGAAAAAGA CCACAGTGCACTCAACACACAGTACAA	1167
TPP	Trehalose6-phosphate phosphatase	Up-regulated under drought on IR64 panicle arrays; 2 loci on CH2 are best top hits to E. coli sequence that on transformation enhances survival	ostps1L ostps1R	AGCGGAAGGTCCCGCAATAAGGTA GCTTCAAAATTGTCGCCTCGGAAA	1509
14-3-3	Membrane associated signal cascade		os1433L os1433R	AGCCGTGGTAATGAGGATCGTTGC CCTGAAGCCGCACACATGGAATTT	1499
MAPK	Transcriptional control of stress response		osmapkL osmapkR	GCCGGAAGCGTTGTACAAGGTCAA CGGCAAGAAAGCATTTCAGGCATC	1499
Extensin	Cell-wall protein for growth expansion		osextenL osextenR	TGTTTGCCTTCCGTTAATGCCAC AGCGCCCCTAATCCGAACCAAAG	1433
SucSase	sucrose synthase		osssynL osssynR	TGAACGACAGGATTCGCAGTCTCG ACTGCCCCACAGTTTCCTTGCTGA	1492
BZIP	Transcriptional contro		osbzipL osbzipR	GTGAGATGGCATCGGAGATGAGCA CTGGCTGCCACCCCTATTTGCATT	1495

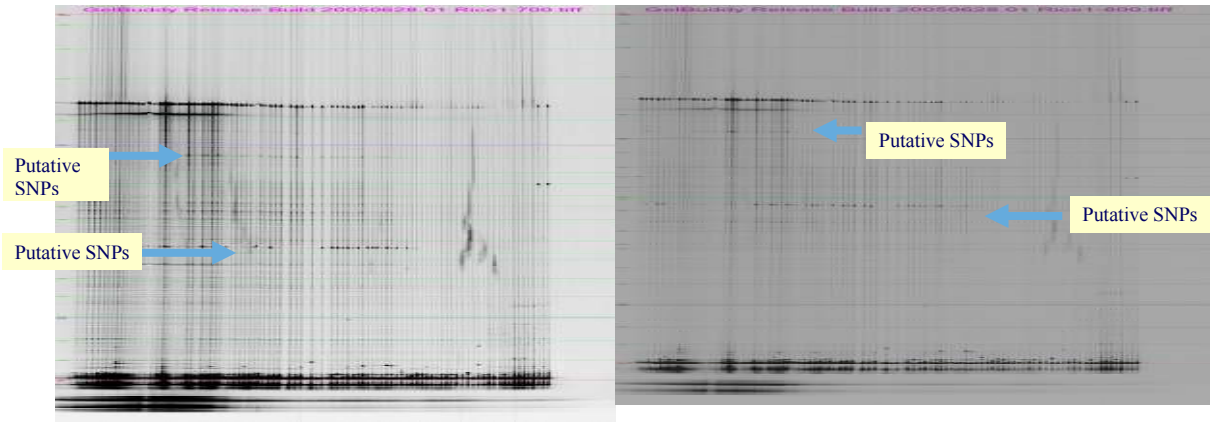


Figure 1. Two gel images showing each of the dual channels corresponding to 2 dyes showing the amplicons of a region of the Extensin gene in rice genotypes. Arrows identify the heteroduplex mismatches corresponding to putative SNPs.

Mutagenic rice population for the identification of induced mutations

An Iranian basmati rice variety, Fajer, was mutagenised with the aim of using the population for validating TILLING methodologies. This is aimed at identifying induced mutations, single nucleotide polymorphisms – SNPs – variations, using a reverse genetics high throughput screening technique, TILLING.

The seeds were treated with the chemical mutagen, ethyl methane sulphonate (EMS). This mutagen, EMS, has been used extensively to induce mutations in the genomes because of its effectiveness in inducing a high frequency of point mutations. It has therefore become a well established mode for producing mutagenic populations. It generates G to A and C to T transitions. Optimizing the mutagenic doses is imperative in induced mutagenesis experiments and is even more critical when the attainment of high mutation frequency, as is the case for TILLING, is required. Optimal mutation does must therefore be empirically determined. If the mutation frequency is too low, too many plants will be required to find out the mutation in target gene and if it is too high, viability and or sterility might pose problems to the development of a suitable mutagenic population.

Seeds were treated with different EMS concentrations in order to determine the optimum concentration of EMS (0.5%, 1.0%, 1.5%, 2.0% and 2.5%) by 6 hours and 10 hours duration of the treatments for achieving high mutation frequency suitable for generating mutagenic populations that would be amenable to TILLING. The kill curve due to different treatment is shown in Fig. 2. In present study seedling treated with different EMS concentration of 6 and 10 hours of duration of treatment did not survive. By reading off the EMS concentration corresponding to 50% reduction in 1seedling height of the control, Concentration of 1.5% was determined to the optimal concentration – by 6 hours duration combination for achieving optimal induced mutagenesis. In practice, a range around this value is normally used for bulk treatments.

A total of 5000 seeds were treated with different concentrations ranging from 1-2% with treatment duration times of 6-10 hours. They were sown in flat beds and seeds from over 1000 plants from the ensuing first mutagenic generation (M_1) harvested individually. Pictures of some of the observed phenotypic mutations in the M_1 population, e.g. chlorophyll mutants, earlier flowering and grain morphology are shown in Photo 1 and 2.

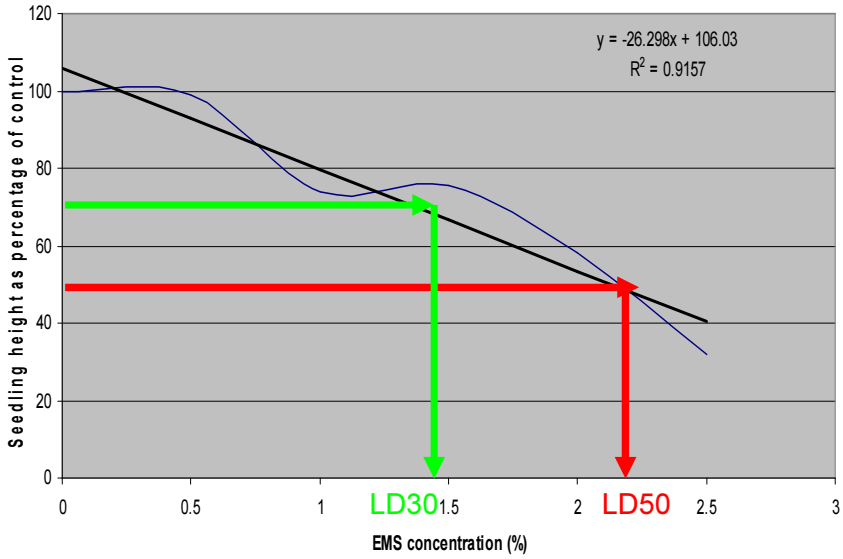


Figure 2. Percentage plant height reduction of rice seedlings from seeds treated with EMS (compared with seedlings from non-treated seeds) plotted against EMS concentration.

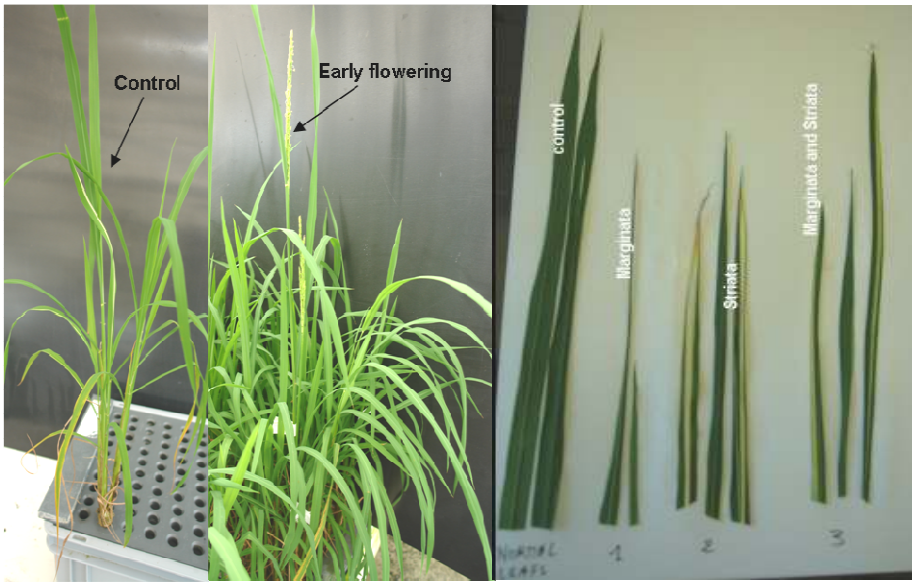


Photo 1. Chlorophyll and early flowering putative mutants from mutagenic populations of Iranian rice variety, Fajer. Note the marginata and striata types of chlorophyll mutations.

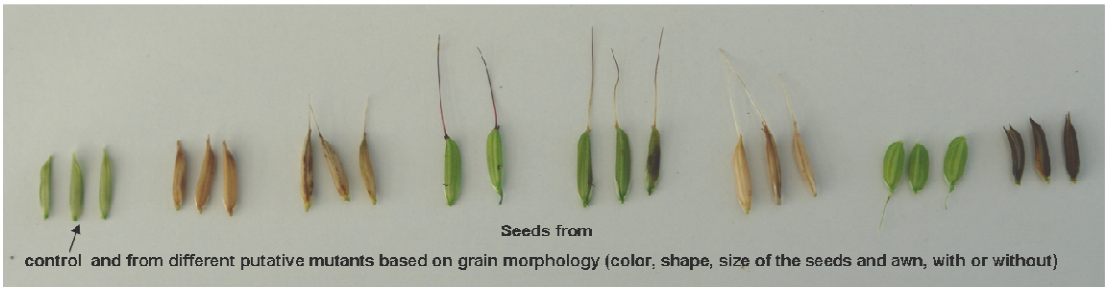


Photo 2. Identified putative grain mutations from the mutagenic populations of Iranian rice variety, Fajer.

2.3. Towards the establishment of TILLING in Cassava

Cassava genetic materials

Seventeen elite cassava accessions from IITA, Ibadan, Nigeria were selected for use in validating methodologies for the high throughput reverse genetic method, EcoTILLING, for detecting spontaneous point mutations, SNPs. These were elite well-adapted cassava clones suited for broad agroecologies of sub-Saharan Africa and which form the bases for the genetic improvement of this crop in this region. They were provided by the Cassava Breeding group at IITA.

Genomic regions surveyed

The genes used for theses assays were those that have been implicated in critical steps of starch biosynthesis in cassava, the granule-bound starch synthase (GBSSI) and starch branching enzyme. The primers designed from the 2 genes are shown in Table 1. The genomic regions for primer design were identified using the web-based platform, CODDLE platform (http://www.proweb.org/input). CODDLE, the acronym from 'Choosing codons to Optimize Discovery of Deleterious Lesions' is an interactive platform that permits the prediction of regions of genes with probable mutations of the G/C to A/T transitions types and which consequently would lead to identifiable phenotypic changes. The web-based primer design software, Primer3 (http://frodo.wi.mit.edu/) was used for picking the sequences of the PCR primer pairs. These primer pairs were synthesized by VBC-GENOMICS, Vienna, Austria (www.vbc-genomic.com).

Table 2. Sequences for the primer pairs for amplifying target regions of genes implicated in cassava starch biosynthesis used for EcoTILLING assays for African cassava accessions.

Gene		*Size(bp)*	*Sequence*
Granule – bound starch synthase	X74160	2168	F5'ACCACAACCACCAGCGGAACCTATTTT 3' R5' TGGAACCAGCATAAAGTCAGCACCAG 3'
Starch branching enzyme	X77012	2843	F: 5' GCGCCTGATTTTGGGTCTTTATCACCT 3' R 5' ATGGCCATTGCTAGGCGATAGTCAAAA3'

Identification of naturally occurring allelic variants – EcoTILLING

The primer pairs from regions of the cassava Granule Bound Starch Synthase and Starch Branching Enzyme genes were used in PCRs to amplify the target genomic regions of the cassava genotypes to validate the fidelity of these two primers for use in TILLING. The PCR products were visualized on ethidium bromide stained agarose gels after electrophoresis (Figure 1). Both primer pairs amplified the expected genomic regions (based on DNA ladder sizing) of almost all the accessions. They will subsequently therefore be used in EcoTILLING analyses of the cassava genome.

Figure 1. Image of ethidium bromide stained agarose gel visualised under UV light after electrophoresis. The DNA bands are from PCR amplification of cassava genotypes using two primers designed from regions of 2 cassava genes, Starch Branching Enzyme and Granule Bound Starch SynthaseI. (Lane1:ladder, Lane 2:TME419 (8,), Lane 3: TME 419 (1), Lane 4: 01-1277, Lane 5: 94-0330 (4), Lane 6: 94-0330 (5), Lane 7: 98-0002 (2), Lane 8: 98-0505 (1), Lane 9:98-0581, Lane 10: 98-2101, Lane 11: I30572, Lane 12: I4(2)1425, Lane 13: I82—0058 (6), Lane 14: I82—0058 (62), Lane 15: I95-0289 (9), Lane 16: I96-1632, Lane 17: TME1, Lane18: TME2 (32), Lane19: TME2 (3), Lane 20: 01-1317.

Generation of cassava mutagenic population for use in high throughput detection of induced point mutations

A critical requirement for the generation of mutagenic populations is always the use of optimal mutagen dosage. In the case of physical agents, this is merely a function of the length of exposure and the dose rate. Essential dose parameters of a chemomutagen are the concentration, duration of the treatments and temperature during treatments. Efficient treatment procedures must also include the chemical's ability to reach the genetic target area. Additionally, the concentration required to obtain high mutagenic efficiency depends on physico-chemical properties of the mutagen as well as on the anatomical structures, the physiological state, metabolic condition and genetic composition of tissues of given biological systems. Experiments were therefore carried out to determine the optimal concentration and duration of EMS treatment to achieve high mutation frequency in the experimental cassava genotypes.

Four different treatments involving the use of EMS of 0.25, 0.5, 0.75 and 1.0 % concentrations with treatment durations of 3 and 6 hours were used, giving a total of 8 treatment combinations in addition to the control, the carrier 2% DMSO. The aqueous EMS solution (2% DMSO and EMS) was prepared under sterile conditions just prior to the onset of the treatments to minimize the hydrolysis of the mutagen. Seventy nodal segments per concentration were used and immediately after treatments these segments were washed with sterile water a minimum of three times.

There were significant differences in the response to the EMS treatments. There was a sharp decrease in survival rates with increased EMS concentration in both treatment durations (Figure 2). EMS concentration of the range 0.5% to 0.75% and duration of 6 hours led to significant reduction of up to 10% in survival rates. Very severe treatment parameters are known to induce multiple mutations and might also result in drastic change of characters in mutant lines derived there from. These extremely high treatment parameters could also result in lowered frequency of mutation that would be of practical use particularly for TILLING assays where the objective is to identify SNPs. Low chemomutagen concentrations improve mutagenic efficiency and the increased mutagenic efficiency result in higher proportions of desired types of mutation. In this study, LD_{50} for treatment of 6-hour and 3-hour durations were in a range of 0.4 to 0.8% EMS concentrations. Based on these results, and

is usually the practice, the nodal segments will be exposed to EMS within a range of 0.75 to 1.0% with a duration of 3 hours for bulk treatment in order to generate appropriate populations for TILLING assays. Two cassava accessions, IITA 419 and IITA 98/2101 will be used for this study.

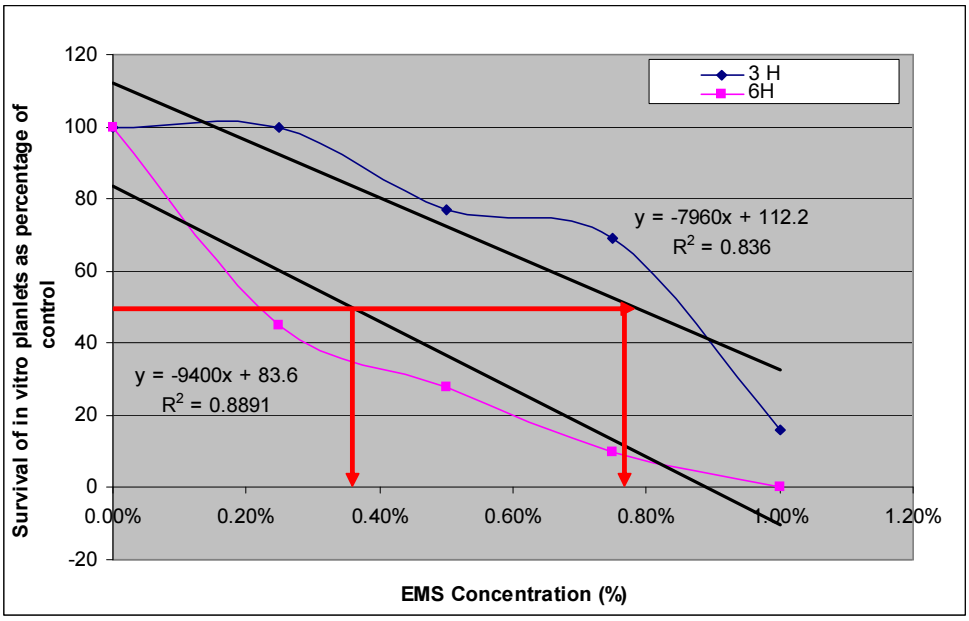


Figure 2. Percentage survival of *in vitro* cassava plantlets from nodal segments treated with EMS (compared with in vitro plantlets from non-treated nodal segments) plotted against EMS concentration.

2.4. Methods Development – *In vitro* techniques in rice improvement

An induced semi-dwarf rice mutant from a tall salt tolerance indica landrace

Abstract

Doubled haploid production has become an important tool in cereal breeding and has helped in accelerating the development of improved cultivars. .Genetic variation for salt tolerance is rare in contemporary semi-dwarf (*sd-1*) rice germplasm (cultivars and breeding lines). Some rice landraces exhibit greater tolerance to salt, but are agronomically unacceptable because of their tall stature. One such landrace, Pokalli was subjected to anther culture in an attempt to induce gametoclonal variation for agronomically important traits such as plant height. Over 100 green plants were regenerated from 2,000 cultured anthers. Among these, two doubled haploid lines (DHs) exhibited semi-dwarf stature. Mutations (induced during the culture procedure) in the semi-dwarfing gene *sd-1* were confirmed by PCR using locus specific primers. The semi-dwarf DH lines were multiplied and checked for response to salinity in hydroponics (10 dSm^{-1}) and field tests at IRRI, Philippines. The work demonstrates the effectiveness of combining doubled haploid protocols with mutation detection

Key words: rice, semi-dwarf mutant, salt tolerance

Introduction

Rice is pre-eminent as a world crop species providing food for about 3,000 million people (FAO:AGCP:CropGroup:Field-Food Crops:Rice). Production however is threatened by environmental factors, particularly salinity and drought. Salinity is a significant limiting factor to agriculture productivity, impacting on about 900 x 10^6 ha of land (Flowers 2004). The existing problem is becoming more acute as a result of poor quality irrigation water. It is estimated that one third of the potential irrigated rice production area is damaged by salt (Subhashini and Reddy, 1989; Szabolcs, 1992; Ghassemi *et al*. 1995; Flower *et al*. 1997). In the past relatively unproductive lands were not required for agriculture, but recent years have witnessed unprecedented rises in the human population which have heightened food security concerns. It is estimated that the global population will increase from about 6 billion in 2001 to more than 9 billion in 2050 (http://www.unfpa.org/swp/2001, Wollenweber *et al*. 2005), but will not be matched by food production owing in part to diminishing areas of good agricultural land. It is therefore likely that more marginal land will be brought into agriculture.

Variation for salt tolerance is available in tall landrace rice material e.g. Pokkali and Nona-bokra, (Akbar, 1986; Khan *et al.* 1987, Xie et al 2000), which are traditional pure line selections very well adapted for local stress condition. However they have not been bred for improved grain yield or short stature. Developing improved varieties that can withstand salt affected soil remains an open and major challenge to the breeders. Breeding for salinity tolerance in rice is difficult. Considerable conventional breeding effort to increase salt tolerance has been made (Moeljopawiro et al, 1981, Gregorio et al., 1993), but the progress in developing tolerant rice has been slow because of the complex nature of the physiological mechanisms involved (Flowers, 2004). The genetics of salt tolerance is also complex showing heterosis, dominance and additive effects (Shannon, 1985) and is governed by two or more quantitative genes (Akbar and Yabuno, 1977) that significantly interact with the environment. As a consequence salt tolerance exhibits low heritability with values as low as 19% (Gregorio and Senadhira, 1993, Lee, et.al., 1995). A project was therefore set up to induce a mutation for semi-dwarf stature in a salt tolerant landrace that would provide a more acceptable form for rice breeders. The project took advantage of the fact that semi-dwarfism is a relatively

common phenotype arising from induced mutation and would, therefore, be relatively easy to obtain (Ahloowalia et al., 2004, Mandal et al., 1999). In this work we attempted to induce a semi-dwarf mutation through somaclonal variation using anther culture. An additional advantage here is that the doubled haploid (DH) plants regenerated are completely homozygous, i.e. any mutation would be fixed immediately. Somaclonal variation is observed in many plants regenerated through tissue culture, especially those with a prolonged callus phase. In general viable and fully fertile variants do not appear to involve gross changes in their genetic make-up, rather somaclones are usually characterised by one or a few altered traits compared to the donor plant phenotype (Mandal et al 1999, Bouharmont et al., 1999). Here we report the development of semi-dwarf mutants derived from the salt tolerant landrace Pokkali via anther culture. Since the callus from which plants were regenerated originated from uni-nucleate gametic cells, microspores, the variants regenerated are referred to as gametoclonal variants.

Materials and Methods

Pokkali

Pokkali is a tall Indian landrace known for its tolerance to salt. Seed was obtained from the Plant Breeding, Genetics and Biochemistry Division of IRRI, Los Baños, Philippines. Plants were raised in soil-filled pots in a lit glasshouse at the IAEA laboratories, Seibersdorf, Austria, with a photoperiod of 8-11 hours (min-max), an irradiance of 220-860 $\mu mol^{-1}s^{-1}$ during the months December–April (1996/1997), day/night temperature of 33ºC/18ºC and a relative humidity of 80%. The panicles were harvested at the booting stages with the distance between the sub tending leaf and flag leaf being 7-10 of 8-12cm. Light microscopy investigations showed that this stage contained microspores at the mid to late uni-nucleate stage. Harvested panicles were subjected to a cold pre-treatment of 8°C for 8-10 days, after which they were surface sterilized by immersion in 70% ethanol for less than a minute, then 10% Clorox (commercial bleach, with a sodium hyperchlorite, NaOCl, concentration of 5.2% w/v) for 10 minutes and washed three times with sterile distilled water before removing anthers for culturing.

Anther culture

Anthers were excised and cultured on liquid N6 medium (Chu, 1975) supplemented with 2 mgl^{-1} 2, 4-D and 5% maltose for callus induction, cultures were kept in the dark at 25ºC. After 6-7 weeks in culture, calli with a size of 2mm were transferred to a regeneration medium: modified MS (Murashige and Skoog, 1972) with 1 mg/l BAP, 0.5 mg/l NAA, 200 mgl^{-1} myo-inositol and solidified with 0.45% agarose (Sigma, Type I-A: A-0169). Regenerating cultures were incubated in the light at 25ºC under a 12h light photoperiod supplied by cool white fluorescent lamps (66 umol m^{-2} s^{-1}). Regenerated plantlets were labelled with respect to callus origin. Generally one callus produced one plantlet, but occasionally multiple regenerants were recorded. Once regenerated and large enough with both roots and shoots, plants were transferred to soil-filled pots in glasshouse conditions.

Glasshouse evaluation

Regenerated plants were grown in pots to maturity and assigned a line number. Out of 125 plantlets, 59 plantlets were spontaneous DHs. There was no attempt to use colchicine to induce chromosome doubling in the other lines as three semi-dwarf types were found in the 59 sub-set. The three semi-dwarf gametoclonal variants showing plant height reduction were identified in the first generation (DH_1). Semi-dwarf DH lines were selfed and the progeny

re-evaluated for height reduction and other agronomic traits in subsequent generations (Table 1). Seed were also sent to IRRI for field testing to confirm glasshouse phenotypic assessments.

Rapid, seedling screen for salt tolerance

Salinity tolerance of the gametoclones was assessed in the glasshouse using a seedling hydroponics test developed by IRRI (Gregorio et al., 1997) and further modified for large scale screening of mutant populations by the Plant Breeding Unit, FAO/IAEA Laboratory in Seibersdorf, Austria (Afza et al., 1999). Seedlings of the DH_2, DH_3 and DH_4 generations of the three gametoclonal lines (GVP1, GVP2 and GVP3) were tested. Seed were germinated on a seedling mesh plate suspended over hydroponics. Salinity was introduced into the solution three days after germination by adding 640 mg^{-1} NaCl ($10dSm^{-1}$) to the nutrient solution as described by Yoshida (1976). The pH was adjusted daily to 5.5. Salinity injury rating was based on visual symptoms (1- tolerant and 9-sensititive) at seedling stage, according to the modified Standard Evaluation System of IRRI Gregorio et al (1997): five evaluation grades were used: 1. Normal growth, no leaf damage; 3. Nearly normal growth, but lowest leaf is desiccated, 5. Growth is severely retarded, and the two basal leaves are desiccated; 7. Complete cessation of growth, only the young, apical leaf survives; 9. Glasshouse conditions consisted of day/night temperature of 30ºC/20ºC with 70% relative humidity. In addition to the semi-dwarf GVP lines, the salt tolerant tall landraces, Pokkali and Nona-Bokra, and a salt susceptible semi-dwarf cultivar (IR-29) were included as checks for salt tolerance and salt susceptibility, respectively, as well as plant height. Results are given in Table 2.

Genotyping for sd-1 *mutations*

The semi-dwarf GVP DH lines exhibited a similar stature to standard *sd-1* cultivars. Since *sd-1* has been cloned (Monna et al., 2002; Spielmeyer et al., 2002) and sequenced (Ellis and Spielmeyer, 2002) it was possible to check for mutations in the GVP lines. DNA was extracted (Doyle and Dickson, 1987) from leaves of GVP3, and GVP35 and portions of the *sd-1* sequence amplified by PCR using primers targeted at specific regions in the *sd-1* locus. PCR reactions were carried out in 15µl containing 50ng DNA, 1.5µl PCR buffer, 0.75 µl 1MmdNTP, 2 µl of each of primers forward (Sd1-F: 5'- CAC GCA CGG, GTT CTT CCA GGT G-3) reverse (Sd1-R: 5'- AGG AGA ATA GGA GAT GGT TTA CC-3) and 0.3 µl of taq polymerase was prepared to amplified the *sd-1* gene from selected population. The PCR profile was as follows: 94°C for 5 minutes for denaturation, 94°C for 30 seconds, 55°C for 30 seconds then 72°C for fifty seconds for 35 cycles and extension for 72°C for 7 minutes. After amplification, the amplified product was resolved in agarose gel by electrophoresis, the gel was stained in Ethidium bromide and viewed using UV lights. DNA profiles were compared to a standard *sd-1* cultivar (IR8) and a none *sd-1*, tall type (Pokkali).

Allelism testing

To study the mode of inheritance and allelism, the GVP3 was selected and crossed reciprocally onto standard *sd-1* cultivars, IR8, IR64 and parental lines. The F_1 with the *sd-1* parents and subsequent selfed generations were all short, indicating allelism with *sd-1.* The F_1 with the tall parent (Pokkali) were all tall and segregation for height was observed in subsequent generations.

Results and Discussion

Of the 125 plants regenerated from anther culture three DHs showed a semi-dwarf stature: GVP1, GVP2 and GVP3. Since the semi-dwarf phenotype was stable over several DH generations the phenotype can be attributed to a stable genetic change. The three gametoclanal variants were

identified originated in the first DH generation and were obvious in exhibiting plant height reduction. The three plantlets originated from two calli clusters, with GVP1 and GVP2 arising from the same callus, they may therefore be clones of each other. The DH_2 generation from self pollinated panicles was evaluated for tolerance to salt and other agronomic traits. In addition to their semi-dwarf stature the three GVP lines retained the salt tolerance characteristics of their tall landrace parental line, Pokkali (Table 1).

Plant dwarfism is one of the most important traits used in plant breeding, particularly in rice, the semi -dwarfing gene in rice (*sd-1*) is one of the most important genes deployed in rice. The properties of *sd-1* are consistent with semi-dwarf phenotype that results from of a partial block in gibberellin biosynthesis (Spielmeyer et. al., 2002, Sesaki et al., 2002 and Weitzen, 2002, Monna et al., 2002). The *sd-1* gene was first identified in the Chinese variety Dee-geo-woo-gen (DGWG) and crossed in the early 1960s with Peta, a tall variety to develop the semi-dwarf IR8 which produced record yields. Since the 1960 *sd-1* is the predominant semi-dwarfing gene in rice cultivation. (Ellis. and Speilmeyer. 2002, Spielmeyer et. al., 2002). The *sd-1* gene in *indica* rice from Dee-geo-woo-gen (DGWG) was found to contain a 383 bp deletion in the genomic sequence of Os200x2, a gene responsible for the production of a growth hormone a giberellin. In the present study, the GVP lines were tested and confirmed as being *sd-1* mutants by genotyping at the *sd-1* locus and by allelism testing (Figure 1, Table 3) A full length gene product (721 bp) was obtained from Pokkali, in contrast IR8 and GVP lines produced a smaller band revealing a deletion of 383bp within the *sd-1* sequence. The semi dwarf *sd-1* gene is the most common height reducing gene in rice and the presence of *sd-1* is normally determined by phenotypic assessment of final plant height and is therefore influenced by environmental factors throughout the life cycle. Furthermore, *sd-1* is a recessive and heterozygous plants are masked by their tall stature, and can only be evaluated retrospectively by evaluating their progeny. Different studies have been done on the pleiotropic effect, i.e. interaction between environment and dwarfism.

Crosses were also made in this project with advanced semi-dwarf lines, e.g. IR58 and IR 64, which served to test allelism and to make the first steps in an introgression programme to improve the salt tolerance of semi-dwarf rice cultivars. Segregation of seed shape were observed in F_2 generation when crossed with semi-dwarf varieties, but there was no segregation for height, i.e. all of them were dwarf. The F_1 with the tall parent (Pokkali) were all tall and segregation for height was observed in subsequent generations.

The project demonstrates the relative ease in which semi-dwarf mutations can be induced in tall rice lines via gametoclonal variation. The approach has breeding potential and may be used to target other traits found in landrace rice for introgression into, and assessment in, semi-dwarf cultivars. The use of a DH system has advantages in that mutations are fixed immediately in the homozygous (DH) lines generated. Mutation rates may also be increased by the application of mutagenic treatments at various stages in the anther culture process. For example, gamma ray irradiation of anthers has been used to develop salt tolerance DH mutants in wheat (Afza et al., 2006). The development of molecular markers for *sd-1* would also provide breeders with an option to test for the presence of short or tall alleles rapidly, in heterozygous material and at any growth stages. The semi-dwarf lines produced here are to be evaluated for salt tolerance in field conditions in other parts of the world, e.g. Asia, Africa and Latin America.

Photo 1. Plantlets regerenated from anther culture

Photo 2. Height comparison between rice gameto-clones and landrace Pokkali (centre)

Table 1. Salt Screening at seedling stage of gametoclons at DH4 generation

Variety/ lines	No. of seeds tested	Salinization score after	
		8 days	16 days
GVP3	665	3-5	5-7
GVP35	549	3-5	5-7
Pokkali Tolerant control	44	3	7
IR29 Susceptible control	56	7-9	9

Table 2. Agronomic traits of gametoclonal rice mutants and parental landrace,

Varieties	Conditions	HD	Plant height	No. of Tiller	No. of Pro-ductive tiller	No. of filled grain
GVP 3	Non saline	73	129	10	10	668
	Saline	83	118	10	9	470
GVP35	Non saline	73	117	10	10	655
	Saline	79	122	10	10	916
	Non saline	67	114	19	19	2008
	Saline	72	102	13	13	798
	Non saline	66	115	25	25	2828
	Saline	74	110	14	13	1161
Pokkali	Saline	59	187	15	15	1259
	Non saline	61	168	17	16	1215

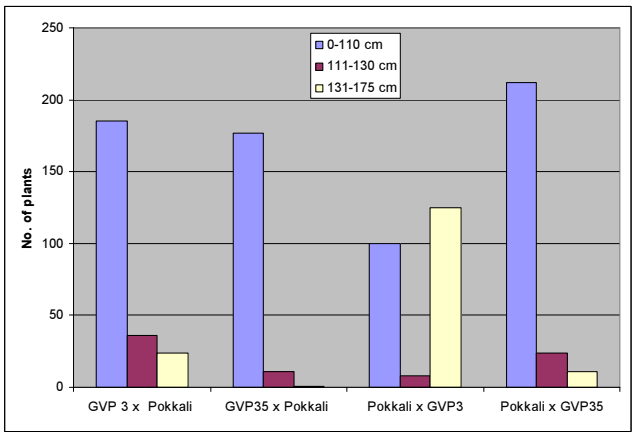


Figure 1. Frequency distribution and segregation of plant height (cm) in F2 generation

Table 3. Frequency distribution and segregation of plant height (cm) in F2 generation

Plant height	GVP3 X Pokkali	GVP35 X Pokkli	Pokkali X GVP 3	Pokkali X GVP35	GVP3	GVP 35	Pokkali
0-110	185	177	100	212-	75	82	
111-130	36	11	8	24			
130-175	24	1	125	-11			146

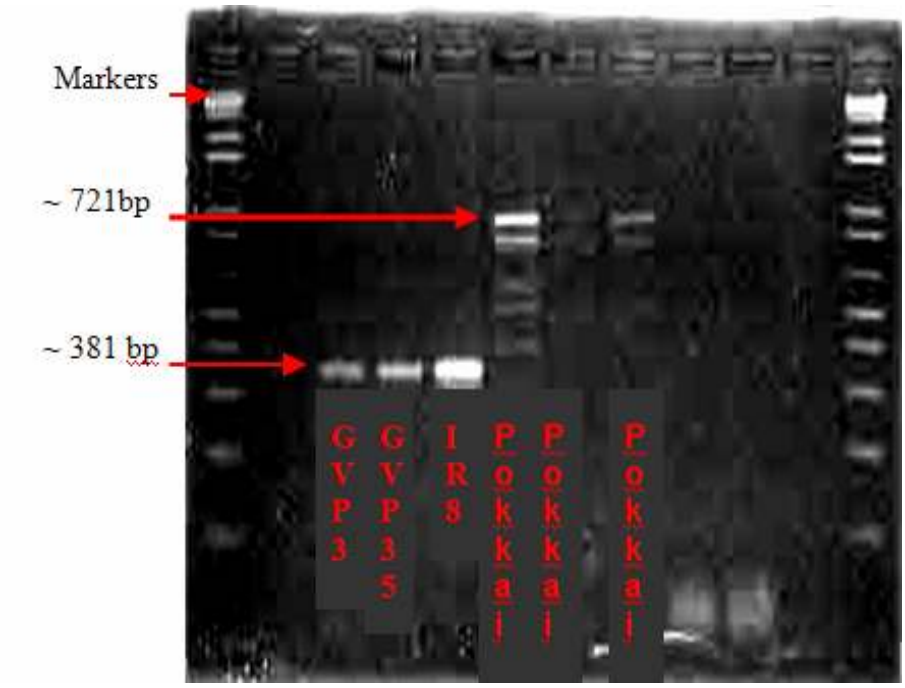


Figure 2. PCR amplified DNA fragments of the *sd-1* locus in rice DH (GVP3, GVP35), semi-dwarf IR8 and tall landrace Pokkali. The profiles for IR8 *(sd-1*) and the DH lines are the same.

2.5. External Collaborations and Partnerships

The development and nurturing of effective synergistic relationships with strategically located and oriented international and national agricultural R&D entities have remained critical for engendering an effective leverage for the Unit's activities. Like in previous years, the Unit's activities in the development and deployment of technologies for the improvement of its adopted 3 'model' commodity crops, bananas, rice and cassava, has been greatly enhanced by the mutually beneficial collaborative activities with centres of the CGIAR and NARS. In the past, such collaborations had led to the development of superior banana variants in Malaysia and Sudan as well as saline tolerant rice genotypes.

We present the partners with active collaborative endeavours during the period under review below. Additionally, we also highlight the status of 2 activities – generation of breeding and genomics cassava resources using African germplasm; and the generation of breeding sunflower materials for the Balkan regions.

Table 1. Research and Development Institutes by crops with ongoing collaborative activities with the PBU, 2006

Commodity Crop	Type of Organization	Name of Institute and contact person
Rice	CGIAR	International Rice Research Institute (IRRI), Manila, Philippines. Dr R.K. Singh Rice Breeder for Salt-affected Areas, Plant Breeding Genetics & Biotechnology Division International Rice Research Institute DAPO Box 7777, Metro Manila, The Philippines Tel: +63-2-580-5600 Ext. 2759/2806 Fax +63-2-5805699 Email : r.k.singh@cgiar.org
		African Rice Authority- WARDA, c/o IITA, Ibadan, Nigeria Dr. Glenn Gregorio E-mail: g.gregorio@cgiar.org
		International Centre for Tropical Agriculture (CIAT), Cali, Colombia Drs. Joe Tohme and Cesar Martinez E-mails: j.tohme@cgiar.org c.martinez@cgiar.org
	NARS	Bangladesh Institute of Nuclear Agriculture -BINA, Bangladesh, P.O. BOX.4, Mymensingh-2200 Tel: 0088-091-52960, 0088-01711938290 Fax: 0088-091-54091 Dr. M.A. Salam E-mail: drmasalam@yahoo.com
		Department of Radiobiology, Centre of Nuclear Techniques, Hochiminh City, Vietnam Vietnam Tel:(84-4)8356568-9202257 Dr. Ph.D. Ass Prof. Le Xuan Than E-mail: thamdalat@hcm.vnn.vn
		National Agricultural Research Coordinating Council (NARCC), Rice Research Station, Rokupr, PMB 736, Freetown, Sierra Leone. Dr. Sydney Donald. Johnson Tel:+23222223282

		Institute of Crops Science, Chinese Academy of Agriculture Sciences, CAAS, China Dr. Prof. Luxiang LIU E-mail: luxiang@263.net.cn
Cassava	**CGIAR**	International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria Dr. Alfred Dixon E-mail: a.dixon@cgiar.org
		International Centre for Tropical Agriculture (CIAT) Cassava Project Km 17, Recta Cali-Palmira AA6713, Cali, Colombia Tel: +57 2 4450000 Fax: +57 2 4450073 Drs Martin Fregene and Hernan Ceballos E-mails: m.fregene@cgiar.org h.ceballos@cgiar.org
	NARS	Crop Research Institute, Accra Office, Root Tuber Crops Improvement Division, P.O.Box 3785 Tel: +223 51 60396 Fax: +223 51 60396 Ms. Elizabeth Okai E-mail: elizabeth_okai@yahoo.co.uk.
		National Plant Breeding Research Centre, Kenya Agricultural Research Institute (KARI), Njoro, Kenya Dr. Miriam Kinyua E-mail: mgkinyua@africaonline.co.ke National Root Crops Research Institute, Umudike, PMB 7006,Umuahia, Abia State, NIGERIA Dr. Emmanuel Okogbenin Tel:+234 805 740 1924 E-mail: e.okogbenin@cgiar.org
Musa	**CGIAR**	International Network for the Improvement of Banana and Plantain (INIBAP) of Bioversity International, Parc Scientifique AgropolisII 34397 Montpellier Cedex 5, France Tel:+33 67 619946 Fax: +33 67 610334 Dr. Nicolas Roux E-mail: n.roux@cgiar.org
	NARS	National Plant Breeding Research Centre, Kenya Agricultural Research Institute (KARI), Njoro, Kenya. P.O.Box 57811, Nairobi. Tel: +254 51 61528 Fax: +254 51 61576 Dr. Miriam Kinyua E-mail: karinjr@africaonline.co.ke
		National Agricultural Research Organization (NARO), Uganda. Institute/National Banana Research Programme Kawanda Agricultural Research Mr. Henry Mwaka, P.O. Box 7065, 256 Kampala Uganda, Fax: 256 41 566381 E-mail: mwakahenry@yahoo.com

2.5.1 Induction of variation through induced mutations for broadening the genetic base of sunflower (*Helianthus annuus* L.) germplasm for crop improvement in Serbia

ABSTRACT

For most of the past century, mutagenesis has remained popular in plant genetic research to introduce novel genetic variation. Induced mutations have been applied for the past 40 years to produce mutant cultivars in sunflower by changing plant characteristics for a significant increase in plant production. The present study focused on the development of breeding materials of sunflower by using of induced mutation in order to improve agronomic traits, resistant to biotic and abiotic stresses. To achieve the goal, seeds of twenty sunflower genotypes of breeding and commercial relevance, were treated with a range of gamma rays and fast neutron irradiation and with ethyle-methanesulphonate of different treatment concentration with different time duration. Radiobiological effects of mutagenesis for M_1 were calculated on the basis of the absorbed dose/EMS in the embryo. The radiation sensitivity studies indicated that all genotype gave wide range radiation damage to gamma rays and fast neutron and in order to obtain same biological effect, higher doses were needed for gamma rays compared to fast neutron. The damage due to chemical mutagen also variety dependant and a damage of growth of 30% due to EMS of different varieties range from 0.1% to 1% concentration. Results also indicated that genotype with higher oil percentage in the seeds are more sensitive in case of gamma irradiation and fast neutron. Mutagenic damage is depends on the biological traits of the variety and every variety has its specific radio-resistance. Based on the radiation damage, bulk irradiation with dose of a growth reduction of 30% was carried out and M_1 plants were grown in field. Different mutations were observed in the field and promising mutants were selected for further testing. Selection of M2 was based on ealry flowereing, short stature, deformations of leaves and heads, appearance of branches, head inclination and sterility.

INTRODUCTION

Sunflower (*Helianthus annuus* L.) originated in North America where it was used and cultivated by Native Americans. Spanish explorers introduced it to Europe in 16th century. At that time it was mostly used as an ornamental and garden flower. Sunflower as an oil crop was mentioned in writing for the first time in 1818 in Russia (*Škorić, 1988*). At the beginning of 20th century Russian breeders enhanced the oil yield from 300g/kg to 550g/kg. By selecting for thin-hulled, oil-rich achenes, Pustavojt (*Pustavojt, 1964*) and other plant breeders developed the modern-day oilseed sunflower which is typified by varieties Peredovic, Armavirskiy 3497, VNIIMK 8931, etc. Seed of the first commercial sunflower varieties contained almost 50% oil. Sunflower growing areas are widely expanding leading to hybrids in commercial production. With the discovery and utilization of cytoplasmatic male sterility (*Leclercq, 1969*) and genes for fertility restoration (*Kinman, 1970*), a new era in sunflower breeding began. The advantages of hybrids are that they exhibit heterosis (hybrid vigour in the F_1 generation), crop uniformity, high genetic potential for seed yield and a simple breeding system for gene introduction, e.g. for diseases resistance. These factors in turn allow for more efficient harvesting and seed storage (*Škorić, 1988*).

Heterosis in sunflower breeding is exploited using single cross, three-way cross and double cross hybrids. Single cross hybrids are used mainy in mass production (*Škorić, 1989*). F_1 sunflower hybrids are derived by crossing cytoplasmatic male sterile inbred line (female line) with male pollinator lines which carry fertility restorer genes.

Sunflower is one of the world's most important oil crops, used for human consumption and industrial processes. It is also used as a confectionary, ornamental plant/flower, and for birdfeed. It currently

occupies over 21 million hectares world-wide. The largest sunflower producers in the world are Russia, the United States, Argentina, China, France, etc. The reason of such outspread is it's fairly modest production requirements (*Škorić, 1988*).

The genus *Helianthus* consists of 49 species, 13 annual and 36 perennial with different ploidy level, diploids (2n= 34), tetraploids (2n=68) and hexaploids (2n=102) (*Schiling, & Heiser., 1981*). The major commercial species are *Helianthus annuus* L. and *Helianthus tuberosus* L. (Jerusalem artichoke). Other wild *Helianthus* species are a rich source of genetic variation which can be tapped via interspecific hybridization, and subsequent backcrossing in introgressing traits. Traits targeted in the wild gene pool include the transfer disease resistance genes in cultivated sunflower (*Georgieva-Todorova, 1976*).

Commercial sunflower seed contains 40-60% oil. It is rich in unsaturated fatty acids, 68-72% linoleic (18:2) and 16-19% oleic (18:1) and poor in saturated, 4-6% stearic (18:0) and 5-7% palmitic (16:0) fatty acids (*Connor & Hall, 1997*). The primary use for the oil is as a salad and cooking oil and the manufacture of margarine. Recently, significant progress has been made in fatty acid modification of sunflower oil (*Fernandez-Martinez et al., 2004*). The high oleic type contains more than 80% oleic acid, and is considered to have high oxidative stability (*Demurin et al., 2004*).

The main objective of sunflower breeding is to develop productive F_1 hybrid cultivars which are stable, high yielding, resistant to biotic and abiotic stresses. Yield is a complex trait, which is the controlled by single and multiple gene effects. The final components of seed yield are: number of plants per hectare (55,000-60,000), number of seeds per plant (>1,500), hectolitre mass (45-50 kg/ ha), thousand seed mass (>80 g), low hull percentage (20-24%) and high seed oil content (>50) (*Škorić, 1989*), and consequently these traits are on important breeding objectives.

Induced mutations have been applied for the past 40 years to produce mutant cultivars in sunflower by changing plant characteristics for a significant increase in plant production **(**Jain, 2005**)**. Mutation treatments have included high-oleics, semi-dwarfs and dwarfs, male-sterile plants and other interesting forms and usually involved seed treatment. (*Soldatov, 1976; Voskoboyin & Soldatov, 1974, Jan & Rutger, 1988*) The treated seed is known as the M_0 generation. Mutation is a random process, and it is difficult to predict the frequency of desired phenotypes, these become apparent in subsequent generations (phenotypic screening normally takes place in the M_3 generation).

Induced mutations have been used successfully for increasing genetic variability of cultivated sunflower (*Miller & Fick, 1997*). Both irradiation and chemical mutagens are commonly used. Thus far, a lot of sunflower mutants with different agronomic traits (earliness, dwarf, thin husk, changed fatty acide composition) have been developed. *Soldatov* in *1976* produced a mutant which has the highest practical importance for sunflower breeding. By treating the seed of VNIIMK 8931 variety with 0.5% DMS (dimethyl-sulphate) solution, he obtained M_3 material possessing a high content of oleic acid in oil. This material was used to make a high-oleic variety Pervenetz. The high oleic content of this variety has proved to be very stable under different temperatures and the trait can be easily transferred in other genotypes by normal breeding processes of crossing. Desirable agronomic traits that may be improved by mutation include: yield, early maturity, short plant height, oil and protein content, fatty acid composition, low hull percentage, resistance to important diseases and herbicides.

The main objectives of this research were to increase genetic variability of sunflower inbred lines and to assess different mutagenic treatments as there is little information on this. The first step was to estimate optimal treatment conditions (doses). Germination of the M_1 seed provides an indication of the sensitivity of the material to the mutagenic treatment applied. Mutant phenotypes are normally assessed in subsequent M_2 and M_3 generations, but the frequency of these is determined by the mutagenic treatment and the survival rate of the M_1 seed.

MATERIALS AND METHODS

Materials

Table 1. List and characteristics of 20 sunflower varieties used for radio sensitivity tests.

Inbred lines	Characteristics
NS-LIN-1, NS-LIN-2, NS-LIN-3, NS-LIN-4, NS-LIN-5	Standard female inbred lines (B analogues), different vegetation period, plant height, oil and protein content and disease resistance. Seed is about 1 cm long, mostly black and thick.
NS-LIN-6, NS-LIN-7, NS-LIN-8, NS-LIN-9, NS-LIN-10	Restorer inbred lines, branched, different vegetation period, plant height, oil and protein content and disease resistance. Seed is long (1.2 cm), clearly black, elongate and medium thick.
NS-LIN-11, NS-LIN-12, NS-LIN-13, NS-LIN-14, NS-LIN-15	High oleic inbred lines, different content of oleic and other fatty acids and tocopherol quality. Seed is 0.8-1 cm long, very thin, brown.
NS-DE-1, NS-DE-2, NS-DE-3, NS-DE-4, NS-DE-5	Ornamental sunflower, different color of ray flowers. It has lot of branches. Seed is 0.8 cm long, medium thickness and different color.

Mutagens used for mutation induction

Gamma irradiation - Fifty seed of each genotype were irradiated with 100, 200, 300, 400 and 500 Gy of ^{60}Co gamma rays at the Joint FAO/IAEA Laboratories in Seibersdorf, Austria. Gamma rays are electromagnetic waves of very short wave lengths (less than 0.01 nm) and are obtained by the disintegration of Cobalt (^{60}Co) and Caesium (^{137}Cs) radio-isotopes. Prior to mutagenic treatment, the seed of the selected lines were kept in a desiccator over a 60 % glycerol/water mixture for 7 days at room temperature for seed moisture equilibration.

Fast Neutron irradiation - Fast neutrons are uncharged particles, which are generated in nuclear reactors or accelerators and Nuclear reactors usually provide a heterogeneous field of radiations e.g. fast and thermal neutrons, gamma radiation. Fifty seeds were treated with five different doses: 10, 20, 30, 40 and 50 Gy at the Atomic Energy Research Institute, Budapest, Hungary. After treatment, 25 seeds of each genotype were sown and germinated to assess Radiosensitivity.. and special containers have been constructed for seed irradiation in different reactor types.

Chemical mutagenesis - The dose can be defined as a particular mutagen concentration for a definite period of time at a particular temperature. For chemical treatment, seeds were pre-soaked in distilled water for 24 hours. Presoaking of seeds enhances total uptake, the rate of uptake and the distribution of the mutagen in the target tissue (*Kodym & Afza, 2003*). The duration of the treatment should be long enough to permit hydration and infusion of the mutagen to the target tissue (*Kodym & Afza, 2003*) The first experiment in chemical mutagenesis was therefore designed to assess the effects of treatment duration. Since there was no previous information on sunflower, we selected 2 genotypes NS-LIN-2 (large seed) and NS-DE-4 (small seed) and treated them with 3 different doses of EMS (0.5, 1 and 1.5%) for 2, 3, 3.5, 4 and 5 hours, these treatments were based on information from other species with similar seed sizes (*Kodym & Afza, 2003*). Once treatment duration was established, we used 5 different concentrations (0.5, 1, 1.5, 2 and 2.5% EMS solution) on all 20 genotypes for 3.5

hours; again treatment concentration was based on studies of other species (*Kodym & Afza, 2003*). After EMS treatment, seeds were washed and sown. The control seeds were treated alike without exposing them to the mutagen.

Germination and seedling evaluations

The treated seeds were sown in the boxes using flat method (*Gaul, 1963*) in greenhouse with environmental control (temp 22-35ºC, lighting of 12h) including control. The parameters used to assess the dose response were the percentage of germination and the hypocotyls length. Germination percentage was estimated by number of germinated seed /total number of sown seed per treatment (results only shown for gamma irradiation). The measurements of hypocotyls length were taken when cotyledons showed up ground and splited up (twelve days after sowing).

Data analysis

Data from these parameters were plotted against the corresponding doses of Gamma ray exposure. Based on the reduction of growth and the other parameters, the dose leading to an average of 30 percentage damage was determined as the optimal dose. This is usually designated as GR30 with LD being the abbreviation for lethal dose. The dose was determined using trend line (Fig.1). GR30 is the dose required to reduce hypocotyl length in compare with untreated control seedlings by 30.

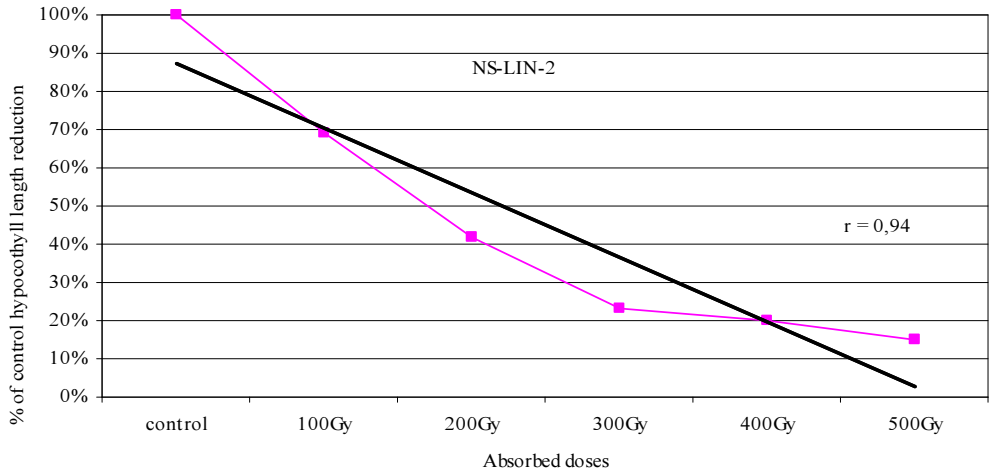


Figure 1. Trend line among irradiated dose and percentage of reduction of hypocotyl length or survival.

Determination of oil contents in seeds:

Oil content was determined using nuclear magnet resonance (NMR) analysis. Nuclear magnet resonance (NMR) is a non-destructive method of determining oil content. Sample plug is put in the magnetic field, Based on the changes in electricity , two parameters are measured; sample mass and oil weight in the sample. Oil content is calculated from these two parameters

RESULTS AND DISCUSSIONS:

The germination percentages of average of 30 seeds of all 20 genotypes were shown in Table 2. From the table it shows that there is no effect of gamma irradiation on the germination of seeds. The germination percentage for control and the treated is on the same range. Micke (1997) reported that in the initial stages of germination, the preformed orgasm that merely respond to water up-take, a process relatively insensitive to mutagenesis. The results confirms that germination rate was not right parameter to measure the radiation damage

Table 2. Mean germination percentage for 20 genotypes.

Treatment	Germination (%)
Control (M_0)	84
100Gy	82
200Gy	80
300Gy	84
400Gy	81
500Gy	83

The length of the radicles, however, showed marked differences among control and irradiation treatments (Figure1). The results showed that radicles length decrease with the increase doses.

Photo. 1. Variation in radicle length of sunflower inbred line NS-LIN-2. (from left to right control, 100Gy, 200Gy, 300Gy, 400Gy, 500Gy).

Hypocotyl lengths in all the three mutagens were decreased with increased dose. For gamma irradiation the GR30 for 20 sunflower inbred lines seeds ranged from 45.5Gy to 269Gy (Table 2). The most sensitive to gamma irradiation following sunflower inbred lines are NS-LIN-4, NS-LIN-5, NS-LIN-6 and NS-DE-1and more radio resistance are NS-LIN-8, NS-LIN-9, NS-LIN-10 and NS-LIN-13.

Table 3. Reduction of hypocotyls length (%) and 30% growth reduction of seedlings height (GR_{30}) in sunflower inbred lines caused by gamma irradiation

Hypocotyl lenth	NS-LIN-1	NS-LIN-2	NS-LIN-3	NS-LIN-4	NS-LIN-5	NS-LIN-6	NS-LIN-7	NS-LIN-8	NS-LIN-9	NS-LIN-10
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
100Gy	77%	69%	74%	33%	45%	48%	64%	98%	74%	80%
200Gy	54%	42%	41%	20%	21%	22%	30%	73%	55%	60%
300Gy	21%	23%	23%	7%	17%	25%	17%	49%	45%	47%
400Gy	9%	20%	17%	7%	17%	5%	6%	32%	38%	34%
500Gy	5%	15%	11%	4%	14%	1%	4%	26%	26%	20%
GR_{30}	153.5Gy	151Gy	158Gy	45.5Gy	96Gy	100.5Gy	132Gy	269Gy	196Gy	216.5Gy
Hypocotyl lenth	**NS-LIN-11**	**NS-LIN-12**	**NS-LIN-13**	**NS-LIN-14**	**NS-LIN-15**	**NS-DE-1**	**NS-DE-2**	**NS-DE-3**	**NS-DE-4**	**NS-DE-5**
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
100Gy	71%	70%	99%	64%	67%	48%	82%	79%	75%	81%
200Gy	37%	42%	56%	33%	52%	30%	42%	70%	62%	59%
300Gy	16%	10%	12%	20%	13%	19%	9%	23%	39%	28%
400Gy	12%	3%	11%	17%	8%	5%	1%	4%	13%	8%
500Gy	11%	2%	10%	12%	5%	4%	0%	1%	4%	4%
GR_{30}	142Gy	144.5Gy	195Gy	131Gy	146Gy	99Gy	160Gy	191.5Gy	192.5Gy	188Gy

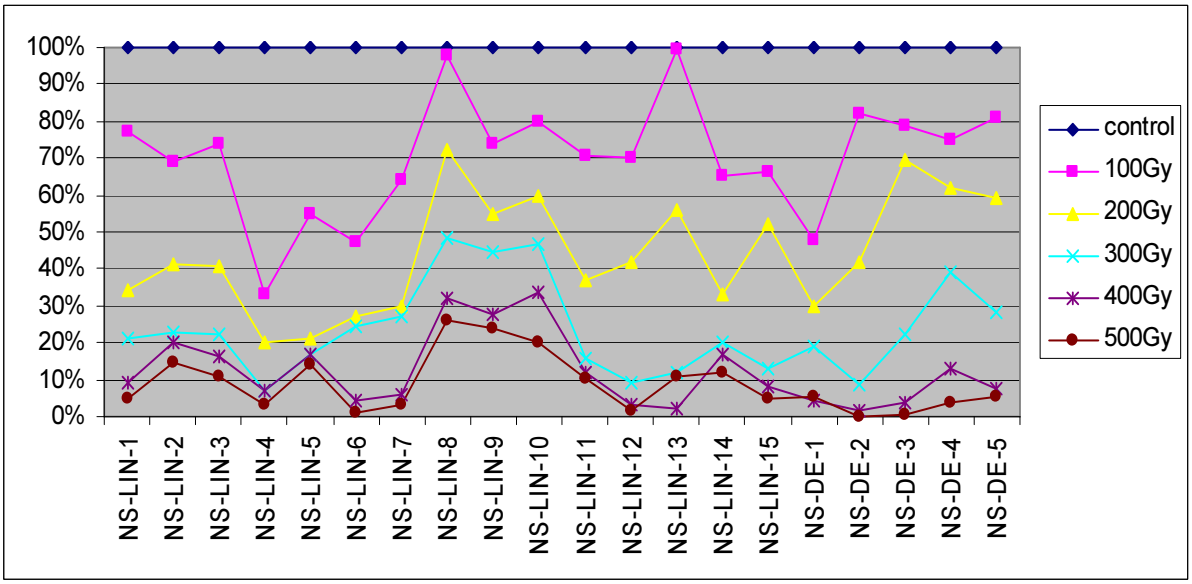


Figure 2. Reduction of hypocotyls length in sunflower inbred lines caused by gamma irradiation

For fast neutron, the GR30 for 20 sunflower inbred line seeds ranged from 2.3Gy (NS-LIN-4) to14.9Gy (NS-LIN-9). Again, it is same like the gamma irradiation, the most sensitive were NS-LIN-4, NS-LIN-5 and NS-LIN-2 and the most tolerant to fast neutron irradiation were NS-LIN-8, NS-LIN-9, NS-LIN-10 and NS-DE-4 (Table 3).

Table 4. Reduction of hypocotyls length (%) and 30% growth reduction of seedlings height (GR_{30}) in sunflower inbred lines caused by fast neutron irradiation

hypocotyl lenth	NS-LIN-1	NS-LIN-2	NS-LIN-3	NS-LIN-4	NS-LIN-5	NS-LIN-6	NS-LIN-7	NS-LIN-8	NS-LIN-9	NS-LIN-10
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10Gy	50%	35%	49%	26%	29%	43%	43%	71%	70%	69%
20Gy	19%	17%	27%	13%	18%	17%	13%	31%	40%	30%
30Gy	17%	13%	18%	0%	14%	11%	6%	19%	23%	17%
40Gy	6%	8%	15%	0%	12%	8%	4%	13%	14%	13%
50Gy	5%	8%	14%	0%	11%	5%	2%	11%	13%	11%
GR_{30}	8.6Gy	4.4Gy	8.8Gy	2.3Gy	2.5Gy	6.5Gy	6.1Gy	13.8Gy	14.9Gy	13.2Gy
hypocotyl lenth	**NS-LIN-11**	**NS-LIN-12**	**NS-LIN-13**	**NS-LIN-14**	**NS-LIN-15**	**NS-DE-1**	**NS-DE-2**	**NS-DE-3**	**NS-DE-4**	**NS-DE-5**
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
10Gy	49%	46%	40%	44%	59%	49%	48%	60%	66%	66%
20Gy	18%	14%	17%	22%	32%	24%	28%	37%	35%	22%
30Gy	12%	8%	14%	12%	24%	24%	8%	5%	15%	6%
40Gy	9%	6%	11%	10%	24%	10%	4%	12%	10%	5%
50Gy	1%	4%	10%	9%	11%	7%	0%	4%	5%	2%
GR_{30}	7.6Gy	6.7Gy	5.4Gy	7.0Gy	12.2Gy	8.7Gy	9.2Gy	12.6Gy	13.4Gy	11.5Gy

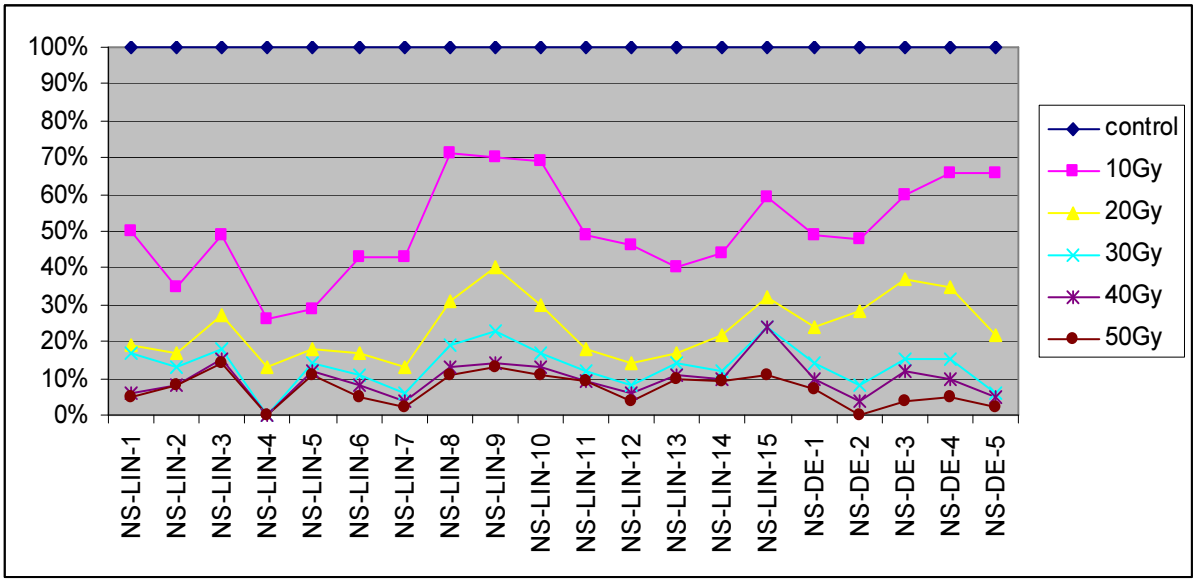


Figure 3. Reduction of hypocotyls length in sunflower inbred lines caused by fast neutron irradiation

For chemical mutagenesis, the results of varying the length of time of EMS treatment on two selected sunflower seed are shown in Table 4. Inbred line NS-DE-4 is more resistant than NS-LIN-2. The results showed that 30% reduction of hypocotyl length of NS-DE-4 was 0.95% compared to NS-LIN-2 which was 0.68%

Table 5. Reduction of hypocotyls length and 30% growth reduction of seedlings height (GR_{30}) in two sunflower inbred lines compared with different time period of EMS treatment.

NS-LIN-2	2 hours	3 hours	3.5 hours	4 hours	5 hours
control	100%	100%	100%	100%	100%
0.50%	100%	65%	70%	52%	23%
1.00%	88%	25%	19%	22%	5%
1.50%	83%	15%	11%	11%	0%
GR_{30}	**2.80**	**0.68**	**0.68**	**0.60**	**0.40**
NS-DE-4	**2 hours**	**3 hours**	**3.5 hours**	**4 hours**	**5 hours**
control	100%	100%	100%	100%	100%
0.50%	86%	85%	81%	76%	34%
1.00%	64%	46%	51%	58%	11%
1.50%	76%	41%	40%	32%	0%
GR_{30}	**1.89**	**0.95**	**0.95**	**0.92**	**0.47**

Photo. 2. Variation in plant height of sunflower inbred lines NS-LIN-2 and NS-DE-4 treated with different concentration of EMS solution 3.5 hours.

The GR_{30} for 20 sunflower inbred line seeds ranged from 0.18% EMS solution (NS-LIN-6) to 0.93% (NS-LIN-4). The most sensitive sunflower inbred lines were NS-LIN-5 and NS-LIN-6 and the most tolerant to EMS were NS-LIN-3, NS-LIN-4, NS-LIN-9 and NS-LIN-12.

Table 5. Reduction of hypocotyls length (%) and 30% growth reduction of seedlings height (GR_{30}) in sunflower inbred lines caused by EMS treatment

hypocotyl lenth	NS-LIN-1	NS-LIN-2	NS-LIN-3	NS-LIN-4	NS-LIN-5	NS-LIN-6	NS-LIN-7	NS-LIN-8	NS-LIN-9	NS-LIN-10
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
0.5%	59%	62%	68%	64%	40%	32%	58%	42%	64%	63%
1.0%	47%	47%	44%	51%	18%	30%	15%	44%	51%	47%
1.5%	40%	42%	38%	54%	16%	28%	35%	40%	47%	39%
2.0%	34%	36%	37%	37%	16%	27%	26%	38%	39%	35%
2.5%	33%	35%	35%	35%	12%	25%	24%	36%	39%	32%
GR_{30}	0.75%	0.80%	0.82%	0.93%	0.26%	0.18%	0.68%	0.78%	0.89%	0.67%
hypocotyl length	**NS-LIN-11**	**NS-LIN-12**	**NS-LIN-13**	**NS-LIN-14**	**NS-LIN-15**	**NS-DE-1**	**NS-DE-2**	**NS-DE-3**	**NS-DE-4**	**NS-DE-5**
control	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
0.5%	56%	67%	47%	57%	48%	48%	49%	61%	59%	68%
1.0%	42%	48%	37%	47%	40%	34%	46%	54%	36%	42%
1.5%	31%	41%	25%	45%	35%	33%	44%	33%	36%	32%
2.0%	29%	38%	20%	34%	30%	27%	31%	24%	34%	29%
2.5%	26%	36%	18%	33%	23%	24%	28%	20%	31%	25%
GR_{30}	0.64%	0.87%	0.50%	0.75%	0.55%	0.50%	0.64%	0.78%	0.64%	0.78%

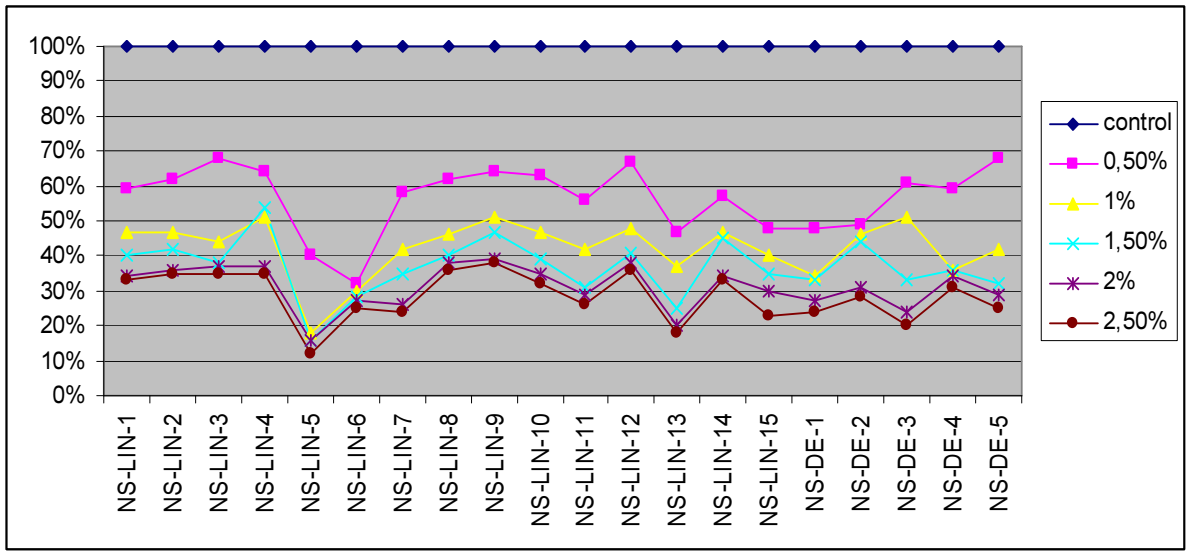


Figure 4. Reduction of hypocotyls length in sunflower inbred lines caused by EMS treatment

The three mutagens agents affected the hypocotyls length which decreased with increasing doses. There is a marked difference in mutagenic damage due to the three mutagen used among the 20 genotypes of sunflower varieties. Based on the radiation damage on hypocotyls length, the GR30 for 20 sunflower were estimated for induced mutation. The growth depressions due to mutagens have been used to determine the doses for mutation induction. It is the most functional parameter which has been used in radiobiological investigation because it is generally considered to be primary injury due to nuclear damage. According to Brunner (1995), Karma and Brunner (1977), seedling height reduction of 30-40% is generally assumed to give high mutation yield.

Doses necessary to reduce M_1 plant hypocotyls length to 30% i.e. GR_{30} are shown in the table 3, 4 and 5. The data also indicated that all genotypes gave a wide range radiation damage to gamma ray doses out of which the variety NS-LIN-4 showed the least radiation damage and NS-LIN-8 showed damage. In case of fast neutron the genotype NS-LIN-4 was the variety having the least radiation damage and the two genotypes NS-LIN-9 and NS-LIN-8 were having the highest damage in the same range. The chemical mutagen revealed NS-LIN-6 to be less damage and NS-LIN-9 again to have the highest damage.

There was a different influence of gamma irradiation, fast neutrons and EMS. The growth inhibition of seedling height was stronger for fast neutron then for gamma irradiation. Gamma rays have a lower relative biological effectiveness then fast neutrons which imply that in order to obtain the same biological effect, a higher dose of gamma irradiation must be given (*Van Harten, 1998; Kodym & Afza, 2003*).

Reduction of hypocotyl length was more in genotype NS-LIN-4 then for others genotypes which reflect a greater radiosensitivity of this inbred line to both gamma and fast neutron irradiation. Adversely, same genotype showed the best resistant to high doses of EMS. Inbred lines NS-LIN-5 and NS-LIN-6 are generally more sensitive than others to all treated mutagens. These can be explained that all three inbreds are early varieties although their seeds have normal size and high oil quantity. Inbred lines NS-LIN-8, NS-LIN-9 and NS-LIN-10 show the best resistance to both physical and chemical mutation. These genotypes are nearly isogenic lines, with different oil quality but low oil quantity. They have large, dark black seed. Generally it can be concluded that mother components of the hybrids, which have large seed, show more sensitivity than father components (restorer lines).

Influence of oil quantity in seed on radiation sensitivity

In this experiment, we compared 30% growth reduction of seedlings height (GR_{30}) caused by gamma irradiation and fast neutron with oil quantity in the seed of chosen genotypes, and found that correlation between them is 0.57 (Fig.5). That means, genotypes with higher oil percentage in the seed are more radio-sensitive and vice versus. Oil percentage in the seed did not effect on the sensitivity of chemical treatments. These results can be related to the differences of radicals produced by the two physical mutagens with organic structure (fatty acid, R-COOH). Singh et al. (1962), working with *Zea maize* (also oil seed) found more radiation-induced free radicals in seeds of low oil content than in seeds of high oil content, but the low oil seeds were found to be more radio-resistant.

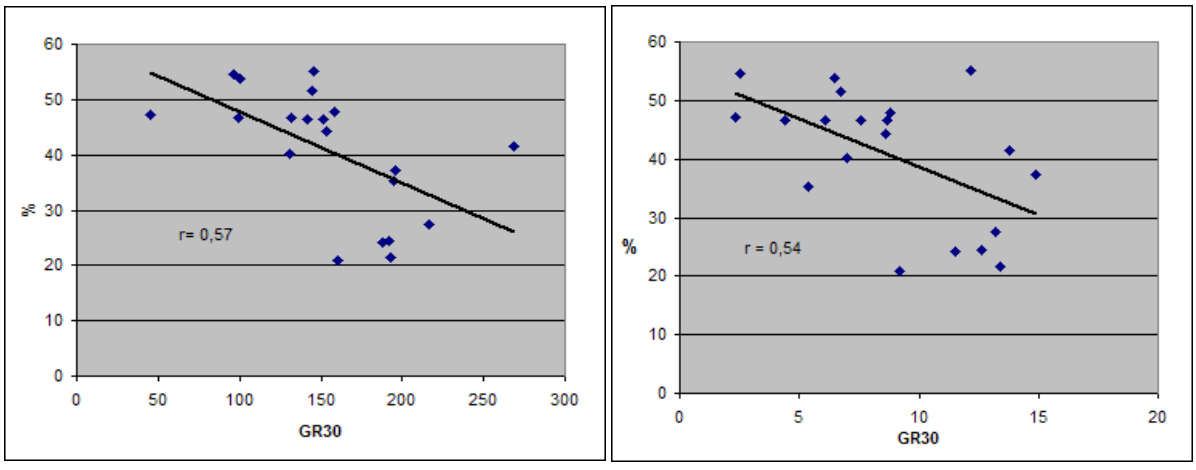


Figure 5. Corelation of GR_{30} gamma (left) and fast neutron irradiation (right) with oil content (%).

The results obtained from this study indicate that the radiation damage due to mutagenesis are not similar even with a same dose in case of 20 varieties belonging to the same species. The same differential responses to radiation among different genotypes in plant species was reported many researchers and is under genetic system control. These intervarietal differences in radiation damage of seeds have reported in rice, tomato and barley to be polygenic system (Y. Ukai, 1970 and Y. Kowyama and al 1987, D. R: Davies, 1962a & b, Y. Ukai and al., 1969), a major geneticic system in einkorn wheat and soybean (L. Smith, 1942, Y. Takagi, 1969), a genetic heterozygosity in maize and peanut (N.K Notani, 1961, M. Stoillov and al. 1966, and D. A. Emery and al 1970). It is a however a fact that plant respond differently to mutagenesis depending on their variety. But the explanation has not yet been fully proved. Mikaelsos and Halvorsen (1953) reported that there are difference in radiation damage when physiological traits like seed size and sort/long growing season. Saric (1961) indicated that radiation damage, depending on the genetic traits differed in their tested inbred line of maize. It was also reported the influences on the some biochemical constituents and metabolites to play important role in inhibition of M1 plant growth. El-Keredy et al., (1976), Kowyama et al., (1987), Gelin et al., (1958) and M. Stoillov et al. (1966) showed the effect of some variations of biochemical constituents in the seed to create intervarietal radiosensitivity, such as indole-3-acetic acid and sulfhydryl compounds in barley, intracellular oxygen content in pea and barley. Therefore, the chemical composition of the seeds is also an important factor.

The different GR_{30} values between genotypes reveal that a dose range of 50-250Gy of gamma irradiation, 2-15Gy of fast neutrons and 0.1-1% concentration of EMS solution will most probably be useful for large-scale field experiments for sunflower. Our results presented in this paper indicated

Photo. 2. Different putative mutants (Flowers. Chlorophyll, seed color, shape of the head). Flower of the control is at the first left corner.

2.5.2 Radiosensitivity and *in vitro* mutagenesis in a vegetatively propagated crop: Cassava, *Manihot esculenta Crantz*

Abstract

Induced mutagenesis holds promise for the subtle manipulation of traits of interest in crop plants. For a vegetatively propagated crop like cassava with severe constraints posed on its genetic improvement by inherent biological systems, the adoption of this methodology seems the more appealing. However, there is scant information on protocols for inducing mutations in this crop. We present in this report the preliminary data on the determination of radiosensitivities for some African cassava accessions as a prelude to a more detailed study that will include other accessions from wider geographical regions where cassava is grown. The implication of genotypic variation in response to gamma irradiation as was found in this study buttresses the need to carry out this larger scale study in order to avail cassava scientists intending to adopt induced mutagenesis of requisite information in this regard. A modified *in vitro* culture medium was also shown to greatly enhance the growth of the plantlets without producing callus.

Introduction

Cassava, *Manihot esculenta* Crantz (Euphorbiaceae) is a dicotyledonous tropical crop cultivated primarily for its edible storage roots on about 16million hectares with a total annual root production of 184 millions tons globally. As a staple crop, it accounts for the daily calorie intake of over 500 million people in the world (Cock, 1985.) Cassava is an ideal subsistence crop for the humid and sub humid tropics because it is well adapted to marginal soils (low fertility, high acidity), has the ability to tolerate environmental stress, has an unrivalled ability to recover from damage by pests and diseases, gives relatively high yields compared to other staple crops, and can be kept underground from 6 - 36 months after planting and is thus always available to the farmer (Uriyo, 1982). Since most processing of cassava into food is done on a small scale in rural areas, it is an important source of employment and income, especially for women. In some parts of the world the leaves are consumed as a vegetable. The starch from the roots is used in a wide variety of products, including paper, textiles, pharmaceuticals, and various foods, such as crackers, flavoring agents, noodles, and cheese breads.

The current and projected future trends in end-user preferred varieties in food, feed and industrial applications indicate a progressively increasing emphasis on the cultivation of cassava varieties whose end-products meet strict industrial and nutritional requirements. The genetic improvement of cassava through conventional means is however severely constrained by the asynchronous and shy flowering nature of the crop (Jennings and Hershey, 1984). Also, being an outcrossing species that is not amenable to inbreeding, the high levels of heterozygousity impact on the ability of breeders to develop lines for use in hybridization schemes. These scenarios therefore make the adoption of alternative means for improving the crop imperative. One such option is induced mutagenesis which accords with the current trends for the crop: maintaining the favorable traits (such as high yield) in elite clones while simultaneously effecting subtle changes to the quality traits in order to develop variants that would be suitable for specific industries (Broertjes and Van Huan, 1978; Broertjes and Van Huan, 1988; Donini and Micke, 1984; Konzac, 1984; Micke et al., 1987). The aim therefore is to produce induced cassava mutants whose end products will be suitable for bio-ethanol production; have contents of waxy starch; root and foliage protein content and profile; high amylose contents; and reduced anti-nutritional factors. One such strategy involves inducing elite well-adapted African cassava varieties to mutate by exposure to gamma rays.

The choice of irradiation doses is critical for the success of mutation induction. For instance, while a high dose may have high mutation frequencies; this is usually accompanied by lots of undesirable mutations in several segments of the genome implying a necessity for elaborate strategies to break

the linkage drags. As there is a paucity of data for induced mutations in cassava, this was the first step in establishiung an induced mutagenesis platform for the genetic improvement of cassava.through the irradiation of cassava *in vitro* propagules. The decision to use *in vitro* propagules derives from the aforementioned difficulty to generate sexual botanical seeds from most of these varieties. *In vitro* propagation from axillary buds and meristems tips is one such method applicable to cassava improvement and also offers possibilities for further manipulation such as for the dissociation of chimeras. The method rely basically on the premise that vegetative explants are treated with mutagens; resulting population of putative mutants are multiplied to dissolve chimerism; and homohistont plants are established and finally screened for the useful traits.

Additionally, mutation induction aims to optimize genetic variation with minimal plant injuries meaning that a balance has to be found between achieving mutagenesis and maintaining the integrity of the majority of the genome constitution of the mutated material. The present study was aimed at determining the optimal doses for irradiation using assessable parameters of primary injury in tissue cultures of different African cassava accessions. These parameters would then be relied upon as being indicative of mutagenic responses and thereby permit the estimation of optimal doses for mutation induction in this crop.

Materials and Methods

Plant materials and source of explants:

Three virus-tested and *in vitro* plantlets of 17 elite IITA-derived cassava varieties and two popular grown landraces in Nigeria were used for the study (Table 1). They were selected on the bases of validated agronomic traits such as high yield (dry matter and starch contents); disease and pest resistance; plant architecture; etc.

Rapid *in vitro* propagation of the cassava accessions

The ex-plants for initiating the cultures were two-node segments obtained by cutting the stems of the *in vitro* plantlets. Different media composition following the protocol of Roca et. al.(1984), Konan et.al.(1997). Groll et al.(2002) and Danso and Ford-Lloyd (2002) for meristem culture of cassava were tested for multiplication of different accessions of cassava *in vitro* and compared with the medium containing 4.4g Murashige and Skoog 1962 basal salts and vitamins and 20g sucrose of pH 5.8. These segments were transferred to conical flasks containing liquid culture medium. Murashige and Skoog basal medium was modified through alterations in strength, and contents of hormonal and other supplements in order to achieve maximum rapid growth of the cultures without callusing.

Determination of optimal doses for irradiation

Radiation sensitivity tests were carried out to determine the optimal doses of irradiation for the exposure of the ex-plants of the 17 cassava accessions. The grown explants were de-leafed and cut into explants containing 2 nodes each. These were placed in petri dishes containing sterile distilled water, with each Petri dish containing 10 explants. These Petri dishes were sealed with laboratory parafilm. Each Petri dish was irradiated with different doses (5, 10, 15, 20, 25 and 30 Gy) while a control batch was not irradiated. Under aseptic conditions (in an airflow cabinet), these irradiated samples were then introduced into conical flasks containing liquid growth medium described above. These were allowed to grow on a horizontal gyratory shaker at about 30 rotations per minute. The culture was maintained at 26 C under continuous light.

After 4 to 5 weeks of growth, 3 parameters, weight of the explants, the average number of nodes, and plant height were measured and scored as a percentage of the control, i.e. untreated material of the same genotype. Data from these parameters were plotted against the corresponding doses of Gamma ray exposure. Based on the differences in height and weight between the irradiated and non-irradiated control, the dose leading to an average of 30 percentage damage was determined as the optimal dose. This is usually designated as LD_{30} with LD being the abbreviation for lethal dose.

Results and Discussion

Culture medium

Formation of callus is one of the critical handicaps for *in vitro* multiplication of some cassava varieties. In the present study, several of the culture media used for *in vitro* multiplication were compared. One that achieved good multiplication rates without callus formation and was also very suitable for multiplication of 42 different accessions of cassava from IITA and from CIAT was identified. This best culture medium was made with half strength of Murashige and Skoog basal medium supplemented with 20gm sugar. The growth hormones were eliminated completely. The medium was constituted as follows:

For one litre of liquid medium, the following was used:

- MS basal medium(Sigma M5519) = 4.4g
- Sucrose (Grade1, Sigma) = 20g
- Sterile double distilled water used to make up the volume to 1000ml
- pH adjusted to 5.8

For one litre of solid medium, the following was used:

- MS basal medium (Sigma M5519) = 4.4g
- Sugar (Grade1, Sigma) = 20g
- Gelrite = 1.8g
- Sterile double distilled water used to make up the volume to 1000ml
- pH adjusted to 5.8

Optimal doses for gamma irradiation

Fig. 1 shows the reaction to variations in irradiation dosage by cassava genotype TME 203. This was estimated as percentage departure of the height and weight of plantlets from irradiated *in vitro* nodal segments from the values of the non-irradiated control. The point corresponding to 30% damage, i.e. 70% mark on the Y-axis (read off from the line of best fit) is considered as the LD_{30}. For this genotype and using these 2 parameters, these points fall around 15 Gy and 18 Gy. This could also be calculated more precisely using the linear regression equation. In practice however, irradiation for generating mutants in crop improvement programmes is carried out over a range of plus/minus 5 of this determined optimal dose. The values for LD_{30} have also been determined for the other cassava accessions (data not shown) and based on these, bulk irradiation of these cassava genotypes have been carried out at 3 different gamma irradiation doses of 12 Gy, 25 Gy and 20 Gy, respectively. This variation would indicate a genotypic effect in the reaction of cassava genotypes to irradiation. In order to substantiate this, a more detailed study is ongoing and involves the evaluation of the reaction of both African and South American cassava accession to gamma irradiation based on these 3 parameters.

Induced putative mutants

Currently, 9252 putative mutant cassava clones, generated from bulk irradiation and the radiosensitivity tests are being further multiplied in vitro at IITA prior to being evaluated for value-added traits. The putative mutant cassava clones currently at IITA are shown in Table 2. These Materials were received in different batches and the first batch has completed post-flask management and is being established in the field for evaluation, while the others are being micropropagated at IITA for another generation to eliminate possible chimeras before establishment. At harvest of the mutagenic plants at 12 months after planting, the physicochemical, functional, and pasting properties and granule morphology of the cassava foliage, flour and native starches will be assessed for targeted products for food, feed and industrial applications.

Table 1. Characteristics of elite African cassava accessions for which optimal gamma irradiation doses were determined

Genotype	Superior characteristics
I30572	High yield and resistance to biotic stresses
I4(2)1425	Good nutritional quality and plant architecture
I82/00058	High yield , resistance to biotic stresses, and good plant architecture
I92/0057	High yield , resistance to biotic stresses, and good plant architecture
98/0505	High yield , resistance to biotic stresses, and good plant architecture
98/0581	High yield , resistance to biotic stresses, and good plant architecture
98/2101	High yield and resistance to biotic stresses
I95/0289	High yield , resistance to biotic stresses, and good plant architecture
I96/1632	High yield , resistance to biotic stresses, and good plant architecture
01/1277	High yield , resistance to biotic stresses, and good plant architecture
94/0330	High yield , resistance to biotic stresses, and good plant architecture
01/1371	High yield , resistance to biotic stresses, and good plant architecture
98/0002	High yield , resistance to biotic stresses, and good plant architecture
TME 1	Popularly grown landrace good nutritional and culinary qualities and resista stresses
TME 2	Popularly grown landrace good nutritional and culinary qualities and resista stresses,
TME 203	Popularly grown landrace good nutritional and culinary qualities and resista stresses
TME 419	Popularly grown landrace good nutritional and culinary qualities and resista stresses

Table 2. Putative mutants of IITA-derived cassava clones and landraces

Clone	No. of plantlets
98/002	2648
TME-1	988
98/0581	4176
TME 2	220
TME 203	252
01/1371	340
01/1277	148
94/0330	176
I95/0289	172
I96/1632	132
Total	**9252**

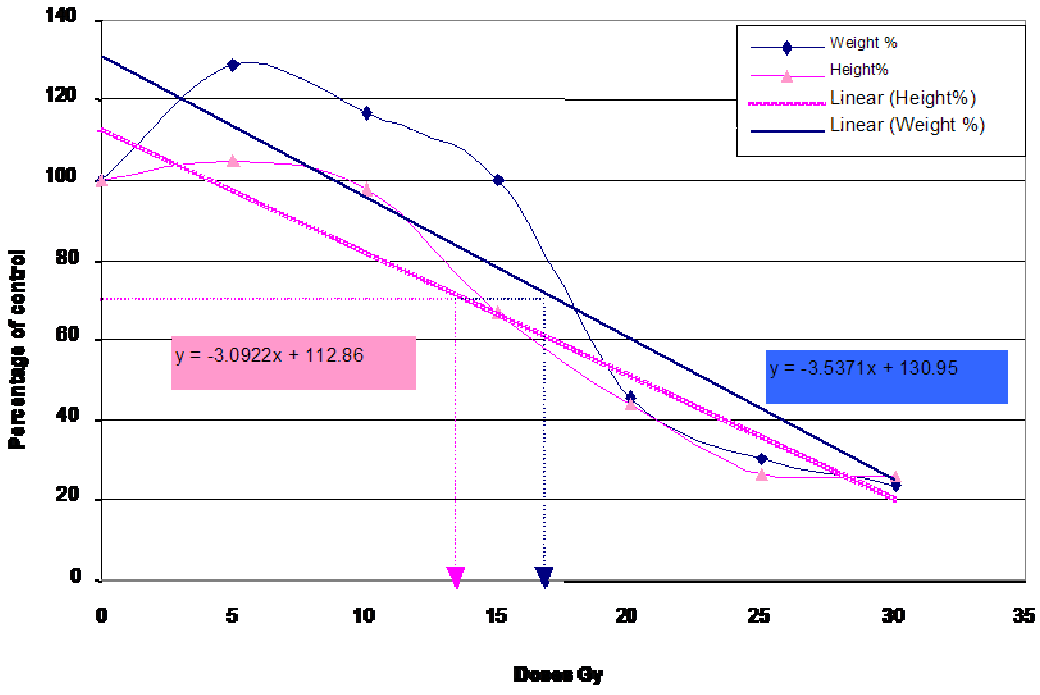


Figure 1. Differences (%) in growth parameters between plantlets arising from non-irradiated and irradiated in vitro nodal segments of cassava genotype TME 203 plotted against irradiation dosage

3. Training Activities

Human capacity development, in groups or individually, along with innovation-driven R&D activities, and the provision of analytical and technical services form the 3 core pillars of the supporting role that the Unit's activities provide to enhance capacity in MSs, especially developing ones, in the use of induced mutations to develop superior crop varieties. Usually, these training activities are aimed at the acquisition by the trainee of skills in induced crop mutagenesis facilitated by *in vitro* techniques and molecular genetics.

Table 1. Fellows and their research activities in PBU for 2006

Name	Country	Period and duration in months for 2006 in brackets	Area of Training
Abdul K. Kadhim	Iraq	September 2005 to February 2006	Induced mutation in vegetatively propagated crops.
Mr. Masoud Rahimi	Iran	1 April to 31July 2006	Induced mutation and related biotechnologies in rice improvement
Mr. SOE Tet Htut	Myanmar	1 May to 30 September 2006	Induced mutation in seed propagated crops and application of molecular marker techniques for germplasm and mutant characterization
Ms. WIN NAY CHI	Myanmar	1 May to 31 September 2006	Induced mutation in seed propagated crops and application of molecular marker techniques for germplasm and mutant characterization
Ms. OWOSENI Omowunmi	Nigeria	1 April to 30 August 2006	Induced mutations in crop improvement, *in vitro* techniques in combination with mutation induction for crop improvement with special emphasis on cassava; application of molecular genetic markers for germplasm characterization
Ms. GREYLING, Ria.	SOUTH AFRICA	1 to 31 July 2006	Induced mutations in seed propagated crops, screening techniques for salt tolerance and phenotypic characterization using grain parameters
DON Pham Ngoc Nga	VIET NAM	14 August to 13 November 2006	Induced mutations in seed propagated crops, application of molecular genetic markers for germplasm characterization and mutant identification, screening techniques for salt tolerance and phenotypic characterization using grain parameters
AMINU A. Zaria	NIGERIA	13 November 2006 to 13March, 2007	Induced mutations in a seed propagated crop (cowpea) for genetic improvement, molecular markers for germplasm characterization, hands on experience with determination of ploidy levels of plant species using the Flow Cytometer.

3.1. Individual training - Fellows

The individual training activities are usually funded by the Agency as Fellowships and Scientific Visits through its Technical Cooperation programme. Other individual training schemes include the cost-free internship and cost-free expert schemes. In 2006, the Unit hosted 8 Fellows with over a cumulative 26 Fellow-months. The details of these Fellows and their research activities are presented in Table 2.

3.2. Group Training

The Interregional Training Course on Mutant Germplasm Characterization Using Molecular Markers is an annual event of the Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture for supporting capacity in developing Member States for the use of induced mutagenesis and relevant biotechnologies for developing superior crop varieties. During the course, 20 young scientists from developing Member States of both the Agency and the Food and Agriculture Organization are exposed to a series of structured theoretical lectures and practical exercises in the laboratory, green houses, experimental fields and computer based analyses.

This course has evolved substantially since its inception 25 years ago in keeping with the trends for the technologies that are relevant to induced mutations and crop improvement. This is reflected in the below series of changing nomenclature since inception in 1982:

- Induction and Use of Mutations in Plant Breeding;
- Advances in Plant Mutation Techniques;
- Advanced Mutation Techniques for Crop Improvement;
- Advances in Technologies for Induced Mutations in Crops; and the present
- Mutant Germplasm Characterization Using Molecular Markers.

Currently, the Course consists of a 5-week programme which is divided into 6 modules:

- Inducing and Detecting Mutants;
- *In vitro* Techniques in Crop Improvement;
- Molecular Genetic Markers;
- Molecular Cytogenetics;
- Reverse Genetics for high throughput detection of mutation events; and
- Population Genetics and Data Management.

The 20 participants in the 2006 edition of the course were from 20 developing Member States (Bangladesh, Brazil Bulgaria, Cameroon, P.R. China, Costa Rica, Cuba, Indonesia, Iran, Jamaica, Libya, Mauritius, Mongolia, Morocco, Myanmar, Nigeria, Sri Lanka, Syria, Thailand and Uganda). Information on these participants, their nominating governments and contact information, along with information on the 6 external lecturers that taught the different modules (in conjunction with sub-Programme Staff Members). Overall, the participant's feedback on the contents of the course; its suitability for application in MSs; resource personnel, teaching materials, and facilities used for the course; as well as exercises during the course; etc. indicated an excellent appraisal.

3.3. Enhancement of Staff Members' capacities - Workshop

Another aspect of capacity building activities in the Unit is the acquisition of new skills by Staff Members. To augument the training activities provided by the Agency for this purpose, the Unit usually organizes an enrichment exercise on themes that are of unique relevance to the Unit's activities. In 2006, one such activity was aimed at introducing Staff Members to novel technologies relating to ploidy determination using the Flow Cytometer. A 2-day workshop on this theme, moderated by Mr. Matthias Steinberg of PARTEC, Germany was held on 15 and 16 November 2006. Ploidy determination using the Flow Cytometer is critical to the successful implementation of the Unit's R&D as well as services-related activities.

3.4. Scientific Visitors to the Unit

Our ability to work with MSs is greatly enhanced through interactions with Scientists and policy makers from these countries. Our Unit therefore regularly plays host to national counterparts with collaborative endeavors usually arising from such meetings. The following were the Scientific Visitors during the year 2006.

Table 2. Scientific Visitors to the Unit

Name of Visitor	Home Institute and address	Subject Area	Period
Mr. Soumana Toudjani	Ministere des mines et de l'energie, National Liaison Officer Niger	Induced mutation in rice and cassava	January, 2006
Mr. Hendrich Theobald	Innovaplant GmbH, CoKG Germany	Induced mutation in ornamental plants	February, 2006
Mr. M.S. Jain	Kyllastomontie 165, 04220 Kerava, Finland	Induced mutation for crop improvement	February, 2006
Mr.SYDNEY D. J	Rice Research Station, Rokupr National Agricultural Research Coordinating Council (NARC), Ministry of Agriculture and Food Security)	Induced mutation for crop improvement	April, 2006
Mr. Asafu-agayei J. N	Director, Crops Research Institute, Kumasi, Ghana	Mutation induction for crop improvement	15-May - 27 May, 2006
Prof. Luxiang LIU	Director, Centre of Space Breeding and Dept. of Mutational Genetics and Crop Breeding, Inst. of Crop Science, Chinese Academy of Agricultural Sciences, 12 Zhongguancun South Street, Beijing 100081, CHINA	Initiation of research collaboration in 'space breeding'.	
Abdul Jabbar KHAN	NIFA, Peshawar, Pakistan	Induced mutations for crop improvement (Chickpea & Wheat); Molecular markers for mutants characterization; Collaboration for germplasm; Screening techniques for drought & salinity tolerance; and Establishment of molecular biology Lab.	25 September to 6 October, 2006

4. Services

Along with R&D and human capacity development, the provision of services aimed at supporting the capacity of MSs to develop superior crop varieties using induced mutagenesis is at the core of the Unit's activities. The services include the irradiation of plant propagules and molecular genetic fingerprinting.

Irradiation

17 crop species were induced to mutate through exposure to gamma rays in a Cobalt-60 source in response to 31 requests from 19 MSs. Additionally, 14,250 *in vitro* putative mutants of vegetatively propagated crops (bananas, potato and cassava) were produced and are being evaluated in MSs.

Molecular genetic fingerprinting

Most activities in this regard have been dedicated to the use of our high throughput facility for in-house needs for DNA fragment sequencing and separation. Two requests for molecular genetic fingerprinting totalling 203 samples (including 11 mutant rice varieties) were received from Member States and promptly executed.

5. APPENDICES

5.1 Publications

Mba, CR, Afza, SM Jain, GB Gregorio and FJ Zapata-Arias. Induced mutations for enhancing salinity tolerance in rice. In: Jenks, MA, Hasegawa, PM, and SM, Jain (eds). Advances in molecular-breeding toward drought and salt tolerant crops. Springer Publishing, Inc., Dordrecht, Germany. (Accepted).

Owoseni O, Okwaro H, Afza R, Bado S, Dixon A and Mba C. Radiosensitivty and in vitro mutagenesis in African accessions of cassava, Manihot esculenta Crantz. Plant Mutation Reports. 1 (2): 32-36.

5.2. Travels

During the period under review, the Unit Head, Chikelu Mba made the following official trips:

Food and Agriculture Organization of the United Nations (FAO), Rome, Italy. 28 February to 03 March 2006

In line with FAO's efforts at charting a course for working with its Member States governments on biosafety, an Expert Consultation on biosafety was convened in order to proffer suggestions for a roadmap to the Organization. This 4-day meeting titled, '**Expert Consultation on Biosafety within a *Biosecurity* framework: contributing to Sustainable Agriculture and Food Production**' was attended by experts in this field drawn from MSs, International Organizations, Conventions, Standards Setting Organizations and FAO Staff Members. The recommendations arising from this meeting covered the areas of collaborations; fostering of research especially in emerging areas; and enhancement of capacity for sharing of information. The discussions on the current non-regulated status of mutants during which a number of attending of policy makers preferred that this situation be reviewed would be of interest to the Agency.

International Centre fro Tropical Agriculture (CIAT), Cali, Colombia. 28 August to 01 September 2006

This trip to CIAT was primarily necessitated by an ongoing collaborative endeavour between both organizations in the use of induced crop mutagenesis to generate cassava mutants as resources for crop improvement and genomics studies. This report details the identification of cassava mutants with immense potentials for enhancing income generation for the growers on account of their desirability in industries. The report also highlights the status of collaborations in induced mutagenesis for rice. Opportunities for involvement in topical global initiatives are also highlighted. Information on the desire of a consortium of Colombian national Agricultural R&D entities to strengthen their activities through nuclear techniques is another important component of this report. The conclusion presents a succinct identification of relevant challenges and opportunities for the Agency while proffering recommendations on how to achieve greater visibility and impact for relevant Agency's activities.

International Institute for Tropical (IITA,), Ibadan, Nigeria. 25 to 29 September 2006

This trip was aimed at reviewing progress, strengthening and planning future activities relating to ongoing collaborative endeavours with IITA Nigeria in the application of induced mutagenesis in the development of elite cassava clones with added value quality traits. Mutagenic populations previously developed at the Agency are severally undergoing further cycles of vegetative propagation and hardening prior to field trialling. The first batch of these populations has been established in the field. Perspectives for advancing this collaboration include strengthening IITA's capacity for assaying for root quality traits in cassava. Other areas of collaboration that were identified in the course of the trip included yam genetic improvement; transfer to the Agency of IITA's capacity for rapid *in vitro* assays for tolerance to biotic stresses using live pathogens; and collaboration with the development of herbicide resistant crop varieties. It is recommended that the Agency collaborates more actively with this Institute on account of its strategic location in the food insecure sub-Saharan Africa with the aim of demonstrating the impact of the Agency's interventions aimed at enhancing sustainable agricultural productivity for vulnerable populations of the world.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