
CHAPTER THREE

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MATERIALS and METHODS

3.1 Culture Initiation

Pisang Berangan (AAA) was the chosen variety in this study. Vigorous and healthy suckers were selected from true-to-type, fruit-bearing mother plants cultivated at the United Plantation. A total of 300 suckers were taken for culture initiation in three batches starting in June 1997.

The outer leaves, leaf bases and corm tissue of each sucker were trimmed to 2.5x 2.5x5 cm and surface sterilized in the commercial bleach (2.5% Sodium hypochlorite, or Chlorox) at 15 % for 15 minutes. After rinsing three times in sterile distilled water the explants were immersed in 70% ethanol for 10-15 seconds and further rinsed with sterile distilled water. Shoot tip meristem (approximately 5x5x5 mm) was removed with a sterilized scalpel, then transferred directly to modified MS medium (Table 3.1). The medium was autoclaved at a pressure of 1.05 kg/cm at 121°C for 30 minutes. The pH was adjusted to 5.5 – 5.8 with 0.2 NaOH or 1N HCL (Appendix A).

The cultures were maintained at 28 ± 2°C and 60-70% relative humidity in a 16-h light cycle with fluorescent light of 1000-3000 lux. Subculturing was carried out after 4-6 weeks or earlier depending on blackening of explants. Shoot initials developed after 3 weeks and were proliferated on induction medium with the BAP concentration reduced to 4.5 mg/L. Multiplication of propagules was carried out by subdividing the newly formed shoots or bud clusters and reculturing them on fresh medium at 4-6 weeks interval up to subculture 6. MS

medium was then supplemented with 2mg/L IAA and 5mg/L kinetin for rooting. Root generation took 6-8 weeks until the plantlets reached the optimal size of 4 -5 cm.

Table 3.1: Composition of culture medium used for banana culture initiation

Chemical	Formula	100X Stock Solution (g/L)
Macronutrients		**100 ml/L**
Ammonium nitrate	NH_4NO_3	16.5
Potassium nitrate	KNO_3	19.0
Calcium chloride	$Ca\ Cl_2\ 2H_2O$	4.4
Magnesium sulphate	$Mg\ So_4\ 7H_2O$	3.7
Potassium dihydrogen Orthophosphate	$KH_2\ PO_4$	1.7
Micronutrients		**10 ml/L**
Manganese Sulphate	$MnSo_4.\ 4\ H_2O$	2.23
Zinc sulphate	$Zn\ So_4.\ 7\ H_2O$	0.86
Potassium iodide	KI	0.086
Cupric sulphate	$Cu\ SO_4.\ 5\ H_2O$	0.0026
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.025
Cobalt(ous) chloride	$Co\ Cl_2.\ 6H_2O$	0.0026
Boric acid	$H_3\ BO_3$	0.62
Vitamin Source		**5 ml/L**
Nicotinic acid	$C_6H_5NO_2$	0.125
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS.Hcl$	0.25
Pyridoxine hydrochloride	$C_8H_{12}N_2\ O_2.\ 2Hcl$	0.125
Myo- inositol	$C_2H_5\ NO_3$	25.0
Glycine	$C_6\ H_{12}\ O_6$	0.50
Iron source		**5 ml/L**
EDTA-salt	$C_{10}H_{14}N_2O_8Na_2H_2O$	3.75
Ferrous sulfate	$Fe\ SO_4.\ 7H_2O$	2.78

- 4.5 mg/L of Cytokinin (6-benzyl amino purine – BAP) - $C_{12}H_{11}N_5$ – was added to the mixture of MS medium as plant hormones.
- Agar (Sigma, St Louis, Missouri, U.S.A) (0.6 – 1.0%) or gelrite (0.1 – 0.3%) was also added.

3.2 Mutation Induction

Mutation induction coupled with *in vitro* technique cause morphological changes and also increase variability in quantitative traits. Physical mutagens such as gamma ray are tools for enhancing and generating genetic variation by inducing mutations at the gene, chromosome and genome levels, in nuclear and cytoplasmic organelle DNA (Larkin, 1998). In *Musa* spp., mutation breeding provides opportunities to improve of one or a few characteristics in an already established cultivar without affecting the desirable traits.

The shoot-tip meristem pieces (about 1cm x 2mm) were aseptically excised from micropropagated plantlets of Pisang Berangan. Each shoot-tip was cut longitudinally into two pieces and then transferred to sterile petri dishes, which contained a sterile moist filter paper. Each petri dish contained 10 meristem pieces and was sealed with parafilm. These petri dishes were carefully packed in a cooler box and transported to the Malaysian Institute of Nuclear Technology Research (MINT) in Dengkil, Selangor, for gamma irradiation. The petri dishes, which contained the meristem pieces, were irradiated in a gamma cell with a Cobalt-60 (^{60}Co) source (GC 400A, 10 Kci). A total of four batches were irradiated. The sample size and dosage are given in Table 3.2.

Table 3.2: Number of meristem tips treated with gamma rays

Dose (Gy)	Batch (date)			
	1(12/6/97)	2(26/9/97)	3 (14/1/98)	4 (20/5/99)
0 (control)	200*	200	200	200
40	250*	500	500	1000
60	250*	500	-	-

(10 meristem pieces in each petri dish)*

The irradiated meristem pieces were washed thoroughly with sterile distilled water and cultured on modified MS solid medium (Table 3.1). The explants were cultured in jar-jar bottles each containing five explants. Subculturing was carried out at 4 - 6 weeks interval until generation M_1V_4 i.e. three sub-cultures. The explants after M_1V_3 were allowed to root in rooting medium containing MS salts supplemented with 2 mg/l IAA and 5 mg /l Kinetin.

After three to four weeks in rooting medium, the plantlets were deflasked and transplanted in the nursery for acclimatization or hardening. The healthy, vigorous and well-rooted plantlets were transplanted into the polybags (5"x7") filled with peat and washed sand at 1:2 ratio. The plantlets were maintained under 70% -80% shade (using shading net) and high humidity. The plantlets were irrigated twice a day. Shade was removed completely after 5 weeks. From the 4th week after transplanting until plants were ready for planting, foliar fertilizer "GROFAS" was applied twice weekly to the plants at a rate of 10 g in 4.5 liters of water. The nursery plants were ready for field planting after 8 - 9 weeks of hardening.

3.2.1 Evaluation of gamma irradiated plants

Five batches of materials were generated for field evaluation trials at University Malaya farm (IPSP-Farm) and the United Plantations Bhd's field (Teluk Intan, Perak) (Table 3.3). The dimensions of the planting hole were 30 cm^2. The plants were grown in rows spaced at 2.5m x 2.5m in a triangular pattern in the U.P. field, and 2.5m x 2.5 m in the IPSP farm.

The three treatments (0, 40 and 60 Gy) were completely randomized in the field, as were in the other 2 treatments (20, 30 Gy). Ground Magnesium Limestone (GML) was broadcasted and mixed into soil a few days before planting. Organic fertilizer Supergro Green (CIRP) –

with a 5:5:5:1 nutrient composition - 75 grams per point- was mixed with soil in the planting hole. CCM65 and CCM44 were applied after that around the base of the stem at doses of 220 and 200 g /point, respectively at monthly interval. Weeding was done at monthly intervals. Unwanted young suckers were pruned 2-3 months after field planting and then at regular interval of 6-8 weeks.

Table 3.3: The number of generated banana plants (irradiated and control) for field evaluation

Dose (Gy) Treatment	Batch (date)					Total
	1 (8/1/98) IPSP	2 (15/3/98) IPSP	3 (21/7/98) IPSP	4 (20/11/99) IPSP	5 Nov. 97 UP	
0 (control)	260	345	361	362	202	1540
20	-	-	-	-	890	890
30	-	-	-	-	908	908
40	274	480	668	866	-	2288
60	71	148	-	-	-	219

3.2.2 Morphological variation evaluated in the greenhouse and the field

The nursery plants were examined for any mutagenic affects on growth and morphological characteristics. The observation was made at the final stage of hardening (7-8 weeks old) before transplanting to the field. Additional observations were made in the field at the vegetative stage and after flowering.

At the nursery stage in the greenhouse, characteristics observed include plant stature (measured from the ground level to the base of the youngest petiole), the distance between petioles and leaves, and the leaf size. Leaf deformation (such as uneven lamina, small and narrow leaves, broad and oval leaves), changes in leaf colouration (discolouration,

darkening green and various forms of variegation), changes in pseudostem characteristics (flat, stiff or bulbous) were observed after the plants reached 30 cm in height. At shoot emergence stage (i.e. flowering) the following characteristics were recorded:

1. Pseudostem plant height (cm): length measured from the soil surface to the bending curve of fruit stalk.
2. Sheath characteristics: glabrous, slightly waxy or waxy.
3. Pseudostem: texture and colour.
4. Petiole margins: erect; incurved or rolled.
5. Petiole bases: spreading or loose; clasping; or corrugated.
6. Leaf characteristics:
    a) Lamina bases: cuneates- rounded or auriculated.
    b) Lamina width: normal or uneven
    c) Crinkling of lamina.
    d) Types of leaf surface: smooth or rough.
    e) The color of the midrib and petiole: normal, red or other.
    f) Variegation on leaves: red sector appearance or petiole edges with red color.
    g) Bending of young leaf.
    h) Twisting of midrib of older leaves
    i) Compact and erect leaves.
7. Proliferation of suckers: high, low or normal.
8. Sheath characteristics: glabrous, slightly waxy or waxy.
9. Girth circumference (cm): girth at 30 cm above the soil level.
10. Bunch characteristics:

a) Number of fruits / bunch

b) Peduncle length: long, short or normal.

c) Bunch weight (kg).

d) Number of combs per bunch

e) Comb weight (g).

f) Number of fingers per comb

3.3 Somaclonal variation in Pisang Berangan (AAA)

The observed variation among regenerated plants and those obtained following mutagenic treatment suggests that somaclonal variation results from a mutagenic process, although the frequency and spectrum of variation was quite different among the somaclones.

The materials used in this experiment were suckers of Pisang Berangan cv. 'Intan'. Isolated shoot apices of 5-10 cm^3 were surface sterilized with 15% aqueous Clorox solution for 30 minutes followed by repeated washing in sterile water. The sterilized explants were cultured on modified MS media supplemented with 4.5 mg/L Benzyl Amino Purine (BAP) plus 3% sucrose, solidified with 0.7% agar. Media pH was adjusted at 5.8 before autoclaving (121°C for 20 min.). The growth regulator was added to the medium before autoclaving.

The culture was incubated at 28± 2° C at an intensity of 12 μ Em^{-2} S^{-1} fluorescent lamps for 16 hours daily and 60-70% relative humidity. For further shoot proliferation, subculturing was done continually every 3-4 weeks by subdividing the multiple shoot clusters and

transferring into a fresh media. After 8-9 subcultures some of the shoots were maintained as the culture stock, and some others were transferred to MS rooting media, supplemented with 0.5 mg/L indole –3-butyrice acid (IBA).

Rooted plantlets were washed in tap water and transplanted into polyethylene trays containing the sterilized media of agricultural substrate: soil: sand (1:1:1). Furthermore, the plastic trays with the plantlets were placed under plastic cover with 50% sunlight reduction, and sprayed with water to maintain relative humidity of more than 70% for two weeks. After acclimatization, each plant was grown in a plastic bag with soil media and kept in the shade for another 2-3 weeks before being transferred to ambient field conditions.

3.3.1 Characterization of variants and evaluation for important agronomic traits

Data were collected for all experimental plants every month, three months after planting for the following:

1. Vegetative characters: plant height (cm), date to shooting, date to harvesting, time to shooting (days) and time to harvest
2. Reproductive characters: bunch weight (kg), number of hands in a bunch, number of fingers in a bunch and finger length
3. Morphological description

The following characterization was carried out when the plants were already established in the field i.e. at about 5 –6 months after field planting. The types of variants recorded were divided into:

1. Types of leaf variants

2. Types of pseudostem variants
3. Types of bunch variants
4. Types of finger variants

The screening for variant plants was done visually since morphological differences were very obvious.

3.4 Analysis of Nuclear DNA Content

FCM is a convenient tool for estimation of nuclear DNA content and ploidy level constitutions in plant (Dolezel, 1991; Dolezel, *et al.*1994). Basically the FCM involves DNA specific flourochrome and the relative fluorescence intensity estimated by stained nuclei. By assumption, the DNA content is linearly related to digitized fluorescence signals. Its validity depends on flourochromes, fluorescence absorption, standard reference and a linear amplification system. Measurement is based on fluorescence intensity of nuclei ratio between known nuclear genome and the nuclei from sample. Dolezel *et al.* (1994) modified the technique for analysis of nuclear genome of bananas and plantains. The method used to screen ploidy levels of plants regenerated from *in vitro* culture was also used to estimate genome size and its variation in *Musa.*

In this experiment Flow cytometry (FCM) allows for measurement of the DNA content of isolated nuclei. If the nuclei are stained by a flourochrome specific to DNA, the size of the genomes of the individuals studied can be determined. When the ploidy level of a control is known and is measured in the same way, the ploidy level of the individuals studied can be detected. Among the advantages of this technique are its rapidity of analysis and computerized data processing.

3.4.1 Plant Materials and Sample Preparation

Samples of mutated Pisang Berangan (cv. Intan AAA) and normal plants (control) were used for determination of ploidy level and identification of the nuclear DNA content by FCM. Nuclei were isolated from young leaves following the method developed by Gailbraith *et al.* (1983). Approximately 50 mg of young leaves from each sample (mutated & *Glycine* plants) were chopped up together with a sharp scalpel blade in a glass petri dish containing 1 ml LBOI lysis buffer (Dolezel, 1991), supplemented with 50 ug Propidium-iodide (PI) and 50 ug RNAse. *Glycine max* was used as internal reference standard for identifying the nuclear DNA content by FCM. An intact interphase nucleus was released from the cut surface directly into 2-ml Lysis buffer supplemented with 0.1 mg/ml of 4,6-Diamidino-2-phenylindole (DAPI). The homogenate-stained nuclei were filtered through 50 µm nylon mesh into the analysis tube and its fluorescence was analyzed by FCM.

3.4.2 Instrument setting and alignment

Fluorescence intensity of DAPI-stained nuclei was measured using a Partec CA11 flow cytometer (Partec GmbH, Munster, Germany), which uses high pressure mercury arc lamp (HBO00W/2L) as an excitation light source. These were filtered through a combination of optical filters: KGI, BG38 and UGI for excitation, TK560 as diachronic mirror, and along-pass emission filter of RG590.

Autoclaved deionized water was used as a sheath fluid. The instrument was aligned using suitable particles for maximal amplitude and minimal coefficient of variation (cv). In order to calibrate the instrument, analysis was started with nuclei isolated from a diploid plant (2n) of *Glycine max.*

3.4.3 Sample analysis

About 2 ml of the homogenate nuclei was analyzed in the Cell Counter Analyzer Cytometer (Model Partec CCA-II). The fluorescence was measured with a 100 W high-pressure mercury arc lamp as a light source filtered as mentioned above. The DNA genome size of the sample was then estimated using the ratio of G1 peaks. The nuclear DNA content is quantified by the following formula:

2C nuclear DNA content (pg) = 2.5/ fluorescence ratio

Symbol C is used for DNA content of haploid set of chromosomes. *Glycine max* has 2C DNA content 2.5 pg (Tiersch *et al.* 1989).

3.5 Molecular Characterization of Mutants

The randomly amplified polymorphic DNA (RAPD) and arbitrarily primed PCR (AR-PCR) approaches have since been widely adopted (Waugh and Powell, 1992). RAPD analysis is a polymerase chain reaction (PCR) based technique, which uses random primers to generate DNA fragments, which can be used as genetic markers (Damasco, *et al.,* 1996 b). The following study was initiated to develop a RAPD technique for the early detection of off-types or mutants.

3.5.1 Plant Material

Mutant plants identified from different gamma-dose treatments (20, 30, 40, and 60 Gy) were selected from *in vitro* plantlets, glass house-grown and field-grown plants. Samples were

taken from the middle part of young but fully expanded leaves and immediately frozen in liquid nitrogen and stored at -70°C until further processing. These leaves were cut from the base by using a sharp knife and removing the upper and the basal parts of the leaf to keep the middle part in liquid nitrogen or ice. Normal (true-to-type) and dwarf plants or stunted Pisang Berangan (Cv. Intan) (AAA) were previously obtained from micropropagation in the tissue culture laboratory. These plants were characterized morphologically. Mutant plants or 'off-types' have been noted for plant stature (mainly dwarf) and very tall variants, leaf variegation and thickness, and fruit bunch characters (small bunches, split fingers, long peduncle and narrow and elongated male buds). The samples sizes from UP and UM field were 450 mutant, 200 from UP (20 Gy and 30Gy) on 17 Nov 1998, 19 March 1999 and from UM on 12 April 1999. The total numbers of samples taken from U.M. nursery and field were 250 mutants (40Gy and 60Gy) and control plants on 15 Feb. 1998 and 9 May 1999 as shown in Table 3.4. DNA was extracted from fresh leaves of *in vitro* plants, glasshouse and field-grown plants obtained from U.M. field and nursery.

Table 3.4: Types and sample size of mutants (variants) used for RAPD analysis

Types of variants	Sample size	Source
Stature	300	UM (40 & 60 Gy) from batch 2 & 4, UP (20 & 30 Gy)
Foliage	60	UM field (batch 1, 2 & 3), and UP.
Pseudostem	25	UM field (batch 1, 2, 3 & 4) and UP.
Bunch	45	UM field (batch 1 & 4).
Control (normal)	20	UM and UP.
Total	450	

3.5.2 DNA extraction

Total genomic DNA was extracted from fresh leaves using a modified CTAB -DNA extraction procedure developed by Gawel and Jarret (1991). Leaf tissues of 1-2 g were frozen in liquid nitrogen and ground in a mortar. To facilitate the grinding procedure, liquid nitrogen in mortar was used. The powder was transferred into 7 ml of prewarmed (60°C) isolation buffer [2%w/v CTAB (Cetyltrimethylammonium bromide)], 1.4 M NaCl, 20 mM EDTA, 100 mM Tris- HCl (pH 8.0), 0.2% B-mercaptoethanol] in a capped polypropylene tube where 1 ml of 0.2% β-mercaptoethanol was added. CTAB is a cationic detergent, which solubilizes membranes, and forms a complex with DNA, while β- mercaptoethanol plays an essential role in inhibiting polyphenole oxidation. The polypropylene tubes with the above mixture were incubated for 30-60 minutes at 60°C in an oven with constant gentle shaking. One volume (7ml) of chloroform-isoamyl alcohol (24:1, V/V) was added, and the tubes extracted for ten minutes on a rotary shaker or by hand. Mixing was done gently but thoroughly enough to ensure emulsification of the phases. Denatured protein and most carbohydrates were removed in this step by using fresh chloroform-isoamyl alcohol and centrifuged for 10 min (5000xg, room temperature).

Heat-treated RNAse A solution [10 mg/ml RNase A in 10 mM Tris- HCl, 15 mM NaCl (pH 7.5) boiled for 15 min., cooled to room temperature and stored at - 20° C] was added to a final concentration of 100µg/ml, mixed and incubated at room temperature for 30 minutes. Ice cold 100% isopropanol (4.2 ml) was added to this tube and mixed gently, but thoroughly by inverting the tube several times. The centrifugation procedure was repeated (5000xg, 4°C) for 10 min with 10 ml washing solution (76% ethanol, 10 mM ammonium acetate) to collect the DNA pellet. The tubes containing DNA pellet were dried by inverting and draining on a paper towel for 10-15 min, but the pellet should not be too dry. Then the pellet

was dissolved in TE buffer [10 mM Tris- HCl, 1 mM EDTA (pH 8.0)] over-night (4°C) without agitation. Ammonium acetate solution (7.5 ml) was added, mixed, chilled on ice for 15 min, and centrifuged for 30 min (10,000xg, 4°C).

Two vol. of 96% ethanol was added to supernatant tubes, mixed by inversion and then incubated for 1 hour at -20°C. Centrifugation was done twice for 10 min (5000xg, 4°C) to wash the pellet. The DNA pellet was resuspended in TE buffer (10 mM Tris, 1mM EDTA, PH 8.0) and the concentration was determined using a spectrophotometer (BECKMAN DU7500). The concentration of each sample was also estimated electrophoretically on an agrose gel with known standards by comparing band intensities by UV absorption. The isolated genomic DNA was stored at -20°C.

3.5.3 Quantification of DNA

DNA to be analyzed (up to 5 μg/track) was mixed with 1/5 volume of loading buffer and electrophoresed on an agrose gel containing 1x TBE buffer [90 mM Tris-borate, 1 mM EDTA (pH 8.0)]. Ethidium bromide (1μg/ml) was included in the gel mix. Molecular weight markers (Gibco-BRL/ Promega) were used as DNA size standards. The DNA bands were visualized under UV, illuvination and photographed.

For quantification of the amount of DNA, readings in a spectrophotometer were taken at 260nm and 280nm, which allowed calculation of the concentration of nucleic acids in the sample. An OD of 1 corresponds to approximately 50μg/ml for double-stranded DNA. The ratio between the reading at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid.

$$DNA\ conc.(\mu g/\mu l) = (OD_{260} x 100(dilution\ factor) \times 50 \mu g/ml)/1000$$

3.5.4 RAPD Primers

The reproducibility of random amplification was determined by evaluating the influence of DNA and $MgCl_2$ concentration across a range of primers. Ten primers of commercially available random oligonucleotide sequences from OPERON Technologies, USA, were screened in different PCR reactions with the investigated plant DNA fragments, for their ability to provide a suitable banding pattern (Table 3.5). Finally, four primers producing the most clear and intense bands were chosen for the RAPD analysis, namely OPA-3, OPA-5, OPA-7 and OPA-9.

Oligonucleotides were used at a concentration of 20 pmol in PCR. This was sufficient for at least 30 cycles of amplification. The stock solutions were always 100 pmol.

Table 3.5: Random oligonucleotide sequences (Primers) for preliminary evaluation

No.	Primer Sequences
OPA-1	G T G C G T A T G G
OPA-2	T C A C G T C C A C
OPA-3	G G G A G A G T C A
OPA-4	G A C A G A C A G A
OPA-5	G A A C G G A C T C
OPA-6	C G C T G T C C T T
OPA-7	G T G A T C G C A G
OPA-8	C T C T C C G C C A
OPA-9	G A C C A G T A C G
OPA-10	G A A C C G G G T G

(OPERON Technologies, USA)

3.5.5 Random Amplified Polymorphic DNA marker for The Analysis of Mutants Method of RAPD-PCR

The PCR reaction was carried out in sterile 0.5µl thin-walled PCR tubes (USA Scientific Plastics). The PCR reaction mix was always prepared as a master mix and aliquoted to each tube prior to addition of the template DNA. The PCR reaction mixture is shown in Table 3.6.

DNA amplification reactions were performed in volumes of 10 µL containing: 10 X reaction buffer (10 mM Tris-HCl pH 8.3; 1.5 mM $MgCl_2$; 50 mM KCl) (Promega). 1.5 mM $MgCl_2$ (25mg/ml) (Promega); 200 µM of each dATP, dCTP, dGTP, and dTTP (Promega). 20 Pmol primer; 50 ng genomic DNA; 1.0 unit of Taq DNA polymerase (Promega) and overlaid with a drop of heavy mineral oil. The PCR was always carried out with two controls, a positive control, and a negative control containing only mixture without DNA template.

Amplification was performed in a HYBAID OmniGene apparatus with an initial denaturation temperature of 96°C for 10 seconds, 35°C for 30 seconds followed by 72°C for 5 minutes and 4°C hold. Then the PCR products were stored at –20°C before use.

The amplification products were separated electrophoretically in 1.0-1.5 % (w/v) agarose gels in 1 X TBE buffer, 70-80 V for a 20x25 cm submerged gel. The gel was then stained with ethidium bromide (10 mg/ml) by immersion for 20-30 minutes. The gel was washed in sterile distilled water for 10 min in a rotary shaker. The amplification products were visualized under UV light (402 nm) and photographed using 667 Polaroid film (Polaroid UK- Ltd, Hertfordshire, England). To improve the precision of the evaluation of the

amplification product patterns, the photographs of the electrophorograms were enlarged to 20x15 cm.

Table 3.6: PCR reaction mixture (reagent) used in RAPD method

Materials	Amount (µl)	Final concentration
10X TBE reaction buffer	1.0	10mM Tris-HCl, pH 8.3 -50 mM KCl -1.5 mM $MgCl_2$ -0.1% Triton X-100
mM $MgCl_2$ *	1.2	
0.5 mM dATP, 0.5 dTTP, 0.5mM dCTP,0.5mM dGTP	0.5	0.1mM each dNTP**
3 µM primer solution	1.0	0.36 µM
Genomic DNA	1.0	50 ng / reaction
Taq DNA polymerase (0.75 units/µl)	1.0	
Sterile H_2O	4.3	

* *$MgCl_2$ (2.5-4.0 mM)*

* * *dNTPs were used at saturating concentration (200 u M for each). Stock solution of dNTPs (50 mM) should be adjusted to pH (7.0) with 1N NaOH to ensure that the pH of the final reaction does not fall below 7.1.*

RAPD bands were scored from photographs as '+' (present) or '–'(absent) of amplification products considered to be relevant, and the data obtained were entered into a binary data matrix. In order to quantify pairwise similarity, a similarity index "S" was calculated from band-sharing data of each of fingerprints according to the formula by (Nei and Li, 1979)

$$S = 2n_{xy} / (n_x \times n_y)$$

Where n_x and n_y represents the total number of bands present in lanes a and b, and n_{xy} is the number of bands which are shared by both lanes.

The degree of polymorphism $(DP) = (1\text{-}BS) \times 100$

Where BS was the 'band sharing'.

3.6 Detection of Dwarfism by using Gibberellic Acid (GA_3)

A particularly valuable approach in such studies will be modulation of plant growth rate by such tools as hormone application and genetic modification. It is important to develop simple assays to detect heritable changes, mainly dwarfism, in early stages of *in vitro* development. Dwarfs are visibly detected *in vitro* and can only be observed in the later stages of nursery production when plants are taller than 20 cm and growing vigorously (Smith and Hamill, 1993). However, the morphological-based characterization of dwarf somaclones during *in vitro* culture is limited. When plants are grown under stress or less than ideal conditions, dwarf off-types often escape detection at high frequencies, hence other methods of dwarf detection are desirable.

The potential of using gibberellic acid (GA_3) to detect dwarf off-types or variants was conducted in this experiment according to Damasco *et al.* (1996a).

3.6.1 The Growth Cabinet experiment

The experiment was conducted in a growth cabinet (HOTECH, MODEL: 624) between 27 April 1998 to 5 Aug. 1998. The materials used include:

Non-irradiated Pisang Berangan (control), irradiated Pisang Berangan, and Pisang Serendah (control of dwarf plants). The total numbers of plants used are given in Table 3.7.

(i) During *in-vitro* culture

Leaves and shoots of single shoot plantlets, were trimmed and then placed on solid (0.7% agar) MS basal medium containing filter-sterilized GA_3 at different concentrations: 0, 29,

and 59 μmol/l. Shoot cultures were incubated in the growth cabinet at 26±1°C for 14 hours photoperiod and photosynthetic photon flux density (PPFD) of 17-35 μmol/m^2.s for a further 30 days and then length of leaf sheath (base of shoot to start of leaf lamina) was measured.

Table 3.7: The Number of plants used for different GA_3 treatment under Growth Cabinet experiment

Treatment	In- Vitro Stage						Deflasking Stage		
	Irradiated Pisang Berangan (40Gy)		Pisang Berangan (Control)		Non-irrad. Serendah (Dwarf, (control)		Irradiated Pisang Berangan (40Gy)	Non-irrad. Pisang Berangan (control)	Non-irrad Serendah (Dwarf, control)
GA_3* (μ mol/L)	29	59	29	59	29	59	289	289	289
Number of plants	20	20	20	20	20	20	14	14	14

*Gibberellic acid (90% pure, Sigma, St Louis, Missouri, U.S.A) was applied at two stages of growth.

(ii) At deflasking stage

Plantlets with well developed roots were planted in pots (10 cm diameter) containing steam-sterilized sand and peat (2:1v/v) mixture. The plants were immediately sprayed with GA3 (289 μmol /L) solution or distilled water (control). Each plant received 5 ml, applied evenly as a spray (droplet size about 200 μmol) and then plants were covered with clear plastic bag to help their establishment in soil, and placed in the growth cabinet (28 / 25 ± 1°C day / night; 14 h photoperiod; PPFD about 200-220 μmol /m^2.s). Leaf sheath of each plant was taken after two weeks (leaf 1) and 4 weeks (leaf II).

3.6.2 The nursery experiment

A similar set of experiments was conducted to evaluate GA_3 effects on plants at *in vitro* stage and deflasking stage in an open nursery (University farm) used for normal hardening process (24 April 1999 – 22 Jul. 1999). The treatments and sample size used are given in Table 3.8.

3.6.3 The green house experiment

Finally GA_3 was applied to non-irradiated and irradiated plantlets of Pisang Berangan at the deflasking stage at 289 µmol /L. The plants were then grown under greenhouse conditions in Rimba Ilmu, U.M. The number of plantlets used was 100 each for irradiated and non-irradiated plants.

Table 3.8: The number of plants used for different GA_3 treatments under nursery Environment

Growth Stage	***In-vitro* Stage**								**Deflasking Stage**			
Treatment	Irradiated Berangan (40Gy)			Non-irrad. Berangan (Control)			Non-irrad. Serendah (Dwarf, control)		Irradiated Berangan (40Gy)	Non-irrad. Berangan (Control)		Non-irrad Serendah (Dwarf, control)
GA_3 (µ mol/L)	0	29	59	0	29	59	29	59	289	0	289	289
Number of Plants	10	65	80	10	20	20	60	60	66	10	24	50

3.7 Screening for Tolerance to Fusarium wilt Disease by "double-tray" system and field evaluation

Fusarium wilt (Panama disease) caused by soil-borne (inhibiting) fungus, *Fusarium oxysporum* f. sp. *cubense* (FOC), which is the major problem in banana production. An approach to overcome *Fusarium* problem is to obtain plants is tolerant to *Fusarium* wilt disease through somaclonal variation using *in vitro* culture and irradiation treatment with gamma ray. These techniques may shorten the time of breeding or improvement programs to obtain new cultivars tolerant to *Fusarium* wilt. All breeding procedures can be summarized as: the introduction or selection of variation followed by selection for useful plant types.

3.7.1 Plant materials

Shoot-tip meristem pieces of Pisang Berangan were used for gamma irradiation at 40 Gy and 60 Gy to produce mutated plants and propagated in MS modified medium for three subcultures until M_1V_4. The plantlets were hardened for 4 weeks before being used for screening of mutants tolerant to *Fusarium* wilt disease. The number of plants produced for evaluations are given in Table 3.9. These plants were grown in the green house until they attained the desired size (age of 45 - 60 days).

The experiment was conducted on 5th Feb, 1999. These plantlets were carefully prepared for inoculation by immersion in the appropriate conidial suspension of *Fusarium oxysporum* f. sp.*cubense* and replanted in the tray for maintenance and observation in the greenhouse. Plantlets were watered using tap water and foliar fertilizer "GROFAS" twice weekly at the rate of 10 gm in 4.5 liters of water.

Table 3.9: The number of plantlets produced for the screening of Foc resistance by 'Double-tray' experiment

Treatment gamma-rays	No. of meristem Pieces	No. of plantlets Produced at M_1V_4	Control	
			Berangan	Novaria
40 Gy	300	500	-	-
60 Gy	200	100	-	-
0	120	200	200	200

3.7.2 Isolates and inoculum preparation

Infected plants of Pisang Berangan and Novaria growing in UP. Bhd's were cut into half longitudinally and clean corm tissues were cut adjacent to the highly infected tissues. Infected tissues were immersed into 5% Chlorox solution in a Macarteny bottle. Plant tissues were removed at different time intervals and dried on sterilized filter paper. Small pieces (1-2 mm^2) cut from infected rhizome tissues were transferred to newly prepared Potato Dextrose Agar (PDA) (Appendix–B) in Petri dishes under the light at 25°C. After pathogen has growon on the surface of the PDA, minute parts of mycelia were cut by using small needles and then introduced into other fresh PDA plates. To obtain pure culture of FOC, a very small plug of mycelia growing on PDA was cut from a 3 day-old culture drawn at the tip of a needle and then introduced in 10ml sterile distilled water in small 30 ml bottles. The bottles were shaken and the water was poured on the surface of freshly prepared sterilized Water Agar (Difco Bacto agar, 15-20 g dissolved in 1L distilled water) in Petri dishes (Booth, 1977; Tousson and Nelson, 1968).

The culture was incubated at room temperature for 16-24 hrs. The tip of a single growing mycelium was selected and cut under the light microscope and transferred by the needle to

another fresh plate of PDA and left to grow for 10 days. About 4-5 plugs (3-4 mm diam) from the edge of PDA containing mycelium of FOC race 4 were cut from the PDA and grown in Armstrong's liquid medium (Brake *et al.*, 1995) prepared in 500-ml bottles, which markedly increased the production of microconidia. Armstrong's liquid medium contained: Sucrose 20g/L; $MgSO_4$ 400 mg; KCl 1.6g/l ; KH_2PO_4 1.1 g/l; Ca (NO_3) 25.9 g/L; $FeCl_3$ 0.2 mg/ml; Zn SO_4 0.2 mg/ml; Mn SO_4 0.2 mg/ml; and sterile distilled water. The flasks were incubated at room temperature under a fluorescent light for 7 days and manually shaken twice daily to enhance growth of mycelium.

Following the protocol of Sun and Su (1984), harvesting of inoculum was carried out by filtering the cultures through two layers of muslin (0.2 μm) to remove the bulk of the mycelium. The concentration adjustment of the filtrated conidial suspension was enumerated by microsyringe method (Ko *et al.* 1973) using a Hemocytometer. The number of conidia determined the concentration of conidial suspension according to Singleton, *et al.* (1992). Two fold dilutions (1/1 to 1/9) were prepared for race 4 of the fungus from $3x10^6$ to $3x10^4$. The concentration of inoculum in the 1/1 dilution was $1.3x10^5$ microconidia/g and the other one was $1.3x10^4$ microconidia/g.

3.7.3 Inoculation procedure

Eight weeks after potting, the roots of the banana plants (Berangan- mutated and non-mutated and Novaria- control) were washed in running tap water and then immersed for 2 hours in the conidial suspension ($3x10^4$ spores ml^{-1}). Inoculated plants were then re-potted in sterile soil (4 sand: 1 clay) for 'Double-tray' system, in tray measuring 43x29x9 cm, which fitted snugly into another larger outer tray measuring 46x31x20 cm. Each tray contained 30 plants. The plants were watered by tap water every other day plus GROFAS

foliar fertilizer once a week. Four weeks later, plants were reinoculated by the addition of 10 ml of a conidial suspension ($3x10^4$ spores ml^{-1}) to each pot followed by cutting of the roots *in situ*. After an additional four weeks plants were excised for foliage symptoms and their corms were also examined for the extent of vascular discolouration.

Table 3.10: The age and number of plantlets inoculated with conidial suspension of FOC.

Inoculation date		Age of the plants	Inoculated Berangan plants(AAA)		Control# (AAA)	
			B(M)*	B(N)*	B(N)#	Novaria
1.	2.5.1999	45days	150	50	25	25
2.	1.6.1999	50days	165	50	25	25
3.	5.7.1999	56days	147	50	25	25
4.	11.9.1999	56days	138	50	25	25
Total			**600**	**200**	**100**	**100**

**B(M) = Berangan Mutants*
** B(N) = Berangan Normal (non-irradiated)*
 # Control = non-irradiated & non-inoculated (-) and Novaria inoculated (+ con)

3.7.4 Assessment of disease symptoms

Leaf symptoms on inoculated plants were observed within 10 to 14 days. Daily observations and visual evaluation on the number of wilted plants and changes in leaves discolouration were recorded up to 1 month after inoculation. Final evaluation on the 5th week was based on the percentage of leaves showing chlorosis and streaks (external examination) and the percentage of rhizome discoloration (internal examination) after longitudinal sectioning. Furthermore, after corm examination, any plant that had very little or no invasion of the corm (<10%) with no symptoms on the pseudostem and leaves were selected and re-planted.

Four weeks later these plants were re-examined. At this stage any plants with little or no invasion of the corm and roots and with no external symptoms were considered tolerant to the fungus. Finally these plants tolerant to the fungus were potted in 90-litre containers to allow for sucker production. The suckers were removed and screened under field conditions in the *Fusarium* hot-spot.

About 12 weeks after inoculation, the disease symptoms of each plant is recorded according to (Waite, 1997) scale as follows (Table 3.11):

Table 3.11: Types and disease symptoms examined in double tray experiment

Types	Symptoms description
Leaf symptoms	Includes yellowing and leaf rosetting.
Pseudostem	Discolouration of stem
Corm (rhizome)	Vascular discolouration %
	10% = light invasion
	10-30% = medium invasion
	> 30% = heavy invasion

Symptoms of leaf and rhizome discolouration were evaluated according to Brake *et al.* (1995) by using different scales for (LSI) and (RDI) as presented in Table 3.12.

Table 3.12: Scales of leaf symptom index (LSI) and rhizome discolouration index (RDI)

Scale	Symptoms of (LSI)	Symptoms of (RDI)
1	No streaking or yellowing of leaves. Plant appears healthy.	No discolouration of tissue of stellar region of rhizome or surrounding tissue.
2	Slight streaking and/or yellowing of lower leaves.	No discolouration of tissue of stellar region of rhizome or surrounding tissue.
3	Streaking and/or yellowing of most of the lower leaves. Discolouration of younger leaves beginning to appear	Trace to 5% of stellar region discoloured.
4	Extensive streaking and/or yellowing on most or all of leaves.	6-20% of stellar region discoloured.
5	Dead plant	21-50% of stellar region discoloured.
6		More than 50% of stellar region discoloured.
7		Discolouration of the entire rhizome stele
8		Dead plant

3.7.5 Field screening for tolerance to FOC disease in the *Fusarium* 'Hot-spot' (Screening of plants derived from gamma irradiated meristems)

A total of 1798 plants derived from gamma-irradiation at 20 Gy (890 plants) and 30 Gy (908 plants) were planted in *Fusarium* free area in the United Plantation Bhd in Nov. 1997 for the evaluation of agronomic traits. Suckers were collected from normal and 'mutant' plants and transplanted to the *Fusarium* 'Hot-spot' in United Plantation on 3 occasions (9 Jun, 1998; 14 Jul 1998; 9 Aug. 1999). Two batches of 133 and 156 plants for 20 Gy and 30 Gy together with 100 control plants were field-planted in a row system with planting distance at 1mx1m. The third and last batch on 9Aug, 1999 was mutated plants at 40 Gy from the third batch,

The third and last batch on 9Aug, 1999 was mutated plants at 40 Gy from the third batch, similar to those used in double tray experiments in the nursery. Survival census was carried out at 3-monthly intervals. After 3-4 months, the surviving plants were dug up and rhizomes were examined for symptoms of *Fusarium* wilt. Those free of infection were again multiplied for confirmation tests.

3.8 Statistical Analysis

In numerical identification, analysis of variance (ANOVA) of each character was compared. Means and coefficients of variation were also calculated. Statistical analysis used the SPSS (Nourisis, 1990) Base 9.0, which is a comprehensive system for analyzing data. To detect differences between mutants at different doses of irradiation and estimation of nuclear DNA content (FCM) (ANOVA- one way analysis of variance) was assessed. In addition, frequency distribution for characters at flowering and harvest stages and Duncan's Multiple Range Test (DMRT) of the means for groups in homogeneous subsets were analyzed by using SPSS 9.0. . Moreover, a 2x2 factorial experiment was carried out to determine the effects of GA_3 on mutant Berangan plants and dwarf Serendah.

The standardization procedure and Partec was used in FCM analysis. The other computer analysis methods were SPSS (Ward's, 1963) method; Hierarchical cluster analysis using Average Linkage between groups, RAPDistance Software Package (Version 1.04) for dendrograms, dissimilarity coefficient and patristic distance for amplification products (phylogenetic tree).