CHAPTER FIVE

DISCUSSION

5.1 General Introduction

Pisang Berangan has good fruit quality, colour, texture, size and shelf-life in addition to its ease of ripening without cold treatment. The plants are however, relatively tall with moderate yield and very susceptible to a number of diseases particularly *Fusarium* wilt cause by *Fusarium oxysporum* f. sp. *cubense*. The *Fusarium* wilt (Panama disease) disease has caused severe economic losses to banana industry. As a soil borne disease, there is no effective chemical control for *Fusarium* wilt except for the use of disease resistant varieties.

As Pisang Berangan are sterile triploid bananas, genetic recombination in parthenocarpic triploids is extremely difficult due to high sterility and meiotic irregularities (Novak, 1992). Therefore, complementary techniques such as tissue culture and mutagenesis are of great potential. For bananas induced mutations become an attractive means to generate genetic variability.

5.2 *In vitro* propagation of Pisang Berangan

Healthy suckers were selected from true to type mother plant with vigorous growth, good fruit characteristics and free of virus at United Plantations Bhd. The shoot-tip explants of Pisang Berangan cultured in modified MS medium supplemented with coconut water and benzyaminopurine (BAP) at 4.5 mg/L induced bud and shoot regeneration. The occasional appearance of shoot primordia between the leaf sheaths was observed, and no green shoots were recorded at this stage. Similar observations were recorded by Ho *et al.* (1996) who

reported the emergence of adventitious buds only after two months in culture. Anatomical studies showed two kinds of bud proliferation systems operating during the micropropagation of banana, one at the axillary position of the explant and the other on the surface of the corm tissue as an adventitious bud (Novak *et al.* 1986; Hamilton 1965; Ma and Shii 1974).

Generally, rapid shoot proliferation was successfully established on this medium showing proliferation potential of efficiencies of the previous work on micropropagation of bananas (Ma and Shii, 1972; De Guzman *et al.* 1982; Cronauer and Krikorian 1984; Hwang *et al.* 1984; Vuylsteke and De Langhe 1985 and Wargantinar *et al.* 1997). For practical purposes the proliferation of shoots rather than shoot primordia was given higher consideration because these shoots were confirmed to form plantlets while the shoot primordia may or may not form viable shoots. But to include the shoot primordia and to assume that they would definitely result in potential plants may be a misrepresentation of results. The occurrence of shoot primordia or nodules were also reported by Vuylsteke and De Langhe (1985); Novak *et al.* (1986). However, De Guzman *et al.* (1982) also reported that the proliferation mass of shoot primordia showed different growth potentials suggesting that not all produced plantlets.

The problems encountered at culture initiation included apical dominance and blackening of explants. The apical dominance could be overcome by cutting the upper parts of meristems and increasing the BAP concentration. Tissue blackening occurs through the action of copper-containing oxidase enzymes such as polyphenoloxidases and tyrosinase, which are released or synthesized, when tissues are wounded (George and Sherrington, 1984). Subsequently, the medium also turned black or dark in colour. Blackening of tissues and media may be minimized by sub-culturing to new medium regularly. After 2-3 subcultures,

blackening and apical dominance disappeared and explants continued their growth normally.

High proliferation rate was observed between the third to sixth subcultures. Few buds were produced in the initial cultures and first two subcultures but considerably increased in later subcultures. The proliferation rate achieved for first two subcultures was only 3-7 shoots/explant while for subculture 3 to 4 the proliferation rate was 8-20 shoot/explant. This finding is in agreement with De Guzman *et al.* (1980) who reported few shoot production in the first two subcultures for Pisang Berangan (AAA) and different Cavendish clones (AAA). The plantlets regenerated from cell shoot-tip cultures provided meristem pieces for mutation induction.

In addition to this high proliferation, there was variation among this cultivar in rate of multiplication. However, it is possible that the difference in the rate of multiplication may reflect variation in the growth rate or some other characters of the cultivar.

5.3 Exploitation of *in vitro* mutation for banana improvement by using gamma ray

In vitro shoot-tip culture had been proposed and routinely used by many workers in mutation induction for bananas (Mak, *et al.* 1995; Novak *et al.* 1987; Donini and Micke 1984; and De Guzman *et al.* 1980; 1982). The advantages of the system included rapid propagation of the explants prior to mutagenic treatment, and the recovery of periclinal and/or homohistonic structures through repeated subculture by the formation of axillary and/or adventitious shoots from the treated apex.

Prior to any bulk mutagenic treatment, the optimal doses of mutagens need to be determined due to the clone × mutagen dose/concentration interactions (Novak *et al.* 1990; Smith *et al.* 1990). Moreover, the optimal dose was defined as that which would result in a high mutation frequency but not one that would severely inhibit growth. Generally, a radiation dose that reduces the growth (the rate of shoot/bud proliferation in the present study) to 50% of the control (i.e. LD_{50}) is considered suitable for mutagenic treatment (Lacey and Campbell 1982; Novak *et al.* 1986; 1990 and Omar *et al.* 1989). Therefore, for mutation induction of *in vitro* cultured shoot-tip meristems of Pisang Berangan by gamma irradiation, the LD_{50} dose of about 38 Gy (Mak, *et al.* 1995) and 30 to 45 Gy by Novak, *et al.* (1990), were used as guide for the present study. In this study the *in vitro* cultured shoot-tip meristems of Pisang Berangan cv. Intan were irradiated by gamma-ray at different doses (20 Gy, 30 Gy, 40 Gy and 60 Gy) to obtain large number of variation and the maximum benefit of gamma ray at wide range for the improvement of this cultivar.

Gamma-irradiated meristem pieces were cultured *in vitro* in MS medium (M_1V_0) and subcultured every 3-4 weeks until M_1V_4. Within 2 to 3 weeks after irradiation the propagules showed browning and blackening, which disappeared after two sub-cultures. After 4 – 6 weeks of gamma-irradiation, the meristem pieces resumed growth. These problems were due to the ionization process, which affected plant growth. A simplified illustration of what happens after radiation treatment of living cells is given in Figure 5.1 (Lawrence, 1971). When radiation passes through plant tissue, interaction with DNA molecules as well as all other molecules present in the system may occur. Van Harten, (1998) revealed that the majority of gamma ray-induced mutations are caused by chromosomal breaks, which often lead to deletion.

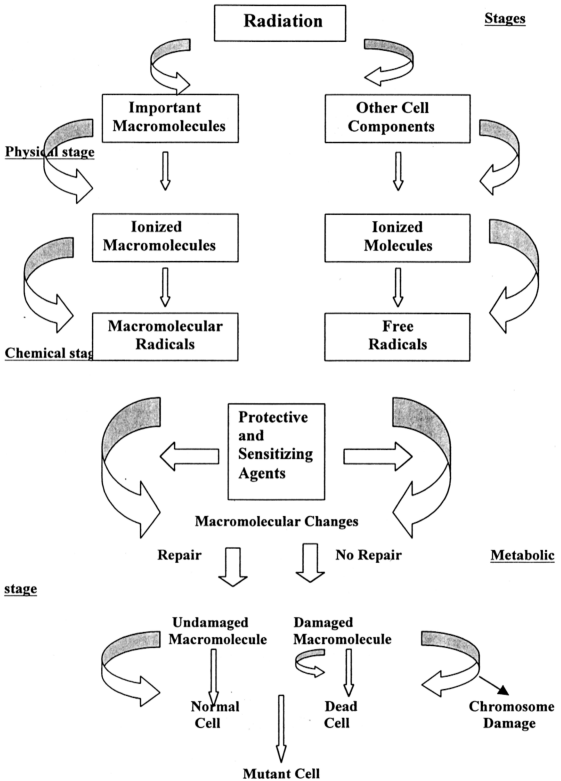

Figure 5.1. : The development of radiation damage in plant cells (Slightly adapted from Lawrence, 1971)

The gamma-irradiated explants showed variable response. Control plants showed earlier initiation while the latest was for explants treated at high dose of 60 Gy. This indicates that high dose of irradiation could cause cell damage and inhibit growth. Similar response was reported by Mak and Ho (1997). Radiation treatment had its greatest damaging effect on the cells located towards the apical meristem with less damage occurring to the cells located away from the meristem. This observation was also reported by Yang *et al.* (1990). Moreover, gamma irradiation caused high mortality rate. Explants at 40 Gy-treatment showed mortality rates ranging from 4.4 to 46% for 1st batch while for the other three batches the rates ranged from 0.3 to 19%. On the other hand, a higher mortality rate was observed for 60 Gy-treatments, ranging from 38 to 69% for the first batch, and 4.4 to 25% for the 2nd batch. Also, explants frequently turned brown or black shortly after isolation and then died. Growth inhibition is most severe in species that naturally contain high levels of tannins or other hydroxyphenols as suggested by George and Sherrington (1984). In addition, growth of explants can be limited by toxic metabolites even in the absence of blackening. If explants did not show any sign of growth after 3-4 weeks, it would be beneficial to transfer them to fresh medium. Transfer became imperative when the medium turned black. Subcultures of explants to fresh medium were done at 10-15 days intervals for all batches to overcome this problem. Browning or blackening is particularly apparent on solid medium where exudates are trapped by the agar and become concentrated in the vicinity of the explant. Broome and Zimmerman (1978) working on blackberry found that tissue blackening could be overcome by moving explants to fresh medium at short time interval. Muriithi *et al.* (1982) also suggested weekly intervals to minimize possible inhibitory effects of phenolic oxidation.

At the early stages of plant growth, at high doses of gamma-irradiation (60 Gy), abnormal plant development such as formation of compact leaf rosettes was observed. In the first and

second subcultures more than 50% reduction in shoot production was observed. Moreover, increasing doses also caused a decrease in regeneration capacity. At 60 Gy, stunted growth and death often occurred. The number of buds/shoot produced per explant decreased with an increase of irradiation dose. The 4th batch of irradiated material differed from the other 3 batches, with a delay on growth initiation, possibly due to the longer exposure of meristems to gamma ray. This caused poor growth and low bud/shoot proliferation in addition to the low survival rate. The multiplication rate was low for the first two subcultures but gradually increased after M_1V_4. This indicates that ionizing radiation affected some cells while the others resumed their growth after 2 subcultures.

The adventitious origin of shoot buds from the superficial cells of the rhizome cultured *in vitro* reveals the possibility that the entire shoots can be raised from a single somatic cell. Whereas, a single mutational event is normally restricted to one cell only. It has been assumed that a mutated cell within non-mutated tissue has the same chance of survival and an equal rate of division as normal cells. Similarly, Van Harten (1998) reported that most meristem pieces contain several initial cells in their growing points; however, a distinct mutation would only occur in one of them. If, however, two initial cells survive, one of them containing a mutant gene, a chimeric M_1 plant arises. The generally regularity is that the higher the number of surviving initial cells, the smaller the mutant sector of the M_1 plant, the lower the proportion of mutant plants in the respective generation.

5.3.1 Post-irradiation recovery and radio-sensitivity

Preliminary tests yielded the necessary information of radiosensitivity of the explant material. Mutation frequency increases with an increase in dosage and dose rate. However, survival and capacity to generate, would decrease with increasing mutagenic doses and

hence reduced the overall efficiency. This agrees very well with Bhagwat and Duncan (1998) working on mutation breeding of the banana Highgate (AAA). Generally, a LD_{50} dose which caused 50% death or growth retardation of plants is adopted. The effect of increasing doses of radiation on regeneration rate of adventitious buds appeared in the low survival rate of explants. On the other hand the slight effect of low doses on plant growth was observed but according to other work in mutation breeding (Novak *et al.* 1990) 20 Gy is not considered as a low dose in banana. However, the 50% growth reduction occurred between 30-40 Gy as reflected by the number of shoots produced, agreed very well with that reported by Novak *et al.* (1990) that gamma- dose of 35-40 Gy was suitable for the triploids (AAA). Furthermore, Mak *et al.* (1997) recorded similar results with Pisang Berangan (AAA).

In the present study, contamination rate recorded four weeks after irradiation was high, probably due to broken parafilm during transportation from the laboratory to MINT. The post irradiation recovery estimated as survival rate of uncontaminated explants was greatly reduced, particularly for 60 Gy where survival rate continued to decrease until the last subculture as shown in the number of buds/shoots (Figure 4.2). Moreover, the blackening of explant tissues was particularly severe for explants irradiated at 60 Gy, which reduced the survival rate. Multiplication rate of 40 Gy treatments also showed significant difference compared to the non-irradiated plants. The mutagenic treatment caused physical damage or injury to the plants often resulting in significant reduction in germination, plantlets growth and fertility of the first generation (M_1).

Many factors affect the mutation rate and one of them was the mutagen dose as reflected by the findings of previous researches such as Broertjes (1968). On the other hand, Novak and Micke (1988) and Novak *et al.* (1990) also found the same phenomenon for their studies on

several banana genotypes whereby an increase of gamma irradiation from 15 Gy to 40 Gy was a corresponding increase in mutation frequency. Beside the mutagen dose, the banana genotype was also found to influence the mutation rate. However, accessions within a genotype did not show any differences as observed for Pisang Berangan in this study. Radiosensitivity varies between plants and depends mainly on the nuclear volume, the greater the DNA contents, the more sensitive; and the ploidy level, the higher it is, the less radiosensitive (Broertjes & Van Harten, 1978). However, Novak *et al.* (1990) reported that diploid clones were most sensitive to gamma irradiation while the tetraploids expressed the lowest level of irradiation damage. Therefore, Pisang Berangan which is a triploid (AAA), exhibited moderate sensitivity to gamma irradiation.

After the application of mutagenic irradiation on shoot-tip meristems, the M_1V_4 plants produced chimeras. I*n vitro* multiplication for three cycles may help to minimize chimeric tissues in Pisang Berangan plants. However, the chances of recovering the induced mutations from these are relatively low. Chimerism is one of the major technical obstacles that have to be dealt within mutation breeding of vegetatively propagated crops (Van Harten, 1998). This problem may be dealt with if plant regeneration from single cells is available. On the other hand, meristem pieces used in this experiment for mutagenic treatment were multicellular tissues. Chimerism automatically occurred. Therefore, an important method for the commercial plant breeders is the adventitious bud technique, which makes use of the phenomenon that (the apices of) adventitious buds, i.e. on detached leaves ultimately may originate from only one cell. Similar finding were reported by Rusli *et al.* (1998) working on the effect of X-irradiation on the adventitious buds regenerating from *in vitro* explants. Also FAO/IAEA (1995) reported that an *in vitro* system of single cell origin for the induction of desirable genetic variation is preferred to overcome or minimize chimerism, and to obtain homohistont shoots. Therefore, it is recommended to investigate

the possibility of using adventitious shoots. Mutation is a single-cell event and solid homohistont tissue is derived from a mutant cell initial that carries a particular mutation.

The technique of repeated *in-vitro* subculture of irradiated meristems may provide good opportunities for cells carrying mutations to reach phenotypic expression. There was high frequency of solid plant mutants obtained after explant irradiation as reported by Novak *et al.* (1989). However, not all variant plants could be eliminated. Many phenotypic changes, about 5.0 – 33.0 % in characteristics such as plant stature, aberrant leaf morphology and pigmentation were observed in some of the M_1V_4 plants. Similar findings were observed by Novak *et al.* (1990).

5.3.2 Mutant Evaluation

The tissue-cultured plantlets (irradiated and non-irradiated) were acclimatized and maintained in the nursery for 10 weeks before transplanting to the field. Generally, the micropropagated banana plants have high survival rate in the field of about 90%. At high doses of irradiation, too many mutational events per cell may be induced, with increased risk that a favourable mutation is accompanied by one or more unfavourable genetic changes. However, in vegetatively propagated crops (bananas), it is impossible to separate favourable from unfavourable mutations by cross-breeding mutants among each other or by back-crossing with the original material. Therefore, the evaluation of the mutants is very difficult genetically unless the use of other techniques such as Flow cytometry for detection of ploidy level and DNA content or RAPD analysis and gibberellic acid for detection of dwarfism. But phenotypically the evaluation of mutants can be possible depending upon certain characteristics when compared with control plants.

Applied mutagenesis is particularly important for the sterile triploid Pisang Berangan. A mutation frequency of 31-37% was achieved, including both morphological and agronomical traits. Novak *et al.* (1990) recorded 7- 42% mutation frequency in their studies on several banana genotypes treated with 30 Gy gamma rays, while Ponce & Orellana (1994) obtained 25% for the Cavendish types treated with 40 Gy gamma rays.

Wide spectrums of variations including both the morphological and agronomically important characteristics were induced by gamma-irradiation (Tables 4.3 and 4.4). These variants include leaves, pseudostem, plant stature, shooting habit, bunch and finger characteristics. The leaf characters involve uneven lamina, crinkling of lamina, rough surface, variegation of leaves (red sectors and petiole edges with red colour), bending of young leaves, twisting of midrib of older leaves, compact and erect leaves and petiole margins and base (Table 4.5). The pseudostem characteristics include colouration, presence or absence of powder and splitting of pseudostem. Bunch characters include fused fingers (>50%) and gap within the bunch at 1st, 2nd and 3rd batches. Moreover, mutation in plant stature (stunted growth & dwarfism) appeared at all doses of irradiated-plants for all batches. Earliness to flowering was observed at 20, 30 and 40 Gy while at 60 Gy this character was not observed.

The effect of gamma-irradiation on explants was observed at the *in vitro* stage, nursery stage and field grown plants. The distributions of these mutagenic changes were quite different at each dose and between different batches. The majority of variants were observed in the first and 2nd batches of irradiation. However, these batches include higher doses (40 and 60 Gy). The total number of mutant variants at 40 & 60Gy's (batch 1 and 2) were 25%, 32% and 37% at *in vitro* stage, nursery and field stages respectively (Table 4.3). The nursery plants showed variants of 17.7% and 14.2% in 40 Gy and 60 Gy treatment respectively, while in

the field about 20% and 17% for each 40 Gy and 60 Gy. The increase of variability in the nursery and the field was due to appearance of some characters in the field more than those observed in the nursery. Both morphological and physiological changes were observed among the nursery and field grown plants. All nursery plants were transplanted in the field, and a higher percentage of variants (37%) were observed in the field than at nursery level. Similar finding was reported by Mak, *et al.* (1995).

However, some variants observed in the nursery stage could not be detected in culture stage. At culture stage, all variants observed were foliage variants such as compact leaf rosette, leaf texture and chlorosis and necrosis beside abnormalities in bud formation. However, some of these variants such as chlorosis and necrosis and variegated leaves disappeared at the nursery and the field stages. Pseudostem characters and plant stature in addition to bunch and finger characteristics and flowering habit were observed only in the field grown plants. This indicated that more variant characteristics would appear in the more mature plants. Mak and Ho (1997) recorded additional phenotypic changes observed in field grown plants such as stunted growth or choking appearance and others, which were increased 4 to 6 fold due to gamma irradiation. Also the mutated plants at 20 and 30 Gy's showed phenotypic changes in field stage due to gamma irradiation at about 37% in all characters.

In comparison with control plants (Table 4.3), irradiation tended to enhance the frequency of mutants and variants both in nursery and field grown plants. Mutagenic treatment induced 2 to 6-fold increase in variant plants. The frequency of variants tended to increase as gamma-doses increased from 20 to 40 Gy except for 60 Gy treatment where a lower frequency was observed in both nursery and the field-grown plants, probably many lethal or abnormal mutants at 60Gy might not have survived in the field (Mak *et al.*1995). More variation was observed at 30 and 40 Gy's.

Moreover, the frequency of 12.3% from the total number of irradiated plants at 40 Gy was variants, which indicated high percentage of mutation rate. However, mutation rate at 20 & 30 Gy were 5% and 6.5% in average and 9.0% at 60 Gy (Table 4.5). Variation frequencies for time to flowering were 0.6 - 1.7%, plant stature 0.7 – 4.6%, total stem morphology 1.7 – 3.5%, total leaf morphology 1.8 – 5.4% while variation for bunch and finger morphology were rare at about 0.4% which were observed at high doses only. Similar variations were also recorded by previous researchers, which included Fortuno and Maldonado (1972); De Guzman *et al.* (1982); Novak and Micke (1988) and Novak *et al.* (1990); however frequency of a variant type differed with genotype. Buiatti *et al.* (1986) suggested that all variations in colour and morphological characteristics could be attributed to the release of variability already present in a heterozygous state in the parental cultivar.

While the contribution of the genotypes to mutation frequency were supported by many researchers, Gaul *et al.* (1968) also suggested that the mutations of individual genes and their phenotypic contribution to the agronomic and morphological characters were influenced by both their genetic background and environmental factors. Increased variability is indicated by the higher values of coefficient of variation (CV). This difference indicated that there was indeed genotype X mutation rate interaction and as such direct comparison should only be made within the same genotype group (Siti Hawa, 1996).

Quantitative traits showed continuous variation; the plant height showed variations ranging from 150 cm at 60 Gy treatments to 390 cm at 40 Gy as compared to control plants especially in the 2nd and 4th batches. The coefficient of variation at these high doses was high indicating the effect of gamma irradiation in these plants; while at 30 Gy treatment the coefficient of variation (CV%) was low (7.9%). Gamma doses higher than 30 Gy caused significant height induction. Out of 2690 plants treated at different doses (20, 30, 40 and 60

Gy's), only 242 plants were relatively short (less than 250 cm). However, 80% from these plants were severely stunted in growth, which is undesirable. Such variations persisted after field establishment, and showed late in maturity, and smaller bunching with fewer hands per bunch. However such short variants are generally inferior in agronomic characteristics, particularly those induced at 60 Gy. Moderately stunted variants grow more slowly than tall (normal), and came to flowering at a much lower height. This may be regarded as dwarfness in stature, a positive trait in terms of ease of management. Short plant stature variants induced mainly by 30 and 40 Gy-treatments (about 5%) which showed desirable agronomic traits might be selected for confirmation trails. Dwarf plants with stunted growth induced at higher dose (60 Gy) accounted for about 50%. However, the frequency of plants exceeding 300cm was 20% while those of less than 290 cm was 10% of population. The other traits that were associated with dwarfness were smaller bunches and fewer hands per bunch.

The C.V. of pseudostem circumference (girth) of plants was high in 40 Gy and 60 Gy treatments. Hence variant would be examined for plants having girth size less than or greater than control plants. There were three different categories including stunted, useful dwarf and extreme tall based on their plant height and girth. The useful dwarf, which considered as desirable traits, showed a balance of height and girth, whereas plant height was between 200 – 260 cm, girth ≥ 65 cm. In this range, very few plants were found in each of 30 and 40 Gy's indicating that large variation occurred at high doses. As girth size is positively correlated with days to flowering and also plant height at flowering, plants of great girth size also tended to be tall and late in flowering.

The distribution of flowering time for irradiated plants tends to skew towards lateness at high doses. Earliness to flowering accounted about 3% at in all population except 60 Gy where all category plants showed delayed flowering. However, 40 Gy-plants showed high

frequency in earliness (1.7%) as compared with 20 and 30 Gy. These variants came to flowering as early as 6 –6.5 months compared to 7-8 months for the control. This finding is quite in agreement with that recorded by Mak *et al.* (1998). Earliness and short plant statures are two of the main objectives for the improvement of Intan cultivar.

Large variation was observed in bunch weight as indicated by C.V. values of 42%, 37%, 34% and 32% for 60, 40, 20 and 30 Gy respectively, in comparison to c.v. value of control (14.8%). The treated plants at different gamma doses showed wide range of bunch weight ranging from 2 kg to 19 kg, while control recorded limited range between 11 kg to 19 kg. The 20 Gy and 30 Gy treatments showed higher bunch weight (19 kg) as compared with higher doses (40 and 60Gy). This indicated the pronounced effect of gamma-irradiation on bunch weight at high doses. The values for both the treated population and the control showed similar distributions but the values for the treated population included more extremes. Yonezawa and Yamagata (1977) reported similar behaviour, and were of the opinion that agronomically promising mutants increased with increased mutation spectrum. However, the more extreme phenotypes at all doses tended to be at lower values of bunch weight which might be undesirable changes, while a small number of extreme phenotypes at 20 and 30 Gy recorded bunch weight of 20 kg more than control.

The effect of irradiation clearly appeared in the comb weight character. Wide range of variations was observed within population of this character as reflected by coefficient of variation values. There were more desirable changes at 20 Gy and 30 Gy, while at 40 and 60 Gy the extreme phenotypes might be undesirable. However, comb weight recorded for 30 Gy-treatment (1.8 kg) was higher than that of control (1.7 kg).

The number of comb/bunch showed wide range of variation due to effects of gamma-irradiation. The population treated with 40 Gy recorded low c.v. value for this character, but high number of combs/bunch was observed with extreme variants more than other doses. However, the effect of gamma- irradiation in this case was considered desirable.

The bunch weight at 30 Gy was positively correlated with number of comb/bunch at the same dose, while bunch weight at 40 Gy was negatively correlated with number of comb/bunch at each of 40 Gy and control plants. Gaps within the bunch and fused fingers were observed in the 2nd and 3rd batches for 40 and 60 Gy-plants with frequency of 0.2% (Table 4.5). The occurrence of the gap and fused fingers were rare which appeared at low frequency. These variants reduced the bunch weight and affected the shape of the bunch.

The morphological and physiological changes observed in the treated materials were also evident in the control plants, but at very low frequency. These variations ranged from 2.1% to 4.4% for all stages of growth in this study. Such phenomenon of occasional phenotypic variants called somaclonal variation is common in tissue cultured bananas (Mak *et al.*, 1995; Larkin, 1998; and Cote *et al* 1993). But not all variations observed were genetic and only a part of the observed variation could be heritable as reported by Van Harten (1998) as epigenetic change (Rieger *et al.*, 1991).

Phenotypic contributions to the agronomic and morphological characters could be influenced by both their genetic background and environmental factors. However, many researchers (Gaul *et al.* 1968) have supported the contribution of the genotypes to mutation frequency. Obvious characters that were recorded to be stable or carried over into the next generation included the striped leaf and brown/black streaking of the pseudostem. While for

other morphological characters deduction could only be made from previous findings based on some parallels between somaclonal variation and mutation induction.

The degree to which a mutation breeding programme will be successful depends to a great extent on the types of desired mutants and the ability to detect a specific mutant. It was observed that phenotypic variation for continuous traits (quantitative) are larger in irradiated populations than in the control populations. In other words, selection for both 'plus' and 'minus' mutants outside the limits of the original frequency distribution of the untreated population should be feasible. However, all desired mutations tend to be accompanied by undesired mutations especially when the dose used is not very low as is the case for 30 Gy gamma irradiation. Therefore, to strike a balance between positive mutations and optimal dose is actually an art in mutation breeding. For example, the selection of plants with short stature, early maturing but with low yield and poor tolerance, or they were of normal maturity with relatively good yield but of long stature and susceptible to *Fusarium* wilt. Kawai (1977), in his review, also found positive correlation earliness with reduced height and those early maturing mutants tend to give lower yield. However, Hwang and Ko (1988, 1990); Hwang (1990) and Hwang and Tang (1996) managed to overcome the problem for their initial selected Giant Cavendish somaclones, which possessed moderate to high resistance but also carried inferior agronomic traits through a combination of *in vitro* propagation and selection over several cycles. Subsequently variants with superior agronomic traits were identified. Thus, by repetitive culturing, selection for the desired character or elimination of the undesired traits could be carried out from the population of the regenerants. This proven technique would be most useful for the further improvement of the selected genotypes with superior traits. In facts, all plant breeding techniques are complementary to each other and mutation breeding like other breeding schemes will

always include selection and will also frequently involve hybridization, as reported by IAEA (1972).

There are indeed many characters of importance for Pisang Berangan that need to be improved to achieve the ideal cultivar. These include earliness to shooting, short plant stature, good post harvest traits, higher yield and tolerance/resistance to *Fusarium* wilt. But breeders seldom have the opportunity for selecting the ideal variety because more often than not, the hybrids, clones or variants have combinations of good and not-so-good traits for the desired characters. The improvement of the latter traits would be given more emphasis in the subsequent screening and evaluation exercise of the respective vegetative progenies.

For the evaluation of different characteristics, some techniques were adopted for example the screening of dwarfism by using gibberellic acid, and screening for *Fusarium* wilt by using "double-tray" and "Hot-spot" techniques. Furthermore, detection of the difference in genomic DNA content was carried out by using Flow cytometric technique and polymorphisms in these mutants by using RAPD.

5.4 Detection of Dwarfism in Mutant plants

Detection of dwarfism in plants with shorter height is tedious in the field. In addition, the environmental influence on growth also makes such selection difficult. Hence, the possibility of using exogenous gibberellic acid (GA_3) treatment to induce differential response between dwarf and tall plants (Damasco *et al.* 1996a, Smith and Hamill, 1993) was adopted in this study.

The verification experiments demonstrated that application of gibberellic acid (GA_3) to Pisang Berangan (Tall cultivar) and Serendah (dwarf cultivar) at the *in vitro* stage and deflasking stage showed a significantly greater leaf-sheath elongation in response to GA_3 for tall cultivar (Pisang Berangan) than for dwarf Serendah. The increased elongation of leaf sheath was 1 to 2-fold at *in vitro* culture stage and 2 to 3-fold at deflasking stage. The results were similar to that reported by Damasco *et al.* (1996a). Several weeks after subculture on GA_3 medium (*in vitro* culture stage) or after spraying of GA_3 on deflasked seedling (deflasking stage), tall plants reacted by internodes elongation while dwarf types remained insensitive to GA_3. Dwarf types showed shorter petioles and internodes and lower leaf ratio.

The response of leaf sheath at *in vitro* culture stage in gamma-irradiated and non-irradiated plants, increased with increasing GA_3 concentrations. The majority of irradiated and tall plants (Pisang Berangan) were responsive to GA_3 while the dwarf cultivar (Serendah) was non-responsive. Table 4.25 show highly significant differences between irradiated Berangan tall control and dwarf Serendah at all GA_3 concentrations (0, 29 and 59μ mol/L). However, with respect to leaf petiole and pseudostem length, tall control plants showed greater response to GA_3 than mutated materials and dwarf Serendah. Gamma-irradiation might have affected indigenous GA of mutated plants, resulting in their slight difference in response to GA_3 compared to non-irradiated tall control. Or gamma-irradiation has resulted in many dwarf mutants in plants with stunted growth.

Generally, tall control plants and mutated plants showed positive response to GA_3, while the dwarf control Serendah did not respond, and in some plants showed response very similar to non-GA_3 treated control. This differential growth response could be the basis for a selection method for detecting dwarfism from mutated populations. Matsukura *et al.* (1998) suggested that GA_3 increased the length and the number of cells in the first leaf only slightly.

However, physiological and genetic control of elongation has not yet been fully elucidated. The elongation of the second leaf-sheath of dwarf mutant plants may be estimated by exogenous GA_3 application. Therefore, according to suggestion by Damasco *et al.* (1996), the leaf-sheath length of the first leaf that emerged after GA_3 treatment was the best discriminating parameter in identifying dwarf and tall plants.

The observed increases in growth after application of GA_3 could be due to the inherent attribute of GA_3 in increasing impaired cell division and cell elongation. This is in agreement to that reported by Matsukura *et al.(*1998) and Barratt and Davies (1997) who concluded that GA_3 promote cell division and cell elongation. However, GA_3 is the major physiologically active gibberellins (GAs) which promote cell division in intercalary meristem of young plants, due to the activation of histone (HI) kinase and cyclin genes at the initial stage as investigated by Sauter *et al.* (1995).

Non-irradiated Berangan plants (control) induced 2-fold elongation in pseudostem compared to dwarf Serendah and only one fold in mutated plants. The elongation or subsequent growth of the stem was stimulated by GA_3 (as a result of production of more than twice the number of cells, which were twice as long).

In the growth cabinet experiment, the *in vitro* GA_3-treated plants showed leaf sheath increase as the concentration of GA_3 increased (29 and 59 umol/L). However, there were some plants producing leaf sheath length similar to control. Gamma-irradiated Berangan produced leaf sheath length ranging from 3.8-7.8 cm and 3.9-8.9 at 29 and 59 μmol/L respectively, while dwarf Serendah produced 3.9 cm and 3.6 cm, which indicated that some plants (3.8 and 3.9 cm) were non-responsive to GA_3.

At the deflasking stage there were three categories of leaf sheath length (based on the range of mean values); tall, intermediate and short. However, leaf I and leaf II showed significant differences in leaf sheath length among these three categories. Some Berangan-irradiated plants showed no response to GA_3 as appeared from the minimum range similar to that produced by dwarf Serendah.

Analyses of effect of GA_3 on irradiated, non-irradiated control and dwarf Serendah showed that low concentration of GA_3 (29 umol/L) did not induce significant response and the best separation of normal and dwarf plants population was seen at 59 umol/L. *In vitro* leaf sheath at 59 μ mol/L showed only 10% of mutated plants overlapped with dwarf Serendah plants compared to 28% overlapping at the lower concentration of GA_3 at 29 μ mol/L. Therefore, 59 μ mol/L is the preferred concentration of GA_3 for application to mutated plants in order to detect dwarf plants from the population. This finding is in agreement with that reported by Damasco *et al.* (1996a) who detected dwarf off-types in Cavendish bananas by using GA_3.

At the deflasking stage the best separation between normal and dwarf population was seen in leaf I where no overlap was observed, while leaf II showed 48% of mutated plants overlapped with dwarf plants. The young explants treated by GA_3 showed no root growth, because gibberellic acid frequently inhibits root formation or might prevent eventual root development (Murashige, 1961; and Vine & Jones, 1969). Therefore, plantlets formed on media containing GA_3 may need to be moved to a medium containing auxins before they could be rooted (George and Sherrington 1984).

In the nursery experiment 70 % within the mutated population showed positive response to GA_3 at 59 umol/L while 57% at 29 umol/L, indicating that the response to GA_3 increased as

the concentration increased. This finding is similar to that reported by Barratt and Davies (1997). On the other hand, Serendah produced 10% elongation in 59 umol/L and 9.2% in 29 umol/L and about 90% non-responsive to GA_3. However, such increase might be due to normal growth during the time of incubation (one month) and not due to response to GA_3. Similarly the response to GA_3 in deflasking stage at leaf II produced a 3-fold increase in response to GA_3 in non-irradiated plants and 2-fold increase in mutated plants but only one-fold increase in dwarf plants. However, separation of mutated and dwarf plants was complete in leaf I where no overlap was observed, while in leaf II about 50% of mutated plants overlapped with dwarf Serendah.

The *in vitro* treated plantlets showed three unequal groups for leaf sheath length at 29 umol/L, and only two unequal groups at 59 umol/L. The leaf sheath length also correlated positively with leaf petiole and pseudostem height. This indicate that leaf sheath is a suitable parameter for the detection of dwarfism at this stage of growth (Damasco, 1996a).

At the deflasking stage (nursery experiment), the elongation of leaf sheath and pseudostem was very pronounced after four weeks (leaf II). However, mutated plants showed response to GA_3 at 289 umol/L with long, intermediate and short groups observed. However, it was difficult to differentiate between long and intermediate plants. The short category in mutated plants was similar to dwarf plants. Similarly, the mean leaf sheath length measured in mutated plants was comparable to that in dwarf plants. Mutated plants showed 2-fold increase in leaf sheath length and plant height more than dwarf plants and 3-fold increase in leaf petiole length. Matsukura, *et al.* (1998), who worked on dwarf mutant of rice, also found that GA_3 enhanced the growth of the second leaf sheath.

Plant height in many cases correlates with GA_3 level, and the mechanism of GA-induced growth has been reported by several researchers such as Barratt and Davies (1997), who suggested that GA_3 might control both cell division and cell elongation. GA_3 has long been documented to induce cell division in both apical and sub-apical meristem, and has also been implicated in control of elongation by altering mechanical and biochemical wall properties. In addition, GA_3 increased the extensibility of cell walls in the elongation zone of the leaf sheath (Matsukura, *et al.* 1998). Gibberellic acid could be useful to differentiate plant types early in the tissue culture and deflasking stages. It is effective at the deflasking stage by simply spraying the plantlets in the nursery. Applying GA_3 at tissue culture might give rise to problems of inhibition of root formation leading to poor nutrient uptake and weak plants with low survival rate.

Some work also reported the use of RAPD for detection of dwarfism. However, this technique is more expensive, and difficult for screening large commercial populations (Israeli, *et al.* 1995; Shoseyov *et al.* 1998). Damasco *et al.* (1996a) concluded that misclassification occurred in 5 -10% of cases even when the screening was applied under the most stringent conditions.

5.5 Screening for disease tolerance to *Fusarium* wilt disease in Pisang Berangan

Pisang Berangan is very susceptible to *Fusarium* wilt (FOC) race 4 (Liew, 1996). In this study the use of induced mutations for "Intan" cultivar was carried out with the hope of increasing the degree of tolerance by accumulating mutagenic changes of minor genes for *Fusarium* wilt tolerance. Imelda *et al.* (1999) reported that irradiation of gamma ray with 2 Gy dose could produce some tolerant clones of banana Raja Sere. According to this report, high dose of gamma-irradiation in addition to high inoculum concentration resulted in more

inhibited growth due to more damage and low tolerance to *Fusarium* wilt. However, the optimum irradiation doses were able to increase growth and tolerance to *Fusarium* wilt disease of banana (Toruan-Mathius and Nurhaimi 1999).

Screening for *Fusarium* wilt tolerance by using double tray method for young plantlets resulted in the elimination of more than 95 % of susceptible plants. Inoculation by immersing roots into the conidial suspension proved efficient. Plants showed the disease symptoms in 2 –3 weeks whereas in field screening it required a long period of time. The localised reaction observed in field screening could be due to the different concentration level, micro environment of the soil, the root system and mode of entry of the inoculum.

Inoculum concentration influences disease severity. Results indicated that a concentration of at least $6x10^4$ spores/ml microconidia was required for infection to occur. Seedlings of Pisang Berangan were susceptible and the symptoms appeared early. Moreover, inoculation efficiency appeared to vary with the age of the plants used. The mortality of the plants inoculated at the age of 40 to 55 days was higher compared to those inoculated at the age of 59 or 60 days. This is similar to that reported by Liew *et al.* (1998) who explained that age of plants, vigour and state of development of the plant influenced disease development and expression.

Novaria, a known susceptible Cavendish to *Fusarium* wilt Race 4, succumbed to the disease within 2-3 weeks, followed by susceptible of Pisang Berangan and all plants of 60 Gy irradiated plants. A few 40 Gy mutants showed mild leaf yellowing after 2 months, but all succumbed to the disease eventually.

The suckers taken from field grown gamma-irradiated plants evaluated in the *Fusarium* hot-spot were susceptible to FOC. About 50% of control plants were dead after 3 months while mutated plants survived until 5 months with slight symptoms, and those of 40 Gy treatments continued their growth in the field until the 9th month. However, eventually all succumbed to the disease and died in the hot-spot. From all the screening trials, it appeared that the inoculum concentration in the *Fusarium* 'hot-spot' might be too high to allow the survival of moderately resistant plants.

For *Fusarium* wilt on bananas, the interaction between the pathogen and host is complex. Wardlaw (1930) noted the formation of tyloses in banana in response to vascular colonization by this fungus. In this study, gamma-irradiated plants showed slight tolerance to *Fusarium* because the plants continued their growth in the nursery after inoculation for two months and in the field 'hot-spot' for more than 9 months, but failed to give rise to tolerance/resistance to FOC. Banana plants were not immune to *Fusarium* wilt. In fact, immunity is not a realistic goal to aim for in a *Fusarium* wilt breeding program in banana because regardless of the cultivar used, the fungus is able to penetrate and become established in the vascular system of root. Resistance may break down when the plants are stressed and the pathogen is active.

A problem common to all methods of *Musa* improvement aimed at producing a FOC resistant cultivar lies in the requirement for field screening. The detection of useful genotype by this method is handicapped by the lack of rapid, reliable screening techniques for determining disease reactions. Moreover, field-testing is costly and time-consuming and requires large areas of land. How the plant reacts may vary depending upon the environmental factors such as soil type, temperature, moisture availability and the inoculum levels. Given that FOC is a diverse pathogen, and because of the influence of the

environmental factors, determining whether new genotypes possess durable resistance to FOC will be difficult. Therefore, the greenhouse screening methods such as 'double-tray' technique would be extremely useful and more appropriate. This method has important advantage that each and every plant received the inoculum treatment, which was difficult to achieve under the field conditions.

A total of 1000 plantlets inoculated and evaluated by the double-tray system include 600 gamma-irradiated plants (40 Gy & 60 Gy), non-irradiated Novaria and control Berangan. Out of 600 gamma-irradiated plants only (8%) from 47 plants treated at 40 Gy showed slight tolerant to FOC according to leaf and rhizome scales for *Fusarium* wilt screening. However, their planting in the infested field (hot-spot) only showed tolerance for 6 months and died eventually. On the other hand, plants treated with other doses (20 Gy and 30 Gy) did not even survive in the field and none of 60 Gy treated plants survived at double-tray stage.

5.6 Further characteristics of the mutants

Mutagens cause random changes in the nuclear DNA or cytoplasmic organelles, resulting in gene, chromosomal or genomic mutations and hence create variability. Taking into account the structure of DNA, genetic information can only result from the sequence of nucleotides. Theoretically, all changes that occur in the DNA sequence may result in changes in the genetic code i.e. mutation (Van Harten 1998). Therefore, it is possible to classify the various types of changes which can lead to an alteration of this sequence such as loss of one or several nucleotides (deletion) as in case of this study of gamma-irradiated Berangan plants. Moreover, gamma irradiation may affect the system controlling the direction of mitotic divisions within a meristem. Some induced variants such as short plant stature or stunted

growth, late flowering plants (late maturity) and abnormalities in bunch characters were selected to study possible changes at the DNA level. In this study, Flow cytometry (FCM) was used to determine ploidy levels and DNA content in gamma irradiated variants.

5.6.1 Flow Cytometry analysis

The most reliable conventional method of ploidy level analysis is the counting of chromosomes of metaphase plates. However, the preparation and microscopic analysis is time consuming, and in some species rather difficult due to high number of small chromosomes. This has led to the development of Flow cytometric techniques for rapid routine ploidy determination in *Musa* (Dolezel *et al.* 1994; Van Duren *et al.* 1996). In addition (Novak 1992), Flow Cytometry is recommended for the accurate estimation of nuclear DNA content. Dolezel, (1997) suggested that the more recently introduced image analysis could not compete with FCM in speed and convenience.

The majority of cells in full-grown plants is not participating in cell division and resides in a so-called G_0 (G is gap) stage of the cell cycle. However, in this stage the nuclear DNA content reflects the ploidy state of the plant. Cells, which are involved in divisions, start from a comparable so-called G_1 state and subsequently pass through S (DNA-synthesis), G_2 (an interphase nuclear stage with a doubled DNA content preceding the actual nuclear division). Accordingly nuclear DNA content in absolute units (genome size in picogram-pg DNA) was adopted for all samples used. Such estimation requires comparison with a reference standard having a known DNA content. In this study, *Glycine max* was as an internal standard because its genome size is relatively constant (Dolezel *et al.* 1994 and Vinderlov *et al.*1983). Moreover, this internal reference standard was used also to avoid bias

due to staining and instrumental changes when estimating nuclear DNA ploidy by Flow Cytometric analysis.

The precision of the ratio between the standard and the sample (Pisang Berangan) reflects the accuracy of DNA content measurements. The results showed that there were no significant differences in ploidy level between all samples used (3n), while differences were found between gamma-irradiated Berangan variants in DNA content. Similar finding was reported by Dolezel (1995) working on the application of karyology and cytometry in mutation breeding of bananas.

The estimated values of peak ratio of these samples could be used to discriminate between gamma-irradiated variants and non-irradiated plants. The peak appeared in the histogram, G_1 phase for Pisang Berangan and G_2 for *Glycine max* indicating that *Glycine* has more DNA content than Berangan. The mean of coefficient of variation (CV) of plant samples and internal reference standard ranged from 1.3% to 2.34% for Pisang Berangan and 1.02% to 1.7% for *Glycine max* cv. Palmetto. The higher CV in Berangan indicated that variation due to mutagenesis occurred within these samples. The discrepancies between observed and expected values in some samples could indicate the occurrence of changes in DNA content due to effects of gamma-irradiation (Barcaccia, *et al.* 1997). Gamma-irradiated Berangan plants showed differences in DNA content from control. All selected variants (short stature, late to flowering and bunch abnormalities) showed DNA content, different from control plants. The values of genomic DNA content (bp) of gamma-irradiation variants decreased as the dose of irradiation increased from 20 to 60 Gy indicating that the high dose of gamma-irradiation on (DNA-bp) resulted in significant deletions, which may be harmful.

It is commonly observed that the estimates of phenotypic variation for quantitative traits are larger in irradiated populations than in the control populations. Accordingly, FAO/IAEA (1982) pointed out that mutations for quantitative traits are more difficult to detect, evaluate and handle than those for qualitative traits. Because of their small phenotypic expression some kind of accumulation and rearrangement is required in order to make use of mutation. The structural mutations may arise as a result of a chromosomal break due to gamma-irradiation. Moreover, a change in the DNA sequences may also result in differences in DNA content of mutated plants. The gamma-irradiated plants showed 1 to 2-fold less DNA content than the non-irradiated control. Variants of short stature showed significant changes in DNA content (1.9 – 2.3 pg) at 40 Gy and 60 Gy suggesting that more changes in DNA content produced more phenotypic variations as in short variants which showed severely stunted growth. The changes were less at the low doses (20 and 30 Gy) where phenotypically the plants were considered short with height less than 170 cm. Other variants such as late maturity and bunch-abnormalities also showed changes of genomic DNA ranging from 2% to 4%, suggesting that the DNA amount has phenotypic effects via its influence on cell size and mitotic cycle time (Van Harten, 1998). It is estimated that at least 90% of the radiation-induced mutations refer to deletions (Van Harten 1998).

The difference in amount of DNA content due to irradiation indicated that gamma ray had direct effect at the DNA level, hence phenotypic changes were higher at 40 Gy and 60 Gy might be due to broken segments lost or became attached somewhere on a chromosome (translocation). However, the broken ends on both sides of the deletion normally are reunited again and at meiotic pairing the homologous chromosome may show a loop corresponding to the deleted part. However, mutations are oven lethal for the plants (Yatou and Amano, 1991), therefore, the more drastic phenotypic changes were associated with high dose of irradiation.

5.6.2 Random Amplification of Polymorphic DNA (RAPD) By Polymerase Chain Reaction (PCR) of Mutated Plants

RAPD analysis was used to evaluate variability of different traits within and between irradiated and non-irradiated samples. In addition, some genetic changes cannot be observed at the morphological or physiological level because the structural difference in the gene product may not alter its biological activity sufficiently to produce an altered phenotype. Analysis of genetic variation using RAPD was conducted on mutated Berangan plants at different irradiation doses. RAPD analysis was used to detect such variation in gamma-irradiation induced mutants of Cavendish cv. Grand Naine (Kaemmer *et al.* 1992).

Ten primers were used to study polymorphism. Four primers (OPA-03, OPA-05, OPA-07 and OPA-09) gave positive amplification products in all samples used. However, primers with high GC content yielded more bands. RAPD-PCR DNA fingerprinting generated a wide variability in the patterns of amplification /products. Jarret and Gawel (1995) also reported that RAPD is suitable for detecting variation in plants. These four primers produced polymorphic bands ranging from 63% (at OPA-09) to 83% (at OPA-03), when compared with the control. The banding pattern was affected by primer concentration, which affected band numbers and reproducibility i.e. with an increase in primer concentration (10 pMol) the number of bands also increased.

Low concentrations of DNA (50 ng) resulted in a good amplification, while any further decrease in concentration resulted in few or no bands due to increase in stringency of reaction. This finding is similar to that reported by Damasco, *et al.* (1996 b) who observed that the suitable concentration of DNA template for high amplification was between 25 to 75 ng. In addition, $MgCl_2$ concentration of between 2.5 - 4.0 mM produced good amplification. Low annealing temperature at 35°C also resulted in more intensive bands

than higher temperature at 45°C. This is in agreement with Kulkarni, *et al. (*1999) who used RAPD for analysis of wild and irradiated bananas.

A wide spectrum of variation was observed between mutated and non-mutated plants and within each mutation group. From the analysis, the four primers differentiated all the samples used by producing different types with molecular sizes ranging from 0.2 to 1.7 kb. The DNA patterns of mutated Berangan biotypes were obtained by each primer. Primers: OPA-05 & OPA-09 yielded more polymorphism than the other two primers.

PCR amplification produced clear scorable bands ranging from 0.2 to 1.7 kb in size. The bands were either present or absent in irradiated (mutated) plants as compared with non-irradiated control. The RAPD bands, namely $OPA\text{-}03_{1700}$, $OPA\text{-}05_{1600}$, $OPA\text{-}07_{1500}$ and $OPA\text{-}09_{1700}$ were consistently present in all normal and absent in all dwarfs. The results suggested that only one type of dwarf mutation exist in all of these mutated plants. However, from the profile generated by primer OPA-03 a major band of 1.7 kbp was absent in four samples irradiated at 40 Gy (M1, M2, M3, and M7) compared to control and other samples irradiated at 20 and 30 Gy. The profile based on Primer OPA-05 showed a major band of 1.5 kbp band were found missing in four samples of 60 Gy treatments compared to normal and those treated at 20 and 40 Gy's. A similar case was also observed for primer OPA-07 and OPA-09, where a major band of 1.5kbp was found absent in all samples irradiated at 60 Gy compared to the control and the remaining samples at 20 and 30 Gy's respectively. The results indicated that morphological changes induced by irradiation and *in vitro* culture were associated with appearance/ disappearance of bands. This polymorphism may be due to single base pair changes. In addition to deletion or translocation discussed earlier. Kang *et al.* (1993) suggested that changes in the size of the PCR products depends upon single base pair changes, deletions of primers sites, insertions that increase the

separation of primer sites over the 3000 bp limit and small insertions/deletions. The results of this experiment revealed that information generated from DNA-based polymorphism provides the best estimate of genetic diversity. The ability to fingerprint strains (cultivars) genetically through the establishment of diagnostic molecular profiles was possible for scientific applications.

From Dendrogram (Fig.4.6-E, Appendix C), independent repetitions of the analysis revealed that amplification patterns obtained in given plants were highly reproducible, provided the amplification conditions were strictly the same. The present study clearly suggested that RAPD-PCR provided a very fast and suitable tool to obtain an overview of phylogenetic relationships of DNA polymorphisms. Moreover, the results showed that RAPD analysis could generate significant information on polymorphism between samples.

The majority of polymorphisms generated were not associated with dwarfism. RAPD marker specific to dwarf plants from micropropagation was identified after analysis of normal and dwarf plants. 12 dwarf plants showed the absence of RAPD OPA-03_{1700} band which was present in normal plants suggesting that the rate of dwarf induction with the mutation would be 100%. Same result obtained with primer OPA-09 .The finding is quite in agreeable with that reported by Smith and Hamill, (1993) and Damasco *et al.* (1996b). The results suggest different dwarf mutations may have occurred in mutated Pisang Berangan (AAA).

The results demonstrated that RAPD analysis could be used to detect genetic variation in gamma-irradiation induced mutants of Pisang Berangan. Higher variability was observed at 40 Gy and 60 Gy doses. The majority of these variants were very short in stature, stunted in

growth or with very low bunch weight. The relation between higher doses and short plants could be an indication that variants were associated with dwarfism.

5.7 Somaclonal Variation Studies

The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of cell. The expected result was clonal multiplication of genetically uniform plants. This expectation has formed the basis of the micropropagation industry and provided a technical basis for genetic manipulation in plants. The occurrence of uncontrolled variation during the culture process was largely unexpected and undesired. Several authors (Cote *et al.* 1993; Smith, 1988; Vuylsteke *et al.*, 1991and Sirisena and Senanayake, (2000) have documented that *Musa* plants produced by shoot-tip culture have shown somaclonal variation rates of 0-70%.

The banana is especially interesting for investigating somaclonal variation as a tool in crop improvement because of its vegetative propagation and the relative difficulty of breeding the crop by conventional means. The pre-existing variation and variation generated by *in vitro* cannot be separated and together account for the occurrence of somaclonal variation.
In this study, analysis of somaclonal variation was carried out to assess the amount of variation due to this factor compared with that derived from mutation induction. However, 1755 primary explants from tissue culture and suckers were planted in the field to detect somaclonal variations and to compare tissue-cultured plants with suckers. Different off-types could be identified at various stages of plant growth. The agronomic and morphological changes include plant stature, abnormal foliage, extreme mosaic, variegated leaves, deformed lamina, pseudostem disclouration, inflorescence and fruit variations. Tissue culture-derived plants showed variation that was akin to mutations in cultured cells.

Various explanations may account for (true) genetic variation that is found following *in vitro* culture. The pre-existing genetic variation may be revealed by *in vitro* multiplication. Furthermore, growth hormones are frequently added to the medium in order to stimulate growth and differentiation of the explants in culture. These growth hormones (auxins/kinetins), in particular when added in high concentrations, also caused genetic instability. Some of the genetic variation that arises *in vitro* is of potential value to the plant breeder.

In the present study phenotypic changes in *in vitro* banana plants (Pisang Berangan) observed in the nursery and the field grown plants varied from gross abnormalities to very discrete changes in morphology. The variations include dwarfism, changes in leaf, pseudostem and flower sizes and shape, and colour. These phenotypic variations could be distinguished in the field, as all plants were grown under uniform condition. Most of the variants about (2.84%) described in this study have been reported by other workers (Israeli and Nameri, 1985; Israeli *et al.* 1991; Smith, 1988; Vuylsteke, 1998; Vuylsteke *et al.* 1996). Of the total variants, dwarf off-types accounted for 1.4%. These plants produced smaller bunch and fruits, which were of no commercial value. And 20 % of variant plants were observed as early flowering individuals (5-6 months) with desirable bunch weight.

Somaclonal variation can be considered as the amplification of natural mutation. On the other hand, most types of somatic mutation listed by Stover and Simmonds (1987) can be found in somaclonal variation. The frequency of occurrence is much higher than that of natural mutation. Therefore, it is desirable to combine tissue culture and mutagenesis to obtain a wide range of variation as a reliable tool for plant breeding.

Early detection of somaclonal variants is desirable. However, only two variants (extra-dwarf and variegated leaves) could be detected at the *in vitro* culture stage (Fig. 5.2). Practically, the most common dwarf variants were detectable at the nursery stage. Eradication of these plants in the nursery will result in a significantly lower rate in the field. Obviously, variations in the inflorescence or bunches were detected only in the field, but their rate could be reduced by using short-term subculture plantlets.

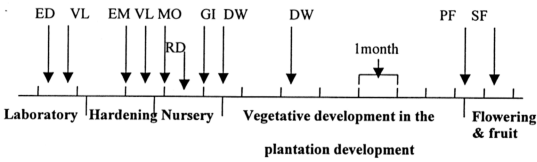

Figure 5.2: Earliest time for the identification of somaclonal variations. (ED = extra Dwarf ; VL = variegated lamina; RD = reddish pseudostem; GI = giant; DW = dwarf; PF = persistent flowers; SF = split fingers). Symbols that appear more than once indicate identification at more than a single stage.

It has been explained that plant breeders should be interested to obtain somaclonal variation of considerable interest. Early fruiting individuals with desirable bunch weight were selected for further investigation. 30% of the whole population flowered earlier (5 – 6 months) than sucker was also reported by Ho *et al.* (1994). We can conclude that in comparison with mutation induction, somaclonal variation accounted for a proportion of the variation detected, although the amount seen was significantly small.