
CHAPTER TWO

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LITERATURE REVIEW

2.1 General Introduction to Plant Breeding

Plant breeding is essentially a selection made by man of the best plant within a variable population as a potential cultivar. In other words plant breeding is a "selection" made possible by the existence of "variability". Plant breeding involves human activities directed towards the production of crop plants which are genetically improved for certain traits or characters and which, therefore, are better suited to human needs (Van Harten, 1998). Hence, any attempt at plant improvement requires genetic variability. Ancient breeders resorted to the genetic variants provided by nature and selected among those which are more suited to their needs in terms of fruit or grain size, taste, ease of harvest, suitability for storage and yield.

The most important factors in producing variability in plants are hybridization, recombination and mutation (spontaneous or induced) (Donini and Sonnino, 1998). All variability in living organisms are ultimately caused by mutation, the interaction with the environment and their recombination, through hybridization and selection, which are all essential components of evolution. Plant breeding, which could be called guided evolution, depends on the same principles.

Mutations provide the raw material for plant breeding. The term "mutation", was introduced by De Vries (1901) to designate sudden change in the hereditary material of a cell, and therefore, transmissible to the progeny. The genus *Musa* consists of two groups of plants:

the cultivars, which are clones maintained exclusively through vegetative propagation; and wild plants, which are diploid and genetically reproduced.

2.2 Classification of banana germplasm

The system of classification was based on the pioneer work of Simmonds and Shepherd (1955). Bananas belong to the genus *Musa* and the family *Musaceae*. According to Simmonds (1955, 1962, 1966) and Simmonds and Shepherd (1955), the family *Musaceae* contains two genera (*Musa and Ensete*). Within the genus *Musa* four sections are recognized, namely: Australimusa, Callimusa, Eumusa and Rhodochlamys. Eumusa contains the great majority of edible bananas (derived from *Musa acuminata* and *Musa balbisiana*). They appear to have evolved through a complex of hybridization events resulting in a diverse group of cultivars.

Edible bananas belong to section Eumusa, which have 22, 33, or 44 chromosomes. The basic number in this section is n =11, so that these cultivars are diploid, triploid or tetraploid. Triploid cultivars are generally the most numerous; diploid somewhat less so and tetraploid forms are very rare. However, triploids (2n = 3x = 33) which were formed by chromosome restitution at meiosis, have more vigorous growth characteristics and are more productive than diploid (Schoofs, 1997). The Australimusa series, which are grown mainly in the Pacific region, have 20 chromosomes (n = 10) and the cultivated forms have apparently arisen from a number of species. The Tahiti (Fehi) cultivars are the only ones that are well known (Williams, 1975). The two sections: Callimusa (n = 10) and Rhodochlamys (n = 11) include species of ornamental value or of no economic interest.

All edible cultivated clones belong to the Eumusa and have traditionally been classified according to their presumed contribution of two species *Musa accuminata* and *Musa balbisiana* to their genotypes (Williams, 1975). Simmonds (1966), designated cultivars the symbols "A" and "B" indicating the contribution of *accuminata* and *balbisiana* genes determined from morphological features'; thus:

AA = accuminata type diploid

AAA = accuminata type triploid

AAAA = accuminata type tetraploid

AB = accuminata x balbisiana diploid hybrids

AAB and ABB = triploids drive from both species.

ABBB = tetraploid derived from both species.

The more the "A" genome, the sweeter the fruit and hence it is useable as a dessert fruit. The more the "B" genome, the more starchy it is; hence it needs cooking. The following examples are common cultivars found in Malaysia:

AA type: *Pisang Mas, Pisang Lemak Manis, Pisang Masam* and *Pisang Jari Buaya.*

AAA type: *Pisang Berangan* (e.g. Intan), *Pisang Udang, Pisang Serendah, Pisang Buloh* and Cavendish sub-group such as Novaria.

AAB' type: *Pisang Rastali, Pisang Raja, Pisang Tanduk, Pisang Seridu, Pisang Helang, Pisang Nangka* and *Pisang Relong.*

ABB type: *Pisang Awak* and *Pisang Abu.*

More recently, Simmonds and Weatherup (1990) used methods of numerical taxonomy to classify cultivated bananas according to the ploidy level and genomic contributions of A and

B genomes. In general, this approach concurred with a simple numerical scoring system developed by Simmonds and Shepherd (1955), and did not provide any evidence of the existence of more *M. balbisiana* cultivars, i.e. edible BB and BBB. Perrier (1993) appropriately observed that the classification approach by which all known types are grouped into a limited number of classes is not completely satisfactory. His observation was shared by many researchers such as Valmayor *et al.* (1991).

The emergence of new techniques for banana classification has been rapid especially in the area of biotechnology. The improved techniques include the use of morphotaxonomic descriptor, which uses both biological and agronomic evidence. However, its serious drawback is that the descriptors are influenced by environment (Swennen, 1988). The use of biochemical markers followed, using enzymes and phenolic compounds (Horry and Jay, 1988; Jarret, 1990). Outstanding progress came with the potential use of molecular marker (RFLP and RAPD) in determining genetic diversity as described by Carreel *et al.* (1994) and Jarret *et al.* (1992), although its widespread usefulness has yet to be determined.

In cultivated banana, *Musa* spp., there are now four known genomes, A, B as previously described as well as S and T. These correspond to the genetic constitutions of wild Eumusa species *M. acuminata*, *M. balbisiana*, *M. schizocarpa* and the Australimusa species, respectively (Hont, *et al.* 2000). Most cultivated clones are triploid or diploid, and have been classified into genomic groups according to chromosome numbers and morphological traits. Genomic *in situ* hybridization enabled the differentiation of the chromosomes of these four genomes. Furthermore, the total repetitive DNA content of the *Musa* genome could be assessed and used as classification criteria. Within the Eumusa section, *M. acuminata* species-specific DNA elements identified by Baurens *et al.* (2000) as either short

interspersed elements or copia-like interspersed sequences. The banana 'A' genome is composed of 77% repetitive elements and 23% single copy sequence.

2.3 Banana Breeding Systems and Genetics

The genetic system of *Musa* is extremely complicated to study because of the serious problems of sterility, interspecific hybridity, heterozygosity, and polyploidy that are common in most of the clones (Novak *et al.* 1993). Bananas and plantains have a very narrow genetic base, being derived from a small number of clones that have been vegetatively propagated. Most cultivated bananas and plantains are triploids (2n = 3x), and triploidy leads to problems during meiosis due to uneven numbers of chromosomes. In addition, the genetics is complicated by a high level of sterility and parthenocarpy. A detailed overview of these problems has been reviewed by Ortiz, *et al.*, (1995). Consequently, plant multiplication is by vegetative means, which is inherently slow.

Triploidy itself is not the only cause of sterility in triploid cultivars. Agarwal (1987) conducted meiotic studies in eight male sterile triploid banana cultivars. He found that pollen mother cells degenerated at various stages of development and divisions did not take place after anaphase 1 in any of the cultivars. Similar cytological behaviour was found in diploid bananas by Raman *et al.* (1970), who concluded that genetic causes rather than chromosomal irregularities were responsible for sterility in bananas.

Therefore, the complexity of *Musa* genetics needs a more effective system to support conventional breeding programs, and prospects of using biotechnology in bananas are very high. During the past decade, many *in vitro* techniques of plant tissue and cell culture have been developed and refined as an aid to conventional plant breeding. These include

efficiency in obtaining variation, selection and multiplication of the desired genotypes. Some of these biotechnologies applied to *Musa* include *in vitro* mutation induction (Ho *et al.* 1994 and Novak *et al.* 1990), cell protoplast culture and somatic embryogenesis (Escalant *et al.* 1994; Panis, *et al.* 1993; Novak *et al.* 1989), molecular markers of genetic diversity (Gawel and Jarret, 1991; Bhat *et al.* 1992; Horry and Jay 1988) and *in vitro* screening and selection for disease resistance (Sun and Su, 1984; Ploetz, 1990).

2.4 Propagation of banana germplasm

Shoot-tip culture is a basic technique for *Musa* propagation, conservation and movement of germplasm (Novak, 1992). The basic protocol has been improved by Cronauer and Krikorian (1984) and has been successfully applied to the rapid propagation of dessert AA and AAA bananas, cooking ABB bananas, and, to a limited extent, to AAB plantains and 'Silk' and 'pome' AAB dessert bananas. An excellent guide to *Musa* micropropagation and *in-vitro* conservation has been compiled by Vuylsteke (1989). Proliferating meristems from shoot-tip explants could originate from either axillary or adventitious buds (Novak *et al.* 1990). The morphogenetic response is controlled by the culture medium and strongly influenced by genotypes.

The micropropagation of bananas generally follows the stages as described by Murashige (1974). These stages are: selection of mother plant, culture establishment, multiplication, transplanting of explants from *in vitro* culture to the nursery for acclimatization, and preparation of plants for re-establishment in soil. The possibility of micropropagation of banana through shoot tip culture has been explored by different researchers (Wargantiwar *et al.* 1997; Israeli *et al* 1995). However, several basal medium formulations have been reported to sustain growth and proliferation. The most widely used medium contains the

Murashige and Skooge (MS) (1962) mineral salts. In bananas, the use of growth regulators such as kinetin (KIN) and indol butyric acid (IBA) is essential to culture explants which often develop certain amount of somaclonal variation depending upon genotypes (Ahloowalia, 1998). Therefore, maximum multiple shoot formation was given by (MS) basal medium supplemented with 7 mg BAP (benzylamine/ litre or 5 mg BAP/ litre) + 15% coconut milk. Rooting of the multiple shoots was best in MS medium + 2 mg IBA/ litre + 0.1% charcoal. In addition the plantlets should be kept under a mist chamber for 10 days to enhance survival and growth of plantlets.

Novel techniques in efficient micropropagation of certain popular banana cultivars have been reviewed by Vani and Reddy (1999). Bekheet and Saker (1999) developed an efficient medium for *in vitro* propagation of banana, and found that addition of adenine sulfate to the multiplication medium increased the number of proliferated shoots but decreased the number of leaves and shoot length. Among different types of auxins used for rooting banana shoots, NAA was more effective than IAA or IBA. The effects of culture media on multiple shoot formation and rooting of *invitro* cultured banana explants has also been investigated by Dhumale, *et al.* (1997).

They found that BAP at 7 mg/ litre induced the highest number of multiple shoots, while 2 mg IBA/ L and 2 mg IBA fortified with 0.1 % charcoal resulted in profuse rooting. The main advantages of the *invitro* propagation technique for bananas are: plants can be rapidly multiplied from a mother plant of known desirable characters; selected and screened plants can be maintained free of serious diseases and pests; 98% survival under field conditions; plants from *invitro* plantlets grow faster in the early growing stages than those from suckers; uniformity of flowering; short harvest period; in comparison with the suckers, plants are easier to propagate and transport; and lastly, the material produced are true-to-type

(INIBAP, 1993). Barnum (1998) concluded that plant tissue culture is invaluable when traditional plant breeding cannot generate plants with desired traits; often micropropagation shows faster results than traditional plant breeding.

2.5 Strategies for Banana Improvements

The most important advance in banana breeding has been the development of elite diploid and triploid populations. It is generally believed that triploids are dominant among cultivated bananas because they have been selected for superiority over diploids in term of vegetative vigour and yield. Subsequently the breeders and their improvement programmes have attempted to develop breeding strategies and husbandry techniques to overcome the genetic and practical barriers. A predictably, it remained a challenge to breed from a triploid population, which is largely sterile, and to introduce genetic improvements and heterogeneity (Vuylsteke, *et al.* 1996). Many researchers (Ho *et al.*1994; Mak *et al.* 1997; Novak *et al.*1990) have reported that genetic improvement of banana cultivars is difficult through conventional methods of hybridization due to these problems. Therefore, alternative strategies are needed to create genetic variability. The strategies, which have been adopted, include the collection and evaluation of germplasm; exploitation of somaclonal variation for desirable agronomic traits and disease resistance; *in vitro* mutation induction techniques to generate genetic variability and ploidy manipulation (Ho, *et al.* 1994).

Simple tissue culture techniques, such as shoot-tip and embryo culture, are well developed. Vuylsteke (1998) reported that application of *in vitro* culture techniques has greatly improved *Musa* germplasm handling for the purposes of clonal propagation, uniform production, and breeding. In general, tissue culture methods offer an alternative to the classical breeding of *Musa* (FAO/IAEA, 1990), which is ultimately used to produce

vegetative progeny expressing the desired trait. Several authors (Hwang *et al.*, 1984; Smith and Drew, 1990a) have documented the field performance of micropropagated bananas that are capable of performing equal to or better than bananas propagated by conventional planting materials.

In addition, *in vitro* culture technique facilitates induction, selection and multiplication of mutants. *In vitro* culture in combination with mutation techniques offers several advantages to overcome some of the problems of conventional breeding, e.g. mutagenic treatment of large populations of cells, somatic embryos, apical and axillary buds, micro-sized plants, and multiplication of selected genotypes in a small space.

The international banana trade is dominated by the "Cavendish" group, which is known to differ mainly in height, bunch and finger characteristics, which could be due to somatic mutations. On the other hand, the collection and evaluation of natural populations (e.g. in Malaysia) of Pisang Berangan resulted in the release of an improved Pisang Berangan designated as Intan (Ho and Tan 1990). Banana plants regenerated from *in vitro* culture exhibited various morphological and biochemical variations due to somaclonal variation.

Somaclonal variation is a potential source of novel and useful variability (Novak, 1992; Ortize and Vuylsteke, 1996; Smith and Drew, 1990 b). Study of variation types, frequency, spectrum and transmission of variation is still limited to a few plants (Jain *et al.* 1998). Furthermore, the success of applying somaclonal variation in plant breeding is very much dependent on the genetic stability of the selected somaclones and a close interaction with plant breeders. In the absence of sexual recombination to generate genetic variation in the commercial banana cultivars, mutation breeding is an excellent alternative (Ho, *et al.*1994; Novak, 1991; Smith *et al.*1995; Mak *et al.*1995).

Morpurgo *et al.* (1997) concluded that the progressive development and adoption of tissue culture and mutation induction techniques to support traditional cross breeding programmes has proved to be successful in generating new improved *Musa* germplasm. Biotechnology also provides powerful techniques for the improvement of plants and the increase of their productivity. New advanced genetic markers and technologies (Gawel and Jerret, 1991; Jerret, 1990; Litz, 1990) have been adopted for improvement of *Musa,* coupled with various *in vitro* techniques.

The current status of *Musa* biotechnology as reported by INIBAP (1992) is very close to making a significant contribution to the breeding of new cultivars. Tissue culture techniques which were developed for induction of heritable variation, proved to be successful for *Musa* breeding. *Invitro* mutagenesis contributes to induce variability for exploitation in plant breeding programmes. Micropropagation is widely used for rapid multiplication of elite clones and for conservation of *Musa* germplasm (Novak, 1992; Afza, *et al.* 1994; Akehurst, 1996 and Vuylsteke *et al.,* 1998). *In vitro* micropagation techniques also offer new opportunities for mutation induction (Hirimburegama and Gamage, 1997). Smith and Drew (1990a) indicated that plant tissue culture is an integral part of molecular approaches to plant improvement. The discovery and development of recombinant DNA technology provides man with a powerful tool to change the whole way the organism's functions. As a consequence, it has prompted Malaysia to embark heavily on developing biotechnology research and development (R&D) to improve agricultural productivity.

2.6 Approaches to Banana Improvement

2.6.1 Conventional breeding of cultivated bananas

Conventional plant breeding is based on genetic variation and selection of the desired genotypes. The availability of genetic diversity and genetic variation is the starting point of any breeding programme. In most crops, sufficient genetic variation is present among land races, cultivars and their wild relatives. In conventional plant breeding programmes this is followed by several years of selection and field evaluation before a desired genotype is released as an improved cultivar (Ahloowaia, 1998). In sexually propagated crops, e.g. rice, wheat, maize, barley, the genetic variability is usually recombined through hybridization. The desired recombinants are then selected from the segregating populations in the subsequent generations, which are tested, multiplied and released as improved cultivars. However, a number of important crops, such as banana and plantain, are propagated from vegetative parts and are not amenable to improvement in the same manner as sexually propagated plants. Many of these plants are complex polyploids, some are self-incompatible and there is little or no seed formation. Hence, it is not possible to produce sufficiently large populations required to obtain the desired recombinant in a short duration. In these plants, mutation induction offers the possibility to alter a few characters without disrupting the genome while retaining all the other characters of a clone (Ahloowalia, 1998; Novak, 1992).

Diploid clones of both wild and edible bananas are essential starting material for banana breeding. Novak (1992), reported that functional pollen grains and egg of diploid clones are haploid, however, many of the edible diploids are either male and/or female sterile, e.g. the widely grown "Pisang Mas". This phenomenon has excluded many valuable clones from hybridization. Several diploids form unreduced female gametes (2n) and hybridization may

result in triploid progenies. Other clones, e.g. "SH-2095", are very poor in pollen production which limits their usefulness as male parents.

The first steps of conventional cross-breeding programmes are hybridization and selection of recombinants at the diploid level (Hammerschlag and Litz, 1992). The improved, disease resistant and pollen fertile diploids are then crossed with triploids. This crossing of triploid females with diploid males has a wide range of application for AAA and AAB dessert bananas, AAB plantains and ABB cooking bananas. The fertility of such crossing is usually very low; most pollinated AAA and AAB clones produce less than 0.1 seed per pollinated fruit, while some ABB females yield more than 10 seeds per fruit. Simmonds (1962) concluded that female fertility increased with enhanced contribution of the B (*M. balbisiana*) genotype to the constitution of a female parent. Banana hybridization is complicated by combination of different polyploidy levels of parents and by female restitution associated with the formation of unreduced female gametes. Novak (1992), suggested an alternative procedure for obtaining synthetic triploids by inducing chromosome doubling of promising AA or AB clones and then to make (induced tetraploid) 4N x 2N crosses or reciprocal 2N x 4N (induced tetraploid) crosses. For example, *Fusarium* wilt disease is the most common and widespread in banana plantations throughout the world, in particular the susceptible cultivar "Gros Michel" was replaced by cultivars of the "Cavendish" group. However the need for resistant cultivars to *Fusarium oxysporum* f. sp. *cubense* resulted in the development of banana breeding programmes in tropical America (Rowe and Richardson, 1975).

Recently, an outstanding tetraploid Goldfinger (AAAB) which combines disease resistance and desirable agronomic qualities has been produced from crossing the triploid Dwarf Prata (Lady's Finger) onto a diploid SH3142 in Honduras (Rowe and Rosales, 1993). Ho *et al.*

(1994) stated that, using improved disease resistant diploid bananas for autotetraploid induction followed by crossing with superior diploid could result in horticulturally desirable secondary triploid after crossing 4n with 2n.

2.6.2 Non-conventional Breeding

Non-conventional breeding approaches have been used in banana and plantain improvement during the last decade (Donini and Sonnino, 1998). This combination of traditional cross-breeding with biotechnology could provide a powerful tool for the development of new clones with desirable characteristics such as high yield and fruit quality with resistance to major pests and diseases. Many researchers (Vuylsteke *et al.* 1990; Smith and Drew 1990b; Dheda *et al.* 1991; Novak 1991; Novak *et al.* 1987; 1989; 1994; Bakry *et al.* 1993 and Donini and Sonnino, 1998) have reported and reviewed the techniques potentially useful for banana breeding. These techniques include shoot tip culture, somaclonal variation, somatic embryogenesis, protoplast culture, embryo culture, genetic engineering/transformation and induced mutagenesis. However, mutation breeding, like molecular markers or plant transformation technology, is simply one of the tools which are available to the breeder, and as such it has advantages and limitations.

Application of radiation or mutagenic chemicals to *in vitro*-cultured cells, tissues, somatic embryos, protoplast fusion products and embryos rescued from wide crosses, could generate variation not usually obtained through conventional breeding methods (Donini and Sonnino, 1998).

2.6.2.1 Somaclonal Variation

The increased genetic variation among plants regenerated from *in vitro* culture has been termed "somaclonal variation" (Larkin, 1998; Ortiz *et al.*, 1995; Israeli, *et al.*1995; Vuylsteke *et al.* 1991; Novak, 1992). Smith and Drew (1990 b) stated that somaclonal variants are one means of generating variation that may be lacking in bananas. On the other hand, Vuylsteke *et al.* (1991) suggested that somaclonal variation closely paralleled naturally occurring variability, whereas breeding via somaclonal variation would only become attractive when it is possible to screen at the cellular level, with selection pressure applied *invitro.*

The origin of somaclonal variation in *Musa* shoot-tip cultures may be explained by Novak (1991), (i) genetic changes are already present in the tissue of the explants, i.e. *in situ* variation; (ii) variation is induced by the mutagenic action of tissue culture media; and (iii) variation is a result of stress induced by the conditions of the tissue culture environment. The first two factors are responsible for heritable changes, e.g. numerical and structural chromosome variations, point mutations and changes in the quantitative traits. Many other mechanisms have been suggested which lead to somaclonal variation (Jain *et al.*, 1998) such as activation of transposons, methylation of DNA, changes in plastid DNA, changes in mitochondrial DNA, segregation of existing chimaeral tissues, and non-specified interactions leading to changes in gene expression.

The stability of the phenotypic variants in ratoons and among sucker progeny suggested that somaclonal variation is of genetic origin (Vuylsteke *et al.*, 1991). There is a large body of evidence that somaclonal variation generate either recessive or dominant single gene mutations, polygenically inherited traits, changes in structure and number of chromosomes,

and epigenetic effects (Drew and Smith, 1990; Smith and Drew, 1990 b; and Novak, 1992). Israeli *et al.* (1996); Cote, *et al.* (1993) stated that most variations observed in banana *in vitro* plants were transmissible and can therefore be referred to as somaclonal variants.

In the Cavendish bananas, somaclonal variation is reported to range from 2.4% to 25%. However, such variation can offer considerable opportunity for banana improvement (Ho, *et al.*, 1994). The frequency of off-types in banana has also been reported to vary from a very high 50% and 25% to a low 3% and 1% (Israeli, *et al.*, 1995). Traits affected by somaclonal variation include plant stature (dwarfism), abnormal foliage, bunch and fingers (fruit shape), colour changes in pseudostem, petiole and bract, inflorescence morphology and associated female fertility (Gowen, 1995; Mak and Ho, 1997; Smith and Drew, 1990 b; Israeli *et al.* 1991). In the "Cavendish sub-group" dwarfism is the most common somaclonal variation accounting for approximately 80% of the total variants (Israeli, *et al.*, 1991; Reuveni and Israeli, 1990; Stover, 1987). So far, the somaclonal variation in the banana plantlets derived from tissue culture can be recognized when they are grown in the field. This may cause financial loss to the farmers because they need investment for planting and plant maintenance. Some strategies can be used to determine the genetic integrity of the plantlets derived from *in vitro* tissue culture i. e. cytological analysis, isoenzyme analysis, and DNA analysis with Restriction Fragment Length Polymorphism (Toruan-Mathius and Hutabarat, 1997). However, some of them have limitations because they need a lot of plant tissue, a long period, and have a high cost of analysis.

Although somaclonal variation is considered to be a serious problem in plant storage, this source of variation also offers a means to generate mutants for resistance screening to major diseases and pests (Ortiz, 1995). However, most of the somaclonal variants are undesirable agronomically (Mak and Ho, 1997). Resistance to *Fusarium* wilt was observed (Hwang and

Ko, 1987) through mass screening of somaclonal variants in banana. According to Reuveni and Israeli, (1990); Reuveni (1990); and Brar and Jain (1998), factors affecting the rates of somaclonal variation in bananas include:

a) Origin of the meristem

In vitro growth may occur from meristem cultures, which may form callus or direct shoot formation. Callus is further differentiated into organized structures by somatic organogenesis. Departure from organogenesis growth is a key element in somaclonal variation. Generally, the longer the duration of cell in culture phase, the greater the chances of generating somaclonal variation.

b) Genotype

Differences in the frequency of somaclonal variation have been shown among species or among genotypes within a species. Moreover, Brar and Jain (1998) reported that somaclonal variation is genotype dependent. It is difficult to separate genotypic effect from differences in tissue culture response, e.g. medium, explant, cultural conditions and their interactions. However, genotype can influence somaclonal variation irrespective of regeneration mode.

c) Culture medium and age of the culture

The composition of the culture medium and particularly its growth regulator content are important factors that affect the rate of somaclonal variation. Brar and Jain (1998), suggested that growth regulators can act as mutagens. Most of the evidence indicates that growth regulators influence somaclonal variation during the culture phase through their effect on cell division, degree of disorganized growth and selective proliferation of specific cell types (Gould, 1984). Even in the absence of the callus phase, deformed shoot primordia have been observed in vegetatively propagated plants.

d) Number of multiplication cycles

Duration of culture medium has a marked effect on the frequency of somaclonal variation. Deverno (1995) reported that the frequency of somaclonal variation increased with the

duration of *in vitro* culture, either as callus or cell suspension. Rodrigues, *et al.* (1998), studied the influence of the number of subcultures on the somaclonal variation of banana, and concluded that somaclonal variation appeared from the ninth subculture in 2.2, and 4.35 % of the plants for the 9th and 11th subculture, respectively. The increase in somaclonal variation with the increase in the number of subcultures needs to be taken into account, especially by commercial laboratories, to assure high quality of micropropagated plants. However, plant breeders always look for useful somaclones of practical importance.

e) Source of explant and age

Both explant source and age of callus affect the extent of somaclonal variation. However, available evidence supports the age effect on the increased frequency of mutation, which is primarily due to sequential accumulation of mutations over time, rather than an increased mutation rate in old cultures (Brar and Jain 1998).

The usefulness of somaclonal variation, largely depends on how we can decrease the percentage of useless or detrimental variation *in vitro* (now by far the majority) and, how we can select and transfer useful variation into a stable, persistent and valuable change in adult plants, which in most cases are to be grown under natural conditions.

2.6.2.2 Mutation breeding

Mutation is defined as sudden change(s) in the hereditary material at gene level (gene mutation) or at the genome level (genomic mutation) such as addition or loss of a single chromosome (aneuploidy) or/and doubling of a chromosome complement (polyploidy) (Basiran, *et al.* 1998; Donini &Sonnino 1998). In a breeding context, mutation is a permanent, hereditable change in the primary structure of the genetic material making up the total genome of a cell or a plant (Mantell, *et al.* 1987). The discovery of DNA explained the

concept that mutation is a change in the nucleotide sequence (Lewin, 1994) that leads to a change in the amino acid sequence, thus altering or abolishing the activity of the protein.

The existence of mutations allows us to compare the properties of a wild-type (normal) gene with a defective gene. Protocols of protein profiles contributed more in identifying the genes that were produced from mutation. Consequently the changes that occur in the phenotypic characters of the organism could be detected. Rothwell (1993) suggested that progress in evolution depend on the ability of a gene to undergo occasional mutation. However, all hereditary variation arises from mutant DNA of somatic cells as well as germ line. These alterations include the deletion or addition of DNA material, or the rearrangement of chromosomes by means of inversion or translocation of DNA. However, a change in the primary structure of DNA may result in an altered phenotype called the mutation phenotype, which remains stable through consecutive cell generations at relatively low frequency and should be transmitted in sexual crosses.

In mutation breeding, the frequency of mutation could be enhanced by the use of mutagens. An induced mutation has a wide range of applications in breeding programmes. The methods of using mutagens in plant breeding are shown in Table 2.1.

Mutation breeding aims at altering one or a few characteristics of a generally acceptable cultivar, but otherwise retaining the original genotype unchanged (Novak, 1992; Broertjes & Van Harten, 1988). This approach might be particularly important for sterile *Musa* species where there is no sexual reproduction that could generate genetic variation. Furthermore, *in vitro* induced mutations and somaclonal variation will contribute to cross-breeding programmes as a new genetic pool for recombination and selection (Novak, 1992).

Table 2.1 Methods using mutations in plant breeding

I. Use of point mutations

1. Autogamous species:
   (a) Direct use of mutations: mutants used directly as improved varieties
   (b) Cross-breeding with mutations

      (i) Crossing the mutants with the original parent variety or line
      (ii) Crossing different mutants from the same parent line
      (iii) Crossing different mutants from different parent lines
      (iv) Crossing the mutant with a different variety or line
      (v) Crossing two varieties apparently carrying the same mutant

2. Allogamous species: induction of mutations to increase variability
3. Heterosis breeding: induction of mutations in inbred lines.
   Induction of male sterility (allogamous and autogamous)
4. Asexual plants: induction of "sports"

II. Use of chromosome mutations

1. Use of translocations: for transferring characters from other species and genera
2. Use of translocation (with known breakage points) for production of "directed" duplications
3. Diploidization of polyploids

III. Use of mutagenic agents for special breeding problems

1. Use of radiation to produce haploids
2. Use of mutagens to increase or lower the frequency of chiasma
3. Use of radiation for production of transitory sexuality in apomicts
4. Use of radiation to reduce incompatibility in wide crosses
5. Use of induced mutations special studies of genetics or physiological, morphological, and biochemical processes in crop plants

Source: Manual on Mutation Breeding, IAEA 1977.

2.6.2.2.1 Sources of Mutation

(A) Natural Mutations (Spontaneous)

This type of mutation occurs with low frequency of natural events and without intentional human intervention, these are called 'spontaneous mutations'. Recently, the origin of this mutation has been suggested in several publications, that spontaneous mutation often results from the activity of so-called transposons: mobile genetic elements that can move within the genome from one place to another and affect the activity of the gene in which they are inserted (Van Harten, 1998). Over a period of time nature evaluates these mutations through testing, and saves the most desirable genetic combinations. However, many natural mutations are recessive when compared with the commonly occurring allele (wild type) in the population (James, 1981).

The reason for this may be that the long evolutionary periods in most plant species have led to the identification and stabilization of many favorable alleles. Dominant types having a lower fitness value would be eliminated rather rapidly from the population since they appear immediately in the phenotype. Whereas, recessive alleles could be carried in the population and tested in combination with many other allelic forms at different loci before being completely discarded. In addition, mutations could be identified according to their origin (Basiran *et al.* 1998). The spontaneous rate of mutation is around 10^{-5} to 10^{-6}. If spontaneous mutations occur above the 10^{-3} level, the gene concerned is considered unstable or highly mutable.

However, each individual plant or even each plant cell may carry one or more spontaneous mutations, which were collected during its lifetime. In vegetatively propagated crops such mutations may pile up throughout consecutive generations as a consequence of their mode

of propagation. Van Harten (1998), suggested that not all phenotypically observed variation refers to genetic changes. At the same time one should be aware of the fact that not all changes within the DNA, ultimately result in permanent changes of the DNA. Moreover, even if such changes in the DNA would be permanent, i.e. refer to real mutations, they may not always result in visible or in other way detectable effects.

In vegetatively propagated plants, natural 'sports' are isolated and easily maintained through asexual propagation. For instance, in crop plants such as banana, potato, peach, pear, cherry, plum, lemon etc., spontaneous mutations occur and contribute to the development of the cultivated varieties. However, the rate of naturally occurring somatic mutation is too low to be used in the breeding process for the development of new varieties.

(B) Induced Mutations and mutagenesis

Following rediscovery of Mendel's work, interest increased in genetic variability and the potential for altering this variability at will. Gustafsson (1969) reported induced mutation research in the early 1900s. Later work demonstrated the scientific nature and feasibility of increasing mutation rates with artificial treatments. That means mutational events can easily be produced in the laboratory, therefore, this knowledge has led the plant breeders to consider producing mutations valuable for breeding use (Sigurbjornsson and Micke, 1974).

Conventional breeding strategies may in fact be impractical in all cases where the gene for a desired trait is closely linked to genes with undesirable effects, or if undesirable pleiotropic effects are known to affect the trait, or whenever interspecific or inter-generic crosses are involved (Donini and Sonnino, 1998). However, in all these cases, induced mutations may

represent a convenient approach. On the other hand, if there is no known genetic source for a desirable trait, induction of mutations is the only possible approach.

In plant breeding, mutation induction has become an effective way for extending plant genetic germplasm and improvement of cultivars. Induced mutations are similar to spontaneous ones, but their frequency is much higher. Moreover, the genetic variability resulting from induced mutations appears to be equivalent to that occurring naturally. Therefore, the basic principles for the use of induced variability are similar to those for natural variability. Mutagens are tools for enhancing and generating genetic variation by inducing changes (mutations) at the gene, chromosome and genome levels, in nuclear and cytoplasmic organelle DNA. Two methods are currently in use: physical mutagens (e.g. radiations) and chemical mutagenes. The physical methods include application of different types of radiation. Both x-ray and gamma rays are electromagnetic radiations that are primarily used to irradiate seeds or vegetative tissue. Breeders most frequently use x-rays, gamma rays, ultraviolet rays, ionized atoms and neutrons as mutagenes (Sigurbjornsson, 1983; Venkatachalam and Jayabalan, 1997) which are widely used for vegetatively propagated plants.

For practical mutation breeding, acute irradiation with fairly hard x-rays or gamma-rays is recommended using a dose rate of 100 – 1000 rad/min (and short irradiation times) (Broertjes & VanHarten, 1988). In general, gamma rays have a shorter wavelength and therefore possess more energy per photon than x-rays. Monoenergetic gamma radiation is usually obtained from radioactive isotope cobalt-60 or cesium-137, in contrast to x-rays, which are generally produced in a wide range of energies. The technical aspects of mutation breeding deal with the choice of the mutagen and dose, the starting material and population

size, the handling of mutagenized material, and the selection of suitable breeding methods (FAO/IAEA, 1977).

The dosages of irradiation can be administered by changing the distance between source and plants in gamma fields. Gamma fields or germ houses with gamma-sources have been established in several parts of the world.

Commercially important traits in horticultural plants have been altered in a positive way by the various physical mutagens. The effect of mutagenic treatments on quantitatively inherited characters should be considered because mutations are induced randomly and the mutagen rarely changes only one particular gene. The main advantage of the use of spontaneous as well as induced mutations, is the possibility of correcting one or a few negative characteristics of a cultivar or more, in general, of a specific genotype, without changing the major part of its total genetic set-up. In this way induced mutations have contributed to the development of new cultivars of crop plants (Venkatachalam and Jayabalan, 1997). All known mutagenic agents, applied at levels intended to yield an appreciable amount of visible mutations, also induce a considerable variation in quantitatively inherited characters, although the goal of selection is a certain desirable mutated trait.

(C) Mutation breeding of vegetatively propagated crops including *Musa* spp.

Particularly in vegetatively propagated crops, mutation breeding is recognized as a useful method, especially when further improvements of outstanding cultivars are desired. However, in vegetatively propagated crops several factors may complicate conventional breeding. Generally, there is a high degree of hetrozygosity, often in combination with the

presence of polyploidy, which together account for a complex inheritance and make genetic analysis difficult.

Vegetative propagation of *Musa* maintains the heterozygotic constitution of edible forms (Novak, 1992). This genetic nature is suitable for the application of mutation breeding. The heterozygotic status Aa is expected in loci of diploid cultivars while the heterozygous status can exist in two different forms, AAa and Aaa, in triploids. Spontaneous mutation (sport) is responsible for a large part of the variations present in vegetatively propagated crops.

Mutation induction techniques provide means for the rapid creation and increase of variability in crop species. However, Maluszynski *et al.* (1995) stated that most induced mutations are recessive and deleterious from a breeding point of view. For vegetatively propagated crops normally meristimetic buds are irradiated. Such buds can be found in all kinds of propagated materials, such as tubers, bulbs, cuttings, rhizomes, etc. An alternative method is to induce the formation of adventitious buds after irradiation of plant material (Broertjes and Van Harten, 1988).

In vitro shoot-tip culture composed of a meristimetic dome with two pairs of leaf primordia (1-2 mm in size) have been used as a system for mutation induction in banana and plantain (Novak, 1992). There is an increasing interest in using mutation methods in combination with *in vitro* culture. Utilization of mutagenes in combination with *in vitro* techniques is quite possible to induce specific mutation (Ortiz, *et al.*, 1995). Mak, *et al.* (1995) revealed that mutation breeding in combination with *in vitro* techniques caused morphological changes and also increased variability in quantitative traits. On the other hand, Gowen (1995) reported that the degree of mutation depends on the level and duration of the distress and potential variation increases as the size of the exposed unit is reduced. This variability

can be used as a source for selection and may be the only possibility for improving the highly sterile *Musa* clones.

The mutagenic treatment of the material is only the starting point of a breeding program. The dose applied depends on the radiosensitivity of the species in general and that of plant part and the state of development in particular. Plant parts that still have to produce new (adventitious) roots and shoots are more sensitive than plant parts with existing roots and shoot meristems. Radiosensitivity varies between plant species and depends mainly on the nuclear volume (the greater the DNA content, the more sensitive); the number of chromosomes (plants with fewer chromosomes given a certain nuclear volume are more sensitive than plants with more, smaller chromosomes) and the ploidy level (the higher it is, the less radiosensitive). Furthermore, Novak (1992) stated that differences in radiosensitivity were also dependent on hybrid constitution of the A and B genome.

The suitable doses for exposure of *Musa* shoot tips cultured *in vitro* were calculated on the basis of 50% growth reduction and on morphogenetic performance in reference to the non-irradiated control. The recommended gamma-doses are 25 Gy for diploids, 35 Gy for triploids AAA, 40 Gy for AAB and ABB, and 50 Gy for tetraploid AAAA (Novak, 1992). Adventitious buds, which proliferated from the shoot-tip base, are subcultured to the next vegetative propagation cycle (M_1V_2). Histological observation suggested that these buds are differentiated from single or a few superficial cells of an irradiated rhizome. No callus formation is involved in bud regeneration and shoots are propagated into the fourth vegetative cycle (M_1V_4), and rooted plants are transplanted into soil.

Mutation induction in the cell complex leads to chimerism (as mentioned in 2.2.2.). Hence, methods have to be established to avoid chimerism by treatment of plant structures of

single-cell origin or through mutagen treatment procedures that result in the induction of large mutated sectors in muticellular structures. However, chimerism limits the timely success of *in vitro* mutation breeding in banana because it takes months to produce new shoots (Afza, *et al.* 1994).

2.6.3 Success in Mutation Breeding

Mutation research bloomed after the UN Geneva Conference in 1966 on 'Peaceful Application of Atomic Energy', when the main emphasis was given to the radiobiological aspects of both physical and chemical mutagenes, their relative effectiveness, and their interaction with biological matter. In the late 1960s, greater interest was paid to mutation breeding technologies to be applied in breeding of both seed and vegetatively propagated crop plants. At the end of 1960s, 117 varieties were developed by mutation breeding (Sigurbjornsson and Micke, 1969). In the early 1980s, the list of mutant varieties had greatly increased to 600 (Micke and Donini, 1982) and by the end of the decade it reached 1363 entries. In the last two decades, the number of varieties resulting from mutation breeding has sharply increased. Many researchers have demonstrated the generation of mutants in banana cultivars, which affect plant form, inflorescence morphology, fruit, flower and leaf shape and colour, stem pigmentation and various agronomic characters. However, among the more useful mutants are those that change plant form, yielding capacity and duration, fruit quality and resistance to environmental stress, pests and diseases because these are the characters that would significantly affect the economics of returns of any improved crop in terms of planting density, yield per unit area, maintain cost and consumer acceptance.

In vitro mutation work by Novak and associates on banana (Omar *et al.*, 1989; Novak *et al.*, 1990), successfully regenerated an early flowering mutant from the Cavendish-type banana

cultivar Grand Naine, when treated with 60 Gy gamma irradiation. The vegetative progenies of this mutant in Malaysia were successfully developed from a GN-60A mutant, underwent further selection and were released as Novaria (Mak *et al.* 1996; Tan *et al.* 1993). On the other hand, Kao (1979) and Smith *et al.* (1990) also produced changes in plant stature for the Cavendish types treated with gamma rays. These mutants with changes in plant stature and forms are the results of the action of various genes regulating growth habit, growth rate and differentiation of organs at different phases of plant development (Kawai, 1977).

In Cuba, a cultivar of cooking banana (ABB group) called Burro CEMSA, resulted from mutagenic treatment. Ortiz and Vuylsteke (1996) stated that this cultivar had been used as starting material for investigating the potentials of inducing 'somaclonal variation'. For the yielding ability and fruit quality in banana, the few potential mutants with yielding potential were those reported by Kao (1979) and Tan *et al.* (1993) for the Cavendish types as a result of gamma induction. Smith *et al.* (1990 and 1993) also carried out preliminary treatments with gamma rays on several banana clones to evaluate for resistance to *Fusarium* wilt (Race 4) and reported to have generated potential mutants of Dwarf Parfitt, named 'Giant Parfitt', although release is pending on further confirmation of the stability of disease resistance and consumer acceptance. De Beer and Visser (1994) successfully produced an improved local selection Sodwana HBBJM with good tolerance to *Fusarium* wilt and acceptable bunch, as a result of 60 Gy gamma irradiation.

2.6.4 Constraints in Mutation Breeding

Mutation breeding is one-cell events and therefore mutagenic treatment of multicellular tissues like bud meristems may lead to chimera formation, although the production of adventitious plants *in vitro* resulted in solid mutants (Broertjes and Lock, 1985). Chimeras

can roughly be described by Brocrjes and Van Harten (1988) as plants with two or (more) genetically different components in their somatic tissue. Chimera formation and competition within a plant between mutated and non-mutated tissue are considered as the main bottle-necks in mutation breeding of vegetatively propagated plants.

The chimeric plants are commonly referred to as M_1 plants. Sigurbiornsson (1983) stated that dominant mutations can be expressed in the M_2 plants, but only in that chimerical sector which results from original mutated cell. The M_1 plants will be heterozygous for newly induced mutation genes and will segregate into mutant and non-mutant phenotypes in M_2 generations. Polyploidy causes a further complication for recessive mutations, which are only revealed in the homozygous recessive condition. The mutagenic treatment may cause physiological damage or injury to the plant parts, often resulting in significant reduction in germination, plantlet growth and fertility of the M_1 plants. Mak and Ho, (1997) revealed that the degree of physiological injury caused by the mutation is usually correlated with radiation doses.

2.6.4 Ploidy Effects in Banana Phenotypic appearance

Plants with an increased number of genomes, i.e. with more than two genomes, known as "polyploids" can be found in nature or artificially induced. Moreover, plants have the ability to tolerate addition of entire chromosome sets to the diploid amount. Broertijes and Van Harten (1988) revealed that the ploidy level could be artificially increased. Most cases of polyploidization concern the situation where mitosis is not followed by cell division or division of nucleus. This situation may arise after temperature shocks or artificial wounding of a plant, followed by callus production and regeneration. Because somatic tissues must be treated, a complication that may result is that ploidy chimeras are generated in the same way

as normal chimeras after mutagenic treatment of multicellular apex. Although artificial induction of polyploidy has led to results of direct practical importance, the method does not automatically imply that better varieties can be obtained.

Ploidy affects the phenotypic expression of several morphological traits e.g. root diameters were influenced by ploidy, where triploid roots are 6.2 – 8.5 mm., diploid roots are 5.1 – 5.7mm and tetraploids roots towards maximal vigor in natural plants of *Musa* (Ortiz, 1995). Simmonds (1948) studied the effect of ploidy on the leaf morphology of *Musa* spp. and showed that minimum thickness showed a slight but steady increase with ploidy. However, diploids tended to have right lamina half broader than the left, while the reverse holds true for polyploids. The polyploids had more drooping leaves than the diploid and the effect increases with an increase in ploidy. This could be the consequence of weaker leaf petioles in the triploids than in the diploid and still less in the tetraploids. In addition, percentage of dry matter also declined with increasing ploidy (Ortiz, 1995). The variation in nuclear DNA content occurs both within and among plant species naturally, or due to mutations. Cytologically, most autopolyploidy have meiotic irregularities because of the presence of extra homologous chromosomes. This is well demonstrated by triploid, which is usually highly sterile i.e. the gene dosage is unbalanced in most of the cells after meiosis. Consequently, the pollens of triploids are often very sterile, and seed set is very low. This sterility has commercial value in plants such as watermelon, where almost the seedless fruit is more desirable than that of the fertile diploid.

The second main type of polyploidy is allopolyploid, that investigated by Rothwell (1993) in which the chromosome sets are derived from different races or species. Various lines of cytogenetic evidence leave no doubt that allopolyploid have a major role in the evolution of plants. Furthermore, the author concluded, in flowering plants polyploid is very

characteristic and has played an important role in the evolution of many plant groups, including a number of valuable crop plants. Chromosome counts using root tips (Ortiz, 1995) or pollen mother cells and the determination of stomata size and number (Simmonds, 1962) are the cytological techniques used to determine the ploidy in *Musa* spp.

A characteristic feature of interphase nuclei is the presence of well-pronounced chromocentra, their number partially resembles the ploidy level of a given clone (Novak, 1992). However, the metaphase chromosomes are very small with a hardly distinguishable morphology. This conventional method has many disadvantages. So far, the main obstacles to the use of conventional chromosome counting in practical breeding and taxonomic studies include: the very low frequency of dividing cells; the rare occurrence of well-spread metaphase plates for chromosome counting; and the time consuming procedures to analyze representative amounts of samples (Novak, 1992). However, DNA flow cytometry (Arumuganathan and Earle, 1991) has been developed as an alternative method to determine DNA content.

2.7. Flow Cytometry for Analysis of *Musa* genome

Flow cytometry analysis of nuclear DNA content provides an alternative technique for individual and large-scale ploidy screening (Dolezel, 1991). This technique is a powerful analytical tool based on the use of DNA-specific fluorochromes and on analysis of the relative fluorescence intensity of stained nuclei (Galbraith *et al.*, 1983; Lister, 1990). A Flow Cytometer is an instrument, which analyses optical parameters (light scatter, fluorescence) of particles in flow, moving with respect to the point of measurement (Dolezel, *et al.* 1997). However, the measurement in flow permits analysis at a very high

speed, typically 10^2 - 10^3 per second. Furthermore, the particles to be analyzed are selected randomly from the whole population without any bias.

The samples to be analyzed are introduced into the centre of the flow chamber filled with a fast moving sheath fluid. Through a focus of exciting light, the particles scatter this light, if they contain a natural flourochrome or have previously been stained with fluorescent dye, they will fluoresce (Dolezel, *et al.* 1997). Flow Cytometry was originally developed as a rapid method for analysis of blood cells. At present, flow cytometry is extensively used in basic and applied research (e.g. botany, zoology, microbiology, marine ecology, embryology, immunology, genetics and molecular biology) as well as in clinical diagnosis, medicinal practice and industry-including seed production and plant breeding.

Galbraith, *et al.* (1983), studied cell cycle in intact plant tissues through flow cytometeric analysis. They chopped plant tissues in buffer containing mithramycin, where intact nuclei were released in the suspension. The amount of nuclear DNA in the homogenates of plants were rapidly and accurately determined by flow microfluorometry, and the distribution of nuclei involved in the cell cycle was charted for tissues selected from different physical locations or developmental stages. Dolezel (1991) reviewed the flow cytometric analysis of nuclear DNA contents in higher plants. He stated that the methods of flow cytometric analysis of nuclear DNA content were originally developed for human cells. However, during the last decade this methodology has been adapted to the analysis of plant cells. In this review special attention was given to preparative techniques, methods of instrument calibration and standardization, and interpretation of data.

2.8. Molecular Markers in *Musa* Breeding Programs

Earlier, attempts were made to distinguish banana cultivars by using biochemical markers (Novak, 1992). The detection of nucleotide sequence variability and its exploitation in the form of genetic markers has revolutionized many aspects of plant genetics. Molecular variation has been characterized at both the DNA and protein level using a number of different approaches. In many instances, the observed variation is a DNA or protein electrophoretic band of altered mobility or a chromosome-altered structure (Kaeppler *et al.* 1998). A wide variety of methods are available to characterize plant genomes. These methods include: Restriction Fragment Length Polymorphism (RFLP); Random Amplified Polymorphic DNA (RAPD); DNA Amplification Fingerprinting (DAF); Amplified Fragment Length Polymorphism (AFLP); Short Sequence Repeats (SSR) (microsatellites); Temperature Gradient Gel Electrophoresis (TGGE); and Denaturing Gradient Gel Electrophoresis (DGGE), (Kaeppler *et al.* 1998). Many of these methods have potential for application in the analysis of somaclonal variation. However, only RFLP and RAPD analysis have been applied widely in analysis of somaclonal variation in *Musa.*

A wide range of techniques may often be applied to the same system. For example, Wolff *et al.* (1996) used RAPD, SSR, and RFLP markers to evaluate somaclonal variation in vegetatively propagated chrysanthemum cultivars. Newbury and Ford-Lloyd *et al.* (1993) suggested that DNA-based markers clearly allow the direct comparison of the genetic material of plants avoiding any environmental influences on gene expression. DNA markers can be used to obtain much information about the genes, which influence agriculturally important trait, thus facilitating breeding efforts (Paterson *et al.*1991). DNA oligonucleotide and DNA amplification fingerprinting techniques have been used for the identification of cultivars and breeding materials of banana and plantain (Afza, *et al.* 1994). Techniques such

as isozymes analysis, RAPDs and AFLPs have been used successfully to distinguish polymorphisms between different genotypes, cultivars, and mutant and normal plants of *Musa* (Hammerschlag, *et al.*1992).

2.8.1 DNA Markers

The exploitation of DNA polymorphisms by an ever-increasing number of molecular marker technologies has begun to have an impact on plant genome research and breeding. Polymorphisms have been detected in restricted genomic DNA of plants and have paved the way to the development of molecular markers for plant breeding (Winter and Kahl, 1995). The rapid change to the use of these markers rather than any others is a consequence of the numerous advantages they provide for the researcher. Unlike isozymes, their expression is not necessary for their detection and all markers can be detected with a single technique.

Recently the technique of amplified fragment length polymorphism (AFLP) has been suggested as the most powerful tool in the molecular breeding of plantain and banana. AFLP analysis has the ability to identify a large number of polymorphic bands without any prior knowledge of the organism (Kaemmer *et al.* 1997). This technique uses synthetic simple sequence repeats as probes for RFLP detection (Ali *et al.*, 1986). James and Mayo (1997) used AFLP technology for detection of DNA polymorphisms in *Musa* cv. "Grand Naine" in Mexico. However, these techniques have not been so successful in distinguishing between somaclonal variants (off-types) arising from tissue culture of bananas and plantains. Changes in chromosome number have been correlated with some of these variants (ascending or descending aneuploidy) and may account for some variants.

Chloroplast and nuclear DNA restriction fragment length polymorphism (RFLP) analyses provided the first successful application of DNA markers to bananas and plantains (Gawel and Jarret, 1991; Jarret *et al.,* 1992). RFLPs were successfully used for low density genome mapping (Faure`*et al.,* 1993), molecular taxonomy (Carreel *et al.* 1994), and inheritance of cytoplasmic organelle studies (Faure`*et al.,* 1994). However, this technique is very labour-intensive and non-selective for highly polymorphic DNA. For these and other reasons another RFLP technique, oligonucleotide fingerprinting, was developed in order to increase the informativity of hybridization patterns (Faure`*et al.,* 1993).

In principle, RFLP probes are single-copy markers that detect only one defined genomic fragment each, although multilocus probes, such as repetitive DNA or cDNA, that detect several fragments at a time, are also used. Winter and Kahl (1995) revealed that, restriction endonucleases cut genomic DNA at specific palindromic recognition sequences, generating thousands of fragments of defined length, the number of which depends on the number of recognition sequences in a given genome. RAPD technique has also been successfully used to distinguish between diverse *Musa* germplasm. It is relatively inexpensive, fast, reliable, and utilizes arbitrarily defined oligonucleotide primers that are applicable to serve as "probes" for a wide range of material.

2.8.2 Polymerase Chain Reaction: PCR-Based Fingerprinting of Bananas

Over the last few years, Polymerase Chain Reaction (PCR) technology has had a significant impact in almost all areas of molecular biology and modifications of the basic procedure has allowed the development of numerous assays for detecting variation at the nucleotide level. PCR enables the scientist to selectively amplify specific DNA sequences without using hybridization. Barnum (1998) suggested that any DNA sequence could be isolated from the

total DNA of an organism. Generally, however, the sequence of the region that flanks the DNA to be amplified must be known so that 'primers' used in amplification can be synthesized. Two short oligonucleotide primers are used (Barnum, 1998) that flank the DNA region to be amplified. The primers anneal or hybridize to the target sequence, one on each strand of the double-stranded DNA molecule. The oligonucleotides define the limit of the region to be amplified, and the DNA polymerase replicates the DNA between the primers using all four of the deoxyribonucleotides (dGTP, dATP, dCTP, dTTP) provided in the test tube, the template is denatured by high temperature, the primers are annealed by lowering the temperature, and the DNA polymerase extends the DNA from the primers. Sequentially, repeated cycles of denaturation, primer annealing, and DNA synthesis result in exponential amplification of DNA.

All PCR-based molecular markers appear to detect a high level of polymorphism within a range of *Musa* breeding populations (Crouch, 1998). In addition, PCR-based assays are amenable to the large scale screening of breeding populations. Polymorphism detected by RAPD has proved to be useful for identifying variation at different levels, ranging from that found between genotypes of *Musa,* sweet potato, and papaya. RAPD or any other PCR-based analysis would be an attractive method for detection of dwarfs produced during banana micropropagation, because it needs only a small amount of tissue sample from an *in vitro* grown plant (Damasco, *et al.*1996 b).

Shoseyov *et al. (*1998) demonstrated that the RAPD markers are useful for detecting genetic instability of *in-vitro*-propagated *Musa*. Similarly, Howell *et al.* (1994) used RAPD to identify nine genotypes of *Musa* representing AA, AAA, AAB, ABB, and BB genomes. The pattern of variation observed following the application of multivariate analyses to the RAPDs banding data was very similar to the pattern of variation defined using

morphological characters. Also, this pattern of variation is used to assign *Musa* material into the different genome classes. This technique can also be used to test the genetic integrity of somatic embryo of *Picea mariana* (Isabel *et al.,* 1993), the genetic stability of the plants regenerated from protoplast *Festuca pratensis* Huds (Valles *et al.,* 1993), somatic hybrid between *Brassica napus* and *Barnarea vulgaris* (Craigh *et al.,* 1994 a and b), somaclonal variation on *Oryza sativa* var. indica (Godwin *et al.,* 1997) and analysis of the genetic stability of plantlet *Populus deltoides* derived from micropropagation (Rani *et al.,* 1995).

So far, PCR methods offer several advantages when compared to hybridization-based techniques, especially if large numbers of samples have to be processed (Kaemmer *et al.* 1997). Random priming of DNA has been exploited for the generation of a theoretically unlimited number of DNA markers for *Musa* germplasm identification as well as *Musa* mapping (Kaemmer *et al.*, 1992; Howell *et al.,* 1994; Faure` *et al.,* 1993; Bhat *et al.,* 1995).

2.9. The role and potential of gibberellic acid

Damasco, *et al. (*1996a); Barratt and Davies (1997) suggested that gibberellic acid (GA_3) is a major growth regulator controlling many plant responses including stem and petiole elongation in bananas. GA_3 controls both cell division and cell elongation, and has been documented to induce cell division on both apical and biochemical wall properties. Gibberellins (GAs) are known to promote cell elongation, and induce hydrolytic enzymes in the aleurone layer of cereal seeds (Matsukura *et al.*, 1998). Auxin also promotes cell elongation, and GA has been suggested to increase the endogenous level of IAA (Reid and Davis, 1992; Law and Hamilton 1989). It was shown that GAs strikingly promote cell division in the intercalary meristem of the young plants, and that is due to the activation of histone HI kinase and cyclin genes at the initial stage (Sauter *et al.* 1995). It is also known

that GA controls the direction of cell growth by controlling the orientation of cellulose microfibrils, which seem to be determined by the underlying arrangement of cortical microtubules, and that the orientation of the microtubules is influenced by auxin and GA (Matsukura *et al.* 1998). Both GA- and auxin-induced changes in the mechanical properties of cell walls of stems, coleoptiles and roots have also been reported (Kutschera and kende 1988; Tanimoto 1994), suggesting that an increase in cell wall extensibility results in loosening and relaxation of cell walls, thereby allowing turgor-driven elongation to occur.

In the GA-deficient dwarf mutant of banana, however, gibberellic acid (GA_3) increases 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 and further analysis is needed. In the GA-deficient type of dwarf mutant, GA biosynthesis pathway from *ent*-kaurene to GA_{53} is blocked (Matsukura *et al.* 1998). The elongation of the second leaf sheath of this mutant plant is stimulated by exogenous GA applications. GA_3 enhanced the growth of the second leaf sheath, but auxins did not (Matsukura *et al.* 1998).

2.9.1 Detection of dwarf off-types in micropropagated Bananas

The extremely high percentage of somaclonal variation (off-types) occurring in tissue-cultured banana plants, sometimes in excess of 90%, causes financial hardship for growers and especially for breeders. The most commonly occurring off-type has been "dwarfism". However, other off-types have sometimes been observed at high levels. The appearance of these plants is, therefore, a common phenomenon in bananas and plantains. Zwar and Chandler (1995) found that dwarfism in the GA-deficient dwarf mutant of barley, was due to shorter leaf blade and sheath in seedling, and shorter elongating internodes at the jointing

stage. The shorter petioles and internodes and the lower leaf ratio identify the dwarf off-type.

Dwarfism is controlled by a single dominant gene (DW) in "AAA" bananas (Ortiz, 1995). Rowe and Rosales (1993) also showed that modifier genes affect the degree of dwarfism. Ortiz and Vuylsteke (1993) working in albinism in bananas found that the substitution of the dominant DW by a recessive allele only reduced the height of the plant. Israeli, *et al.* (1995) suggested that, off-type plants might differ permanently or temporarily from the source of the plants. The latter are a result of an epigenetic or physiological effect, and are characterized by a non-heritable change and are reversible. The stage of development at which off-types can be identified is variable. Studies of Daniells and Smith (1993) have shown that at least half of the population of dwarf off-types in a batch of tissue-cultured plants can be identified prior to field planting if left until about 20 cm in height. Selection is most effective when plants are growing vigorously and uniformly.

2.9.1.1 The use of Gibberllic acid for detection of dwarf off-types

The gibberellins (GAs) are a family of compounds based on the ent-gibberellane structure (ring system) (Davies, 1995). Its application to dwarf or rosette plants, dormant buds, or dormant and germinating seeds often results in dramatic and diverse effect on growth (Sponsel, 1995). Gibberellic acid (GA_3) is often used as a supplemental growth regulator to auxins and cytokinins in culture media. In some banana cultivars it is possible to detect dwarfs in the flask by adding GA_3 to the culture medium (Damasco *et al.*, 1996a; Israeli *et al.*, 1995; 1991; Smith and Drew, 1990; Reuveni 1990; 1989; Cote *et al.*, 1993 and Graebe 1987).

Damasco *et al.* (1996a) used the procedure of Smith and Drew (1990) for early detection of dwarf off-types in micropropagation of Cavendish bananas (cultivars New Guinea and Williams) during *in vitro* culture and deflasked plants. For the screening of plants during *in vitro* culture, leaves and roots of single-shoot cultures of banana plants are trimmed and the explants placed on a solid MS medium containing filter-sterilized GA_3 at different concentrations. The screening of plants at deflasking was achieved by spraying GA_3 solution onto deflasked plants and measuring various plantlet responses. Damasco *et al.*, (1996a) concluded that the most useful identification criterion is elongation of the sheath of the first leaf emerged after GA_3 application. Reuveni (1989) found that normal plants respond with fast elongation and reduced leaf production, while dwarf variants are much less responsive. The use of such a selective medium is valuable where it is crucially important to detect off-types as in germplasm exchange and conservation.

Reuveni (1990) measured the reaction of dwarf and normal regenerates of 'Williams' banana to GA_3 several weeks after subculture on GA_3 medium, dwarf and normal plantlets responded differently to gibberellic acid. In normal plantlets, GA_3 elongated internodes while dwarf plantlets were much less sensitive to GA_3. The effect of GA_3 treatment was also tested on 'Grand Nain' *in vitro* plants during acclimatization (Cote *et al.*, 1993) and the same result was obtained. This treatment resulted in faster extension in normal compared to dwarf plants. On the other hand, Shoseyov *et al.*, (1998) reported the disadvantage of these methods that are based on early morphological identification is that they are prone to environmental effects. They are sufficient to detect undesirable dwarfs in a commercial multiplication operation, but are not sufficient when *in vitro* germplasm conservation is considered. Furthermore, Israeli *et al.* (1995) suggested that, this technique does not seem to be applicable to commercial operations because of the added cost.

2.9.1.2 The use of Molecular Marker for the detection of dwarf off-types

In a preliminary study various RAPD primers and polymerase chain reaction (PCR) amplification were applied to obtain polymorphic DNA bands, thus enabling fingerprints of normal and dwarf bananas to be obtained (Damasco *et al.* 1996 b and Williams *et al.* 1990). RAPD technique would be used for detection of dwarfs produced during banana micropropagation because it needs small tissue samples from *in vitro* grown plants. This technique would be rapid, thus dwarfs could be eliminated early in the micropropagation cycle. However, the research adopted in this thesis studied the possibility of using the RAPD technique for detecting variations such as dwarf somaclones of mutated Pisang Berangan cv. Intan (AAA).

Fresh leaves of banana plants were stored at -70°C prior to DNA extraction. By using CTAB DNA extraction procedure developed by Gawel and Jarret (1991), the total genomic DNA could be extracted. Hence, target DNA sequences were amplified by PCR in programmable thermocycler. PCR amplification products have been analyzed by 2% agrose/TBE gel electrophoresis and stained with ethidium bromide. The profiles of amplified products from New Guinea Cavendish and Williams and their associated micropropagation-produced dwarfs were compared by Damasco *et al.*(1996 b), for markers specific to dwarfs. The RAPD band could appear in all normal and disappear in all dwarfs for all accessions of two Cavendish banana cultivars. So far, polymorphisms among normal plants and dwarf plants have been detected by using this technique.

2.10 Constraints in Banana production

In Malaysia, banana is frequently cultivated as an intercrop with oil palm, rubber, coconut and with perennial fruits such as durian, dokong and others. As such the hectareage planted would change according to the different stages of development of these main crops. In addition, land that is planted solely with banana as the main crop is also on the decline. As a consequence, there has been a decrease in suitable land available to agriculture in general. Bananas, which have been cultivated near urban areas, have given way to infrastructural development.

The high cost of agricultural input such as labour, fuel, fertilizers, agro-chemicals, packing materials and transportation has made farming in general less attractive to local as well as foreign investors. There has been a shortage of labour-saving technology and it has not been widely used due to the small and scattered nature of the farm.

The major constraints on banana production are pests and diseases. They cause losses both in fruit production and crop quality. Liew, *et al* (1998) investigated some of the important field diseases that have seriously affected banana cultivation world-wide include: Panama disease (*Fusarium* wilt), Black Sigatoka, Yellow Sigatoka, Moko disease, Banana Bunchy Top and Leaf Freckle (on Berangan). Although Sigatoka diseases were high on the list of concerns, especially in the Pacific, *Fusarium* wilt ranked as the number one problem in most countries (Valmayor, 1991). Jones (1994) stated that this disease seriously affects the cultivation of popular cultivars in Asia such as Silk, Pisang Awak, Pisang Berangan, Gros Michel and was also is a problem on Cavendish types grown for both export and local production.

2.10.1 *Fusarium* Wilt, distribution and spread

Fusarium wilt (Panama disease) caused by soil-borne (inhibiting) fungus, *Fusarium oxysporum* f. sp. *cubense* (FOC), colonizes and occludes the xylem of susceptible cultivars to cause a terminal wilt (Ploetz, 1994). The fungal pathogen of this disease is a facultative parasite, which can survive in infested soils in the absence of the host plant for several years. There is no cost-effective means of chemical control in established field infestations. Liew *et al.* (1998), suggested that the genus *Fusarium* even though it has no known sexual reproductive stage is highly variable functionally and phenotypically.

Fusarium wilt is a typical vascular disease causing disruption of translocation and systemic foliage symptoms, which eventually leads to collapse of the crown and pseudostem. The pathogen develops in the vascular system of the host plant by producing microconidia, which are conveyed upwards in the transpiration stream. Perforation plates in the end walls of xylem elements provide natural barriers but the fungus grows through them to produce a new crop of microconidia.

Fusarium produces a very wide range of toxic metabolites known as phytotoxins. The notable toxins produced are fusaric acid and the closely related compound dehydrofusaric acid. These compounds are considered to be wilt toxins that contribute to the whole fusarial syndrome. It was proposed that the metabolites produced in culture filtrates of the fungus be tested for possible use in mass screening of banana plants (Ho; *et al.*, 1994). Gowen (1995) stated that symptoms generally commence as a premature yellowing of the oldest leaves followed by necrosis and collapse. The examination of petioles of the older leaves may reveal chlorotic streaks or patches and such leaves may collapse before chlorosis of the lamina is apparent, due to folding of the petiole close to the pseudostem.

Moreover, longitudinal sections of the pseudostem will show sign of vascular discoloration and this discoloration will be more distinct in the corm tissue. Most of the cultivars in Malaysia, such as *Rastali, Awak, Embun, Berangan, Mas, Raja, Abu, Nipah* and the introduced Cavendish cultivar 'Grand Naine' and 'William's' are susceptible to the fungus (Nik Masdek, 1991; Yaakob *et al.*, 1994).

Fusarium wilt was first reported in Australia in 1874 on Silk (*Pisang Rastali* type), followed by Costa Rica and Panama in 1890, and soon after in the tropical Americas and Africa (Ploetz, 1994). In Asia, it was first reported in India in 1910, Java in 1916 and the neighbouring countries in the 1920s. While in Taiwan, *Fusarium* wilt was first recorded on Cavendish clones in 1965 (Siti Hawa 1996 and Hwang 1985).

2.10.1.1. Pathogen Variability

The fungus is classified into pathogenic races, which are differentiated, by the resistance or susceptibility response of a selected range of differential banana clones to the fungal isolates. There are four recognized races. Race I have a worldwide distribution and caused the displacement of Gross Michel (Pisang Embun; Pisang Bunga, Silk, Pome, Pisang Awak, Pisang Berangan). Race 2 attacks Bluggoe and other cooking banana varieties (ABB genome) less-widely grown, clones. Race 3 attacks *Heliconia* spp., which is botanically related to the banana. Lastly, the new race from Taiwan designated as Race 4 attacks Cavendish clones e.g. Grain Naine Silk, Pome, and Bulggoe.

Pathogenic variation exists within each of the races described above. It is well recognized that the term "race" in FOC does not imply a defined, genetic relationship with the host, as occurs with other pathosystems (Ploetz, 1994). Races of FOC are simply groups of strains

that are pathogenic on the differential cultivars. *Fusarium* wilt race 4 was first established in Taiwan in 1977 and has since become widespread in all banana-growing areas of Taiwan, attacking the Cavendish cultivars. Outbreaks of FOC on Cavendish cultivars have since been reported from Australia, South Africa, the Canary Islands, the Philippines, Indonesia and more recently Malaysia as reviewed by Stover (1990) and Ploetz (1990 and 1994).

Liew (1997) reported that genetic variation within a non-sexually reproducing fungal population could be studied using suitable genetic markers. Vegetative compatibility is one such genetic marker and the grouping depends on the ability of fungal hyphae from strains with a similar compatibility gene to anastomose and fuse together to form heterokaryons (Liew and Chu, 1992). Using complementation matings between nitrate non-utilizing mutants, isolates can be categorized into similar genetic classes called Vegetative Compatibility Groups (VCGs) (Puhalla, 1985). Isolates in a VCG are often in the same race, and usually share similar geographic ranges and physiological characters. Unlike other formae specialis of *Fusarium oxysporum*, FOC is extremely variable and 24 different VCGs are currently established (Leslie, 1993).

Liew *et al.* (1998) stated that race 4 has two sub-variants, the sub-tropical Race 4 has been reported from Taiwan, Australia, Canary Islands and South Africa, while a more aggressive tropical variant belonging to a different vegetative compatibility grouping has been reported from Peninsular Malaysia, Indonesia (Sumatra and Sulawesi) and the Philippines.

2.10.2 Breeding for Disease Resistance/ Tolerance

Breeding for disease resistance using classical breeding methods remains a difficult endeavour because of high sterility, polyploidy and long generation times of most edible

cultivars (Swennen and Vuylsteke, 1993). The use of disease resistance in bananas against Panama disease of *Fusarium* wilt can serve as a useful case-study in understanding the requirements for long-term, economical and successful use of this method for disease control (Ploetz *et al.*, 2000). Breeding for disease resistance is no different. Variation can be introduced by sexual hybridization, mutation, or by some uncharacterized mechanism of somaclonal variation (Glenn and Joseph, 1984).

Disease resistance is a main target for biotechnology research in *Musa*. Advanced diagnostic techniques are being developed for the detection of *Musa* pathogens and for screening segregating plant populations in resistance breeding programmes. Reliable selection for resistant genotypes may soon be available for juvenile plant stages, while *in vitro* cell selection seems to be a long-term target for basic research before it can be applied in breeding programmes. Somatic cell genetics, especially somatic embryogenesis and cell colony formation in protoplast cultures should facilitate the use of genetic engineering for banana improvement (Hammerschlag and litz, 1992).

Genetic diversity for disease resistance has been maximized whenever the host gene center and pathogen gene center coincide (Glenn and Joseph, 1984). However, new and aggressive genotypes in the pathogen select against new resistances in the host and vice versa. Hence, these sites produce the most virulent pathogens as well as the most resistant hosts allowing the crop to be grown even in the most inhospitable disease areas.

Disease resistance in plants is usually conditioned by one or a few genes with varying degrees of dominance. The breeders can be most effective when the mode of inheritance is fully known. When resistance is conditioned by a single gene with complete dominance, progress in disease resistance breeding can be rapid. Hammerschlag and Litz (1992)

investigated the genetics of the host-pathogen interaction of FOC. They concluded that both major (dominant) and minor genes are involved in governing the expression of tolerance/resistance to the pathogen. From the breeding point of view, a major problem is screening the required trait out of many plants in various degrees of development during the propagation procedure. Primary screening must be done as early as possible and the final screening in the field should involve only a limited number of preselected plants (Smith *et al.* 1990a).

Several systems of different complexity can be used for *in vitro* screening and selection for environmental stress tolerance and disease resistance in *Musa* (Hammerschlag and litz, 1992). These include protoplasts, cell suspension cultures, calli, somatic embryos, meristem tips, regenerated shoots or intact plantlets.

Successful *in vitro* screening for resistance to FOC using meristem culture plantlets has been demonstrated in Taiwan (Morpurgo *et al.* 1994), where both race 1 and race 4 of FOC have been used for the preparation of culture filtrate. Conidia were collected from fungal cultures grown on potato dextrose agar in the light. After 21 days of growth in Czapek-Dox medium, the fungus produces maximum toxicity in its filtrate. Crude filtrates from both races were shown to reduce growth of isolated shoot tips proportionally to their concentration. The pathogen virulence testing procedure on young banana plants of Sun and Su (1984) at the nursery stage under green house conditions using crude filtrates (Ko *et al.* 1973) from both races and the proposed sand-culture (Liew, 1996) have been adopted.

An alternative protocol was used by Brake *et al.* (1995) using conidial suspension isolated from race 4 in Armstrong's liquid media (Booth, 1977). The plantlets screened at the nursery stage i.e. 45 – 60 days in acclimatization, roots washed in running tap water and

then immersed in the spore suspension for 2-3 hours. Inoculated plants were re-potted in sterile soil. Plants could be monitored for one month for recording of the symptoms and evaluations. Immersion of roots into the conidial suspension proved efficient and successful compared to field screening, which requires a long period of time.

Liew *et al.* (1998) suggested that the test plants could be evaluated for response to infection based either on visual symptoms intensity and degree of leaf yellowing or necrosis or based on the amount of discolouration in the dissected corm. Moreover, in the evaluation system described by Brake *et al.* (1995), they established 6 different response level based on visual symptoms, and 5 different categories of corm discolouration. The scale similar to the one adopted by INIBAP for adult plant evaluation in the field for external symptoms as well as internal symptoms at time of harvest.

Field screening under stress environment for fungal evaluation of selected plants. To facilitate disease screening in the field, a quarantine field heavily infested with *Fusarium* race 4 known as "Hot-Spot" was established in United Plantations Bhd. in Malaysia. This facility allowed complete disease screening at nursery and field planting stages for this pathogen.

Cloning of plant genes responsible for pest and disease resistance will firstly help in elucidating some of the biological mechanisms of resistance, particularly with regard to those operating at the molecular level (Mantell *et al.* 1987). Transfer of specific genes from one species has been shown to be of great value in exploiting resistance mechanisms more fully. For example, the genes that determine the production of chitinases, a group of enzymes that degrade a component of fungal cell wall chitin, are considered as general defense factors of plant resistance to pathogenic fungi (Hammerschlag and Litz, 1992). The

authors reported that these genes have been cloned and evaluated as inhibitors of fungal diseases in plants. Transformation of banana cells with chitinase genes should be evaluated for their potential for increasing tolerance to *Fusarium*.