PLANT GROWTH REGULATORS AND SOMACLONAL VARIATION IN CAVENDISH BANANA (*Musa* AAA cv. *Zelig*)

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PREFACE

This thesis was written after an investigation made in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of KwaZulu-Natal, Pietermaritzburg under the supervision of Professor Johannes van Staden and co-supervision of Doctor Catherine. W. Fennell.

I hereby declare that, unless specifically indicated, this thesis is the result of my own investigation. No part of this thesis has been submitted in any form to any University for the purpose of obtaining a degree.

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We agree that the above is correct

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African Biotechnologies Ltd, South Africa, for supplying *in vitro* grown banana plantlets.

Lastly, I would like to praise and thank the Almighty God for making everything possible.

LIST OF ABBREVIATIONS

2,4-D	2,4- Dichlorophenoxy acetic acid
A	Represents the *Musa acuminata* genome
AB	Represents a cross between the *M. acuminata* and *M balbisiana* genomes
AP-PCR	Arbitrary primed PCR
APC	Anaphase – promoting complex
AFLP	Amplified fragment length polymorphism
B	Represents the *M. balbisiana* genome
BA	Benzyladenine
cDNA	copy DNA
CDK	Cycline – dependent kinase
cv.	Cultivar
DAF	DNA amplification finger printing
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate set
FAO	Food and Agricultural Organization of the United Nations
Fv/Fm	Chlorophyll fluorescence ratio
G_1 & G2	Gap 1 and gap 2 of the cell cycle
GA_3	Gibberellic acid
GOT	Glutamate oxaloacetate transaminase
HPLC	High performance liquid chromatograhpy
IAA	Indole-3- acetic acid
IBA	Indole-3- butyric acid
M	Mitosis
MAAP	Multiple arbitrary amplification profiling
MDH	Malate dehydrogenase
MS	Murashige and Skoog basal medium
MWM	Molecular weight marker
NA	Nucleic acids

NAA α-Naphtalinacetic acid

PAGE Polyacrylamide gel electrophoresis

PCR Polymerase chain reaction

PGM Phosphoglucomutase

PRX Peroxidase

RAPD Random amplified polymorphic DNA

RFLP Restriction fragment length polymorphism

S Synthesis (DNA) phase of the cell cycle

SKDH Shikimate dehydrogenase

SSR Simple sequence repeats

ssRAPD Silver stained RAPD

STS Sequence tagged sites

SV Somaclonal variation

Ta Annealing temperature

TDZ Thidiazuron

Tm Melting temperature

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ABSTRACT

Cavendish bananas are the most important sub-group of all bananas. They includes more than 30% of the global banana production and almost all bananas exported are of the Cavendish type. This sub-group is also an important food source. Most of the fruit is consumed locally, such that only 35% enters the international market. To meet the regular demand for domestic consumption and market supply there must be a reliable production strategy. The technique of tissue culture is a better option than conventional propagation techniques. However, the high incidence of somaclonal variation among plants derived from tissue culture is a problem for commercial producers. Several factors such as genotype, tissue source, duration in culture, and the tissue culture technique employed, cause somaclonal variation.

The impact of plant growth regulators on somaclonal variation was studied on Cavendish banana cv. '*Zelig*' obtained from African Biotechnologies Ltd., South Africa. *In vitro* grown plants at the 4^{th} multiplication cycle were supplied for the investigation.

The first component of the investigation dealt with the effect of types of plant growth regulators. Combinations of the auxins IAA, IBA and NAA with the cytokinins BA and TDZ were used to culture the plants for ten multiplication cycles. Plants were then randomly selected to collect leaf material for DNA extraction and RAPD analysis. The second aim of this study was to investigate the effect of the cytokinin BA on plantlets subcultured over 5-10 multiplication cycles. The auxin IAA at the concentration of 2 mgl^{-1} was combined with BA at concentrations of 2.5, 5.0 and 7.5 mgl^{-1}. Plants were analyzed at each level of subculture from the 5^{th} to the 10^{th} cycle for respective cytokinin concentrations. Plants were then randomly selected for the collection of leaf material for DNA extraction and RAPD analysis.

DNA was extracted from leaf tissue of *in vitro* grown plants using a modified CTAB extraction procedure. DNA amplification products were scored for the presence and absence of bands in a particular locus. Results were clustered according to their

similarities. The relationship between multiplication rate and variation was assessed using correlation analysis.

Results of the investigation showed that treatments with higher multiplication rates produced higher rates of variation. A variation rate of 55% was recorded for treatments containing IAA and BA. A higher rate of variation (72%) was identified in the treatment with IAA ($2mgl^{-1}$) plus BA ($7.5\ mgl^{-1}$) over 10 cycles. In all cases the dwarf off-type was the most common type of variant obtained, contributing 87.7% of the total amount of variation.

The dwarf specific marker ($OPJ\text{-}04_{1500}$) reported previously in Williams Cavendish was identified in cv. 'Zelig' in this study. Another band similar in size was amplified by primer OPC-15 and named $OPC\text{-}15_{1500}$. This band consistently appeared in all the normal plants and was absent in all the dwarf types and hence could be used as a dwarf specific marker.

CHAPTER ONE

GENERAL INTRODUCTION AND BACKGROUND

1.1. Introduction

Banana is one of the most important fruit crops in the world in terms of production and consumption. Annual production for the year 2000, for example, was 58332 (1000 Metric Tonnes) putting it second only to oranges (FAO, 2000). Fruits, fibres, vegetables, ornamentals and processed products are the major uses of the different sections of the genus *Musa*. Banana is one of the most nutritious, tasty and digestible foods in nature. Cavendish bananas with about 30% of global banana production (SWENNEN *et al.*, 1998), are the most important of all bananas. HALLAM (1995) indicated that the entire 9 million metric ton of export was from Cavendish. This is less than 35% of the total production that enters the international market. The unique and highly acceptable edible qualities of this fruit, in line with its year round availability, are the primary basis for any promotional attempt to boost production and sales of fresh fruit (ROBINSON, 1990).

Cooking bananas, sometimes called plantains, very often are a staple food and comprise a major part of the calorie intake of large numbers of people in the Caribbean, Central and South America, South-Central and Southeast Asia, and the tropical west, central and east Africa (KRIKORIAN & CRONAUER, 1984; ROBINSON, 1990). Although banana is commonly used fresh as a dessert it can also be processed and used in many different ways such as banana figs, juice, powder, flour, starch, jams and jellies, chips, stem candies, puree and fermented products like ethanol, brandy and beer (SHANMUGAVELU *et al.*, 1992; THOMPSON, 1995). Other products like ketchup, cocoa, coffee and chocolates are also reported to be made from banana (THOMPSON, 1995). However, the proportion of world banana production used for processing is limited (ROBINSON, 1990). To optimise advantages of the varied uses of bananas and ensure their sufficient and regular supply, reliable and efficient propagation procedures are important.

The rapid development of techniques for plant cell and tissue culture in the 1960's and 1970's introduced a new era in plant biotechnology. It is now possible to regenerate plants from a range of explant types of many plant species, including most of the important crops, fruits and ornamental species (DREW, 1997). MA & SHII (1972 in KRIKORIAN 1989) first reported the use of *in vitro* techniques for banana clonal propagation.

Tissue culture offers considerable potential for the production of economically important plants. Micropropagation of banana cultivars from the Cavendish subgroup accelerated the production of good quality planting material. It has been estimated, for example, that 15 to 20 million-micropropagated bananas were produced in 1991 (ISRAELI *et al.*, 1995). In spite of its massive use, for some unknown reasons, the use of shoot tips for *in vitro* culture of banana cultivars often results in severe genetic defects (HWANG & KO, 1987; STOVER, 1987).

These genetic defects result in the production of large numbers of off-types or variants referred to as somaclonal variants hereafter. This high rate of somaclonal variation is limiting the use of *in vitro* techniques for banana propagation in large-scale commercial operations and prevents the widespread acceptance of tissue-cultured planting material in the banana industry (SMITH & DREW, 1990a).

Understanding the cause(s) of somaclonal variation and eliminating off-types before field establishment, preferably at the *in vitro* stage, has great significance for *in vitro* banana propagation. Although there are different factors (culture medium composition, rate of multiplication, primary explant origin, formation of adventitious shoots, increased duration in culture, and certain genotypes) reported to cause somaclonal variation in banana, the reports on effects of growth regulators seem to be inconsistent. Some authors report that these factors have no effect (REUVENI & ISRAELI, 1988) while others report that they affect the rate of somaclonal variation both directly (STOVER, 1987) and indirectly (KARP, 1994; DAMSCO *et al.*, 1998b). Still others say not enough work has been done in this regard (ZAFFARI *et al.*, 2000)

to reach a logical conclusion. Therefore, the effect of growth regulators on somaclonal variation was studied in greater depth in this study.

Visual detection of off-types in micropropagated bananas is time consuming, laborious and expensive since it is done 3 to 4 months after field establishment (ISRAELI *et al.*, 1991). Earlier detection of off-types in the nursery by inspecting individual plants is also possible but it is laborious and needs optimal and uniform growth conditions for all plants (SMITH & HAMILL, 1993). The application of GA_3 to detect dwarf off-types was attempted. However, misclassification occurred in 5-10% of the cases even when the screening was applied under the most stringent conditions (DAMASCO *et al.*, 1996a). Therefore, the development and use of efficient and reliable methods for detecting off-types is of prime importance to the banana industry. Detecting off-types by molecular analysis may offer a better alternative in this regard (DAMASCO *et al.*, 1996b)

1.2. Agriculture in Eritrea

The total landmass of Eritrea is 12.2 million hectares; of which 1.7 million hectares are suitable for cultivation. Presently 417,000 hectares are cultivated under rain fed and 22,000 hectares under irrigation conditions. Eritrea consists of a central highland mass, which divides the country between its eastern and western lowlands. Altitudes vary from over 3000 m above sea level in the highlands to below sea level in the denakil depression (in the coastal area). Eritrean agriculture is based on rain fed farming. There is also potential for irrigation in the low lands; the western lowlands having considerable potential for the production of tropical fruits like banana, citrus, mango, and others (FAO, 1995).

Agriculture is the main contributor to the economy, both as a source of food and raw material for industry; its performance is crucial to overall economic growth of the country. Although banana is one of the most important fruit crops grown in Eritrea with respect to area, production, consumption and export potential, its production is limited and inconsistent due to traditional farming practices, lack of good planting material,

and absence of skilled labour, production inputs and marketing infrastructure (NAYAR, 1999).

1.3. Origin, Distribution, and Taxonomy of Banana

All edible bananas and plantains are indigenous to the warm, moist region of tropical Asia comprising the regions of India, Burma, Thailand, and Indonesia. The Assam-Burma-Thailand area is supposed to be not only the centre of origin, but also the main centre of diversity (SHANMUGAVELU *et al.*, 1992). Historical evidence shows that the Arabs seem to have introduced the banana from India to Palestine and Egypt perhaps in the 17th century A.D (SHANMUGAVELU *et al.*, 1992). It soon became popular in those areas and later spread to the east coast of Africa at a very early date and subsequently throughout the African continent as evidenced from the first reports of European visitors to that part of the world. Generally, greater adaptability, high commercial and food value contributed to its rapid spread around the world (SHANMUGAVELU *et al.*, 1992).

SIMMOMDS (1962) postulates the pre-historic domestication of a range of diploid (AA) and triploid (AAA) *M. acuminata* types originating in Malaysia was followed by spread through Southeast Asia. Their spread to areas where *M. balbisiana* occurred, principally the Indian sub-continent and probably the Philippines, led to hybridisation and the appearance of AB hybrids (now rarely found) and the more vigorous AAB and ABB types so common today.

Banana belongs to the family *Musaceae* in the order Scitaminae. Besides *Musa*, another genus in this family is *Ensete*. This family is characterized by leaves and bracts spirally arranged, male and female (hermaphrodite) flowers separated within one inflorescence; and a many-seeded berry (SHANMUGAVELU *et al.*, 1992). The genus *Musa* comprises four sections namely *AustraliMusa, CalliMusa, EuMusa* and *Rhodochlamys* (SIMMONDS, 1966 SHANMUGAVELU *et al.*, 1992). The edible bananas in the section *EuMusa* had their origin in only two wild species, *M. acuminata* and *M. balbisiana* (STOVER & SIMMONDS, 1987). The section *EuMusa,* the biggest and geographically the most widely ranging section of the genus, has given rise to the

greatest number of edible bananas and is of primary importance in terms of cultivation (SIMMONDS, 1966).

1.4. Morphology

The most conspicuous feature of the banana is that they are very large herbs; *M. ingens* is the largest herb known to science. A few species (members of the genus *Ensete*) are unbranched and monocarpic but the majority (*Musa*, the true banana) are stooling or rhizomatous perennials. Each aerial stem produces, first, leaves then an inflorescence and then dies, though the plants are potentially immortal (SIMMONDS, 1962).

The primary seedling root of the banana dies early and is replaced by a purely adventitious root system. Roots arise usually in groups of four at the surface of the central cylinder of the corm. They are 5-8 mm thick, white and fleshy at first if healthy and, later in life, become somewhat corky. In number they vary considerably, depending on the state of health of the plant. These roots produce fibrous lateral roots that are responsible for water and mineral uptake. The rhizome system of the banana is sympodial. In nearly all bananas horizontal growth is limited since they are predominantly clumped in habit, excepting the species *M. itinerans* and *M. laterita* (SIMMONDS, 1966).

The buds by which growth of the sympodium takes place are borne on the middle and upper parts of the parent corm. Each bud, after it had turned upwards, builds up an inverted cone of tissue where growth takes place in the subterranean stem structures. Ultimately, the growing point at the crown of the corm is transformed to an inflorescence associated with narrowing of the stem and lengthening of the internodes. The aerial stem is white in colour except where it emerges from the top of the pseudostem and there, being exposed to sunlight, becomes green. Mechanically the aerial stem is entirely dependent upon the surrounding mass of leaf sheaths for its support; it is a limp structure incapable of supporting itself and a bunch of bananas and is purely connective in function to provide vascular connection between the roots, the leaves and the fruit. The aerial parts consist of a tightly packed mass of leaf

sheaths (the pseudostem). Each sheath is terminated by a petiole and lamina; new leaves are thrust up the centre of the pseudostem to emerge at the crown and there unfurl (SIMMONDS, 1962; 1966).

1.5. Genetics and Improvement

The sections *EuMusa* and *Rhodochlamys* have 2n = 22 chromosomes while sections *AustraliMusa* and *CalliMusa* have 2n = 20 chromosomes. The basic chromosome number (x) for cultivated and wild banana is x = 11 which is considered as the secondary haploid number (SIMMONDS, 1966; ORTIZ *et al.*, 1995). Most cultivated bananas are triploid (2n = 3x) but their parental species were diploid (2n = 2x) both for cultivated and wild bananas. The occurrence of tetraploid (2n = 4x) bananas was also reported for the genus *Musa* (ORTIZ *et al.*, 1995). Plantains or starchy bananas comprise hybrids of *M. balbisiana* and M. *acuminata* whereas the dessert bananas are invariably triploids of the *M. acuminata* genome (SIMMONDS, 1962; ROBINSON, 1990). The banana cultivars were derived by natural hybridisation between the two diploid species *M. acuminata* and *M. balbisiana*, which contribute the A and B genome respectively. In 1955 Simmonds and Shepherd classified the *Musa* genotypes in the natural germplasm by ploidy level and the relative expression of the two diploid characteristics. According to this method of classification, 'Cavendish' and East African highland banana are categorized as AAA, plantains and 'prata' bananas as AAB, and most cooking bananas as ABB (SIMMONDS, 1986; SHANMUGAVELU *et al.*, 1992).

Naturally evolved seedless bananas are perhaps the most conspicuously sterile of all cultivated fruits (KRIKORIAN & CRONAUER, 1984). Selection by man for fruit has resulted in the most widely used edible cultivars. Because vegetative selection has been said to give minimal improvement of dessert bananas, crosses using pollen from male fertile (AA) cultivars of *M. acuminata* and flowers of male sterile triploid (AAA) cultivars have been the prime strategy of breeders (MENENDEZ & SHEPHERD, 1975). Triploidy *per se* has been considered as one of the potential causes of sterility in banana. But some banana species have been found to produce functional pollen grains showing triploidy is not the only cause of sterility in triploid cultivars (ORTIZ *et*

al., 1995). Several characteristics of *Musa* species make breeding and genetic analysis difficult. The bulk of the cultivated bananas and plantains are triploid (2n = 3x = 33) and sterile. The low rate of hybrid progenies recovered and resulting small sample size are the major obstacles to genetic analysis. But resistance breeding has gained importance due to increased pest and disease pressure (ORTIZ *et al.*, 1995).

1.6. Banana Propagation

Propagation by seed, conventional vegetative propagation (by suckers, bits and stumps) and micropropagation (tissue culture) are methods by which banana can be propagated. The use of seed propagation is limited to breeding and other crop improvement activities due to the highly sterile nature of cultivated bananas. Conventional vegetative propagation and the use of tissue culture are the two commonly used commercial propagation methods for banana. These methods have their own advantages and limitations (ROBINSON, 1993).

1.6.1. Use of conventional planting material

Suckers, bits and stumps are amongst the different types of conventional planting material for banana and plantain. The term sucker refers to a rhizome in which the central growing point is used for regeneration, whereas a corm or bit refers to a rhizome in which the central growing point is absent or removed mechanically leaving an axiliary bud (to regenerate new plants). These planting materials can also differ in size, with varying amounts of rhizome storage material to sustain new growth (ROBINSON, 1993, 1996).

Suckers: Suckers used for planting could be: (1) 'peepers' which have just emerged through the soil surface; (2) large 'sword' suckers which have narrow leaves, a tapered pseudostem and a large rhizome; (3) 'water' suckers which have broad leaves, a narrow straight pseudostem and a small rhizome or suckers with attached mother rhizome for extra reserve. Water suckers are weak and not recommended as planting material. Sword suckers may simply be detached and planted whole with the pseudostem and root attached. More commonly, however, the pseudostem is excised 50-100 mm above the rhizome collar. The roots and the outer layer of the rhizome are

pared away to expose the white rhizome tissue. This is done to detect any damage due to banana weevil, infection by barrowing nematodes or infection by panama disease (ROBINSON, 1993).

Bits: The best quality bits are those from vigorous healthy rhizomes from plants just too large for spear point suckers up to plants just prior to flowering. Removing the whole butt from the soil and cutting the pseudostem off 100-150 mm above the rhizome collar can give ideal bits for planting. Locating the axiliary bud can be done by carefully removing the outer layer of the leaf bases until the 'pink-eye' buds are exposed. But it is advisable to leave a small portion of covering tissue as protection against damage during transport. By splitting the latter it is possible to get two bits with their own buds and apical dominance is transferred to the selected bud by gouging out the central growing point of the divided rhizome (ROBINSON, 1993).

Stumps: These planting materials differ from the others in that they consist of plants from which the bunch has been harvested. In this case the plant is uprooted and all the roots and suckers removed. Then the pseudostem is cut off at a height of 0.7-1.0 m above the rhizome collar. Planting one small sword sucker with the mother plant can be used alternatively. But it is necessary to pare off all the roots of the sucker and mother plant and the pseudostem of the parent plant is cut 0.5 m above the rhizome collar (ROBINSON, 1993).

1.6.2. Use of tissue culture

Micropropagation or, more generally, *in vitro* culture of plants is defined as the culture of a plant's seeds, embryos, vegetative organs, explants, tissues, cells and protoplasts on nutrient media under sterile conditions (PIERIK, 1987). Micropropagation of banana generally follows the stages (Figure 1.1) described by MURASHIGE (1974). Thereafter the plants are transferred from *in vitro* culture to the nursery for acclimatization.

1.6.2.1. The laboratory stage

Mother plant selection, culture establishment, *in vitro* multiplication and preparation of plants for re-establishment in the soil are the different laboratory stages for *in vitro* culture of bananas.

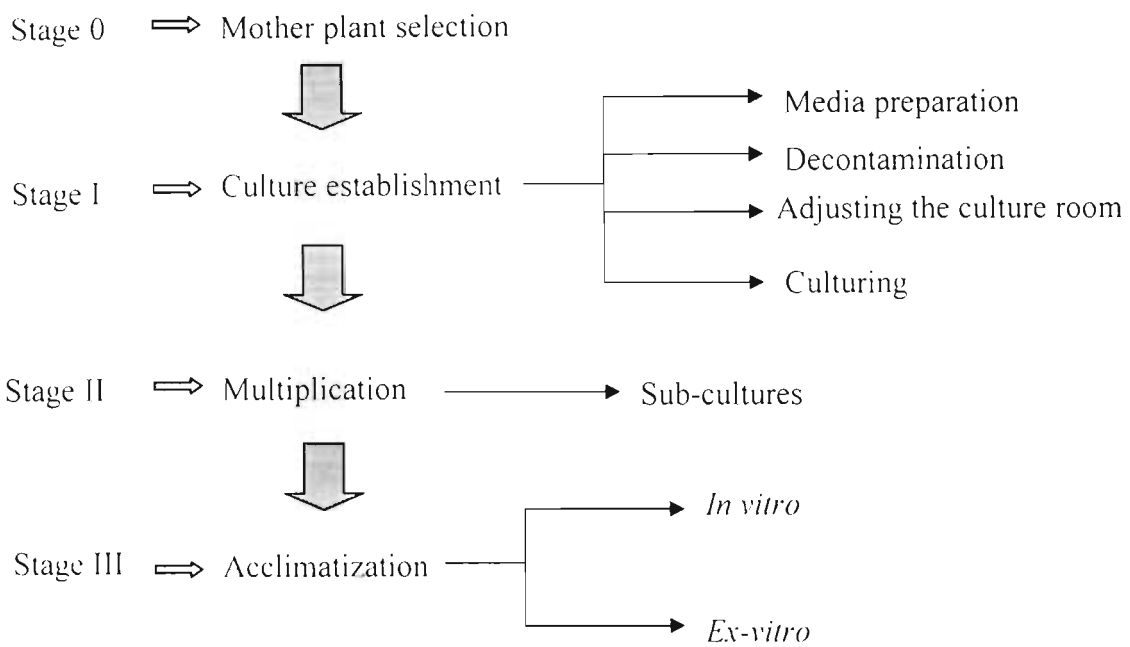


Figure 1.1: Schematic representations of the tissue culture stages (MURASHIGE, 1974)

Mother plant selection and primary explant origin (Stage 0)

A single mother plant can be a source of several buds, which could be multiplied to several thousand plants. This makes careful selection of source plants very important. Trueness to type, vigour and rate of growth, fruit characteristics and sucker health and appearance are useful parameters to be considered while selecting mother plants. For the successful selection of mother plants it is necessary to have a well-managed mother stock plantation. Meristem and shoot tips (meristem plus a few attached leaf primordial) are used successfully to establish *in vitro* cultures (ISRAELI *et al.*, 1995) and hence is recommended as the standard protocol (KRIKORIAN & CRONAUER, 1984). Suckers collected from the field are trimmed and washed before transfer to the laboratory.

Culture establishment (Stage I)

This stage includes preparation of growth media, trimming of the explants for decontamination, adjusting the culture environment and culturing the plant (ISRAELI *et al.*, 1995).

Preparation of growth media: - Although several basal media have been reported to sustain growth and proliferation of banana the most widely used medium contains the MURASHIGHE & SKOOG (MS) (1962) mineral salts. Other supplements include myo-inositol (100 mg l^{-1}), L-tyrosine (200 mg l^{-1}), thiamine-HCl (0.5 mg l^{-1}), adenine sulphate (160 mg l^{-1}), 6-benzyladenine (BA) (5 mg l^{-1}), indol-3-acetic acid (IAA) (2 mg l^{-1}) and sucrose (30 g l^{-1}). The pH is adjusted to 5.8 and the medium is solidified with gelrite (2 g l^{-1}). Test tubes (25x150 mm) with 12 ml of per tube are autoclaved with the medium at 121°C and 103.4 kpa for 15 minutes (ISRAELI *et al.*, 1995).

Decontamination and culturing: - surface decontamination is made for the outer leaves, leaf bases and corm tissues of a selected explant after trimming to 2.5x2.5x5 cm using sodium hypochlorite with a surfactant. Under aseptic conditions, the explants are rinsed three times in sterile distilled water and shoot tips are trimmed to approximately 5x5x5 mm and transferred directly to the culture medium; a further surface sterilization is optional. Within 10 days after culturing the growing apex starts to turn green; shoots appear 2-3 weeks later. Sub-culturing is done after 4-6 weeks or earlier if blackening occurs (ISRAELI *et al.*, 1995). Dipping the explants in antioxidants such as cysteine (JARRET *et al.*, 1985), citric acid or ascorbic acid (GUPTA, 1986) before transfer to the medium reduces blackening as a result of phenolic oxidation. Alternatively, BANERJEE *et al.* (1986) reported that antioxidants can be included in the medium itself. It is also possible to reduce blackening by lowering temperatures, shortening the time of illumination or by adding activated charcoal (0.5%) to the initiation medium (ISRAELI *et al.*, 1995).

Adjusting the culture environment: - cultures are maintained at 28 ± 2°C at 60-70% relative humidity in a 16 hour light cycle with fluorescent light of 1000-3000 µmol m^{-2} s^{-1} (ISRAELI *et al.*, 1995).

Multiplication (Stage II)

After effective culture establishment multiple bud and shoot formation is possible by adding relatively high levels of cytokinin to the medium; the most effective being BA. Propagules could be multiplied by sub-culturing the newly formed shoots or bud clusters and reculturing them on a fresh medium (Figure 1.2). This procedure is repeated at 4-6 week intervals (ISRAELI *et al.*, 1995; KRIKORIAN & CRONAUER, 1984). No significant blackening or contamination is expected at this stage. The rate of multiplication in the AAA 'Cavendish' sub-group is 3-to-5 fold for every sub-culturing. Multiplication depends on genotype, culture medium composition (mainly cytokinin), size of initial explant, its preparation procedure and age of culture (ISRAELI *et al.*, 1995). With low demand, decreasing light intensity and lowering the temperature to 11-18°C can slow down the rate of growth and multiplication. This makes it possible to maintain the explants for more than one year without sub-culturing (ISRAELI *et al.*, 1995).

Figure 1.2: *In vitro* grown, 10-weeks-old, banana plantlets ready for acclimatization

Acclimatization stage (Stage III)

At this stage activities favouring development of individual plants to such a size that they will be able to survive in the soil are undertaken. This is induced by sub-culturing propagules to a medium with a low or a medium free of BA concentration, or replacing BA with kinetin, which reduces axiliary bud and shoot formation. MS medium supplemented with 2 mgl^{-1} IAA and 5 mgl^{-1}kinetin is usually recommended (ISRAELI *et al.*, 1995). Quite often 1-2 mgl^{-1} NAA is used (GUPTA, 1986) and sometimes activated charcoal (KRIKORIAN & CRONAUER, 1984). There is no need for a special rooting medium since shoot elongation and rooting is accomplished using the same medium (ISRAELI *et al.*, 1995). Plants are then transferred to *ex-vitro* condition in the mist house (Figure 1.3) with an appropriate potting mixture.

1.6.2.2. Nursery stage

Acclimatization to *ex vitro* conditions and growing the plantlets to field planting size are performed at this stage by growing the plantlets in the container *ex vitro* and hardening them gradually (Figure 1.4). This stage involves significant changes such as hetrotrophic to autotrophic conditions, increase in light intensity, decrease in humidity, exposure to diurnal temperature changes and pathogens. *In vitro* plants as compared to *ex vitro* have a less developed cuticle, limited stomatal activity, limited mesophyll development and many intra-cellular cavities. Stem and root anatomies also differ and are not adapted to function under *ex vitro* conditions (PREECE & SUTTER, 1991). Gradual acclimatization to *ex vitro* conditions is therefore essential; the most important factors being irradiation and water supply. Acclimatization could be started in the laboratory by opening the flask lids. To discourage pathogen development and avoid interference during planting, rinsing agar residues and pruning roots to 3 cm is important after their removal from culture (DANIELLS & SMITH, 1991; PREECE & SUTTER 1991; ISRAELI *et al.*, 1995;). The potting mixture should be well aerated with a relatively high water holding capacity. Peat and washed sand (1:2 ratio v/v) are used in this case. Local components such as compost made of plant residue and forest soil are economically more preferable. Shade and care during fertilization is important (DANIELLS & SMITH, 1991).

Figure 1.3: Banana plants acclimatized for 4 weeks in the mist house and ready for greenhouse transfer.

1.6.2.3. Advantages and limitations of *in vitro* cultures

Advantages: -

1. Availability, efficiency and planning of planting. The capacity to deliver a large number of plants at a planned time and location;

2. Precocity, vigour and high production - *in vitro* plants grow faster, flower earlier and complete their first cycle before conventional plants (DREW & SMITH, 1990);
3. Uniformity in harvest timing and single cycle plantation; and
4. Pest and disease free plants of tissue cultured bananas are great practical and economic benefits (ISRAELI *et al.*, 1995).

Figure 1.4: Fully acclimatized greenhouse grown (4 weeks) banana plants ready for field planting.

Limitations: -

The most important limiting factor for the practical use of *in vitro* bananas is somaclonal variation, which is the gist of this project. Other limitations include high initial infrastructure investment for construction of laboratories and labour training. Fast multiplication of new selections may encourage the use of cultivars, which have not been tested sufficiently and proven to be superior to the cultivars in use. *In vitro* plants are usually more expensive than conventional planting material. This is so, especially in the tropics where labour is relatively cheap (ISRAELI *et al.*, 1995).

CHAPTER TWO

SOMACLONAL VARIATION: A REVIEW

2.1. Introduction

The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell and hence it should theoretically not cause variation. Clonal multiplication of genetically uniform plants is the expectation. This expectation is usually considered as the basis for the micro-propagation industry. The occurrence of uncontrolled and random spontaneous variation during the culture process is, therefore, unexpected and undesired (KARP, 1994).

Plant breeders have already known about spontaneous heritable variation before the science of genetics was established and the art of plant breeding practiced. Occurrence of "sports" (spontaneous mutations), "bolters", "off-types" and "freaks" in vegetatively propagated crop plants has been observed by farmers in sugarcane, potato, banana and floricultural plants with the commencement of the domestication of plants. Some of the successful cultivars based on spontaneous mutation such as naval orange, dwarf bananas, coloured and striped sugarcane and several potato cultivars are comparable to somaclonal variants and are frequently cultivated. Somaclonal variation, therefore, may be of value in crop improvement (AHLOOWALIA, 1986).

In contrast to spontaneous mutations *in vivo*, *in vitro* generated variations seem to occur more frequently, and are detected more easily since variants can be readily spotted in a limited space and within a short time. This can be verified by considering the occurrence of an albino variant in a Petri dish as compared to that in a field of spaced plants. The exposure of unprotected genetic material to chemicals in the medium and survival of the resulting variants in a non-selective environment increases the mutation rate several fold over that in glasshouse or field grown plant populations. Even if the rate of mutagenesis are the same in cell and tissue cultures as in field-grown plants, the sheer number of occurrences in a cell population (10^6 after 20 cell

divisions) would make accumulation of mutants far greater than in field-grown plants. Hence, somaclonal variants can be detected more frequently in cell cultures than mutations in field grown populations (AHLOOWALIA, 1986).

The *in vitro* culture of plant material can induce or reveal variation between cells, tissues and organs thereby creating variation within cultures, or between the plants derived from them. Some, or all, of the regenerated plants may be physically different from the stock plants from which the culture was derived. Variability of this kind, which usually occurs spontaneously and is largely uncontrolled or directed, can be of two different kinds. These are, firstly, changes caused by cells having undergone persistent genetic change and secondly, those caused by temporary changes to cells or tissues, which is either genetically or environmentally induced (PIERIK, 1987; KARP, 1994).

In banana, tissue culture variation rates, less than 5% are commercially acceptable (STOVER, 1987). Therefore, avoiding or reducing the variation rate to the commercially acceptable rate becomes very important.

2.2. Definition and Types of Somaclonal Variation

Somaclonal variation can be defined as variation originating in cell and tissue cultures (LARKIN & SCOWCROFT, 1981). Currently this definition and/or name is universally adopted or used although there are terms like protoclonal and gametoclonal variation used to describe variants of protoplast and anther culture respectively (KARP, 1994). Somaclonal variation involves all forms of variation in tissue culture. Some scientists add another aspect to the definition and require that somaclonal variation be heritable through a sexual cycle. Unfortunately, it is not always possible or feasible to demonstrate heritability because of complex sexual incompatibilities, seedlessness, polyploidy or long generation times. Therefore, explaining the heritable nature of somaclonal variation for these types of crops could be difficult or impossible (SKIRVIN *et al.*, 1994).

Somaclonal variation is becoming a serious problem for commercial propagators who require extreme uniformity, to the extent that some started advertising that their propagules are not from tissue culture (SKIRVIN *et al.*, 1993). LARKIN & SCOWCROFT, 1981) on the other hand reviewed the extensive application and potential use of somaclonal variation for crop improvement. In this review they enumerated substantive examples of somaclonal variation in crop improvement for sugar cane, potato, tobacco, maize, oats, barley, rice, *Brassica* sp., pelargonium and others. Somaclonal variation, therefore, has both useful and harmful effects.

There are many types of somaclonal variations. These include individuals exhibiting:

- Physical and morphological changes in undifferentiated callus;
- Differences in the ability to organize and form organs *in vitro*;
- Changes manifested among differentiated plants; and
- Chromosomal changes (SKIRVIN *et al.*, 1993).

Off-type plants differ from the source plant permanently or temporarily. Temporary changes are an epigenetic or physiological effect and are non-heritable and reversible. The permanent variants referred to, as somaclonal variants are heritable and are an expression of pre-existing variation in the source plant or are due to the *de novo* variation via an undetermined genetic mechanism (LARKIN & SCOWCROFT, 1981).

2.3. Origin and Sources of Somaclonal Variation

The causes of somaclonal variation are not well understood and have not been elucidated. Although studied extensively the causes remain largely theoretical or unknown (SKIRVIN *et al.*, 1993; 1994). EVANS & SHARP (1986) stated that among the heritable types of variation, single base-pair changes, chromosome deletion, translocation and changes in ploidy levels have been encountered (Figure 2.3). Generally variation in tissue culture could either be pre-existing or tissue culture induced (GEORGE, 1993).

GENGENBACH & UMBECK (1982) demonstrated that somaclonal variation is not limited to nuclear DNA. By using restriction enzyme analysis of isolated mitochondrial DNA they showed variation in mitochondrially controlled male sterility. This could suggest that variation could occur in the whole plant genome.

2.3.1. Pre-existing variation

Multi-cellular explants may vary in their ploidy level because they contain several types of cells. BRIGHT *et al.* (1983) suggested that explants derived from sources other than protoplast be called 'complex cultures' in recognition of their multi-cellular origin.

Chimeras are another source of pre-existing variation *in vitro* (GEORGE, 1993). The arrangement of the genetically different tissues within the plant meristem affects chimera stability. For instance McPHEETERS & SKIRVIN (1983) reported that nearly half of the tissue obtained from tissue culture of a chimeral thornless blackberry were dwarfed and pure thornless. This can best explain the importance of the inherent genetic composition and genome uniformity of the mother plant that is used as starting material for tissue culture. It is more likely that more than one explant be taken from one plant. Therefore, it is imperative to assess the entire plant for genetic uniformity before using it for tissue culture.

Heritable cellular variation could result from mutations, epigenetic changes, or a combination of both mechanisms. The distinction between the two mechanisms is an important one, since genetic mutations are essentially irreversible and are likely to persist in the progeny of regenerated plants, whereas epigenetic changes are not transmitted by sexual reproduction (GEORGE, 1993)).

Thorough characterization and classification of tissue culture-induced chromosome aberrations have led to a more complete understanding of somaclonal variation. Variation in chromosome number and structure has been observed among cultured cells and regenerated plants. Detailed studies have indicated that structural

chromosome changes most accurately reflect the frequency and extent of karyotypic changes. In cultured cells the predominant type of aberration is the result of changes in chromosome structure. Therefore events leading to chromosome breakage, and in some instances subsequent exchange or reunion of fragments, appear to be of fundamental importance (LEE & PHILLIPS, 1988).

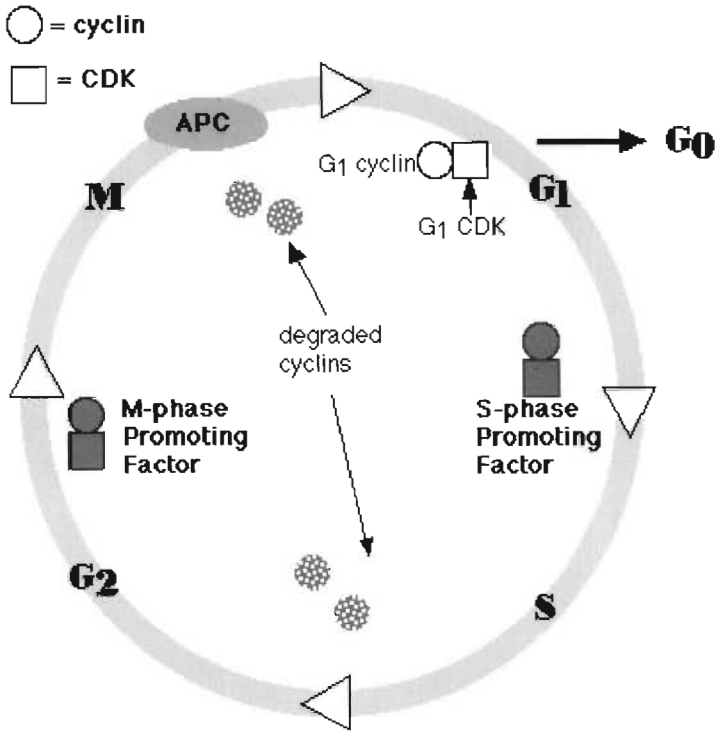


Figure 2.1: Diagrammatic illustrations of stages of the cell cycle. The cell cycle an ordered set of events, culminating in cell growth and division in to two daughter cells. The stages, as pictured to the left, are G_1-S-G_2-M. CDK- cycline-dependent kinese; APC- anaphase-promoting complex (available online at http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/C/CellCycle.html)

BRYANT (1976) indicated that late – replicating heterochromatin and nucleotide pool imbalance are two possible origins of chromosome rearrangement in tissue culture. The former involves the mitotic cell cycle of higher organisms. This cell cycle consists of four phases, G1 (gap), S (synthesis of DNA), G2 (gap), M (mitosis consisting of prophase, metaphase, anaphase, and telophase), each with a species specific and cell type specific duration. Any perturbation affecting the synchrony between

chromosome replication during S phase and cell division would likely result in chromosome aberration. Because heterochromatic regions replicate later than euchromatic segments, their integrity may be particularly vulnerable to fluctuations in the cycle (LEE & PHILLIPS, 1988).

The regulatory mechanism of the cell cycle (Figure 2.1) can play a direct role in plant growth and morphogenesis. KAPLAN, (1992) proposed two opposing views; the 'cell theory' and the 'organismal theory' to address the function of the cell cycle in the growth process. The former considers cells as the building blocks of an organism – any increase in cell number causes growth. While the later considers cell division as a consequence rather than cause of growth. In their review BEEMSTER *et al.* (2003), however, explained that 'cells are not autonomous and their growth and development is governed by a variety of signalling and growth substances that are, in turn, synthesised by individual or groups of cells, either within the same or in a different organ'. As a result they proposed an integrated model of the two theories. This suggests that any effect on a cell or organ could possibly induce an effect to the whole organism.

In view of the complicated role of the cell cycle in controlling the growth and morphogenesis of plants, the problems in cell cycle control can possibly create errors during tissue culture that may alter the normal life phenomenon. KARP (1994) explained that during protoplast cultures, for example, a high frequency of errors in microtubule synthesis, spindle formation, spindle orientation, chromatid segregation and cross wall formation occurs which, result in variation in chromosome number and structure. This suggests that any operation that alters the normal process of the cell cycle can cause somaclonal variation.

Evidence supporting the importance of mitotic recombination in generating somaclonal variation is lacking. However, with a few noteworthy exceptions, mitotic recombination including somatic crossing-over and sister-chromatid exchange could produce several types of chromosome rearrangements (Figure 2.3) observed in tissue culture,

especially if the exchanges were symmetric or between non-homologous chromosomes (LARKIN & SCOWCROFT, 1981).

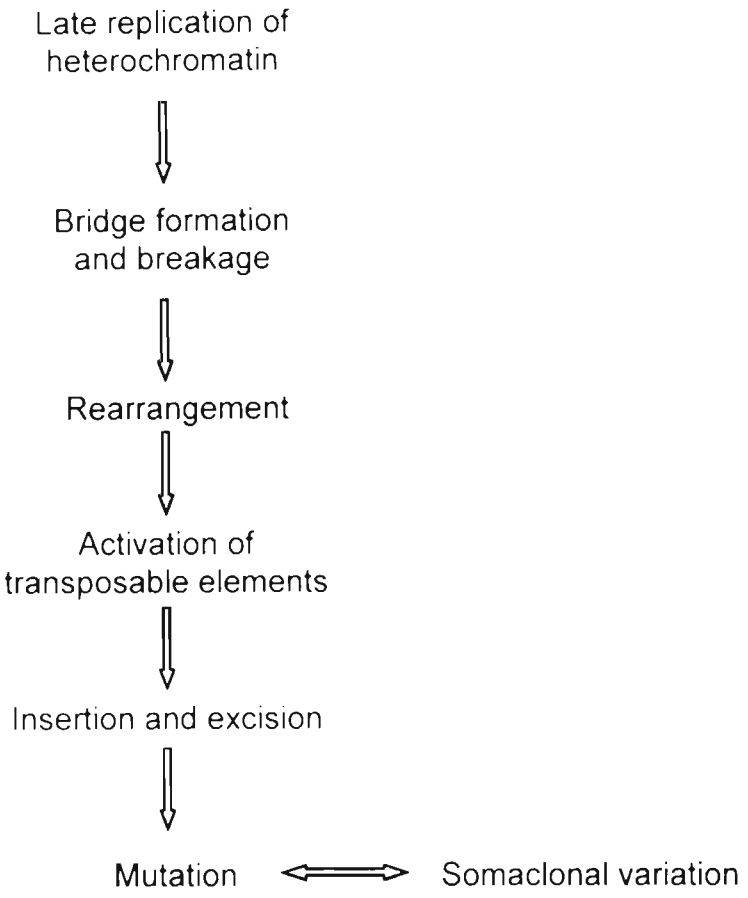


Figure 2.2: Schematic representation of the way chromosome breakage and rearrangement function in the generation of somaclonal variation as proposed by LEE & PHILLIPS (1988).

As proposed by LEE & PHILLIPS (1988), one-way chromosome breakage and rearrangement function in the generation of somaclonal variation occurs as indicated in Figure 2.2. Of course, there are other avenues of chromosome rearrangement and many forms of somaclonal variation. Some examples of somaclonal variation such as morphology of regenerated plants, position effects, qualitative variation and chromosome rearrangements, changes in sequence copy number and gene amplification are involved with chromosome rearrangements (LEE & PHILLIPS, 1988).

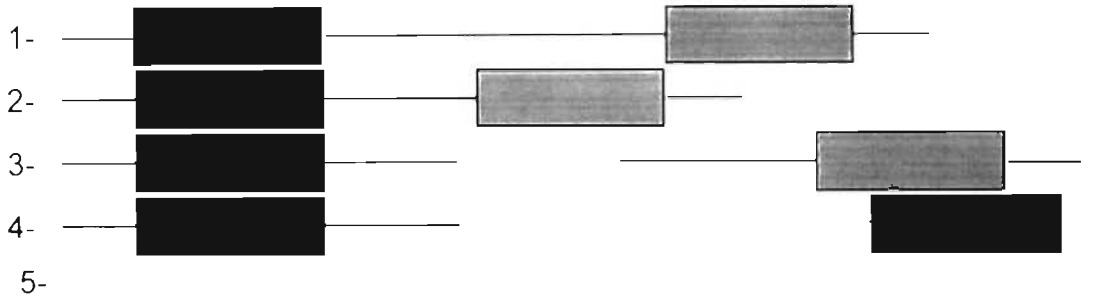


Figure 2.3: Schematic illustration of genomic- instability forms. 1 normal; 2 internal deletion; 3 insertion; 4 translocation; and 5 total or partial chromosome loss (XAVIER *et al.*, 2000).

Activation of cryptic transposable elements is another source of chromosome based somaclonal variation. The discovery of activation of maize transposable elements in tissue culture suggested a possible relationship between somaclonal variation and mobile elements. Chromosome breakage is a means for initiating activity of maize transposable elements (PESCHKE *et al.*, 1987).

To test for pre-existing somaclonal variation, regenerants may be subjected to another round of *in vitro* regeneration. Clones with pre-existing variation should yield more variability in the first generation than in the second and thereafter variation should be eliminated or stabilized. Subsequent variation is more likely to be tissue culture-derived (SKIRVIN *et al.*, 1994).

2.3.2. Tissue culture-induced variation

Although the causes of somaclonal variation are not well understood, it is generally agreed that the method of vegetative propagation used, using chimeral plants, the type of growth regulator, the type of tissue and starting material used, genotype and number and time of sub-cultures are factors determining the chance and frequency of variation during *in vitro* culture (PIERIK, 1987).

2.3.2.1. Methods of vegetative propagation used

Cellular organisation is important in terms of describing origin and cause of somaclonal variation. Tissue culture involves disorganised growth at various levels,

from those systems which least disturb the cellular organization such as meristem tip culture to systems such as protoplasts and explant cultures where regeneration is achieved through the formation of adventitious meristems after a phase of disorganised callus or cell suspension culture. Systems subject to instability and disorganised growth suggested that cellular organization is a critical feature and that somaclonal variation is related to disorganised growth. The longer the duration of the disorganised phase and the greater the departure from organised structures, the greater the chance of somaclonal variation for all organogenic and embryogenic cultures (KARP, 1994). Generally, the more the organizational structure of the plant is broken down, the greater the chance of mutations occurring. It is possible to preserve genetic stability to a certain extent by using a single-node or the axiliary-bud method for *in vitro* propagation. If adventitious shoot formation occurs as a result of the use of regulators then the chance of mutations occurring is increased (PIERIK, 1987). Although the direct formation of plant structures from cultured plant tissue, without any intermediate callus phase, minimizes the chance of instability, the stabilizing influence of the meristem is usually lost when plants are grown in culture (KARP, 1994).

2.3.2.2. Types of tissue or starting material used

The chance of variation is less when the type of starting material used is undifferentiated tissue (pericycle, procambium and cambium) rather than differentiated tissue such as pith. Cell differentiation *in vitro* can result in the production of polyploid cells (D'AMATO, 1977).

D'AMATO (1989) explained that gross changes in the genome including endopolyploidy, polyteny and amplification or diminution of DNA sequences could occur during somatic differentiation in normal plant growth and development. Tissue source therefore can affect the frequency and nature of somaclonal variation. KARP (1994) stated that the process of de-differentiation and redifferentiation may involve both qualitative and quantitative changes in the genome and different DNA sequences may be amplified or deleted during these changes in the state of the cell that is related to the original tissue source and regeneration system. Somaclonal variation, therefore, can arise from somatic mutations already present in the donor plant.

2.3.2.3. Types and concentration of regulators in use

In a review, KARP (1994) explained that evidence for direct mutagenic action of growth regulators is somewhat contradictory and most evidence point to a more indirect effect through stimulation of rapid disorganised growth. Growth regulators can also cause transient modifications of phenotypes, although inherited mitotically during plant growth, and not sexually transmitted they are therefore, epigenetic. Inadequate gas exchange in a closed vessel in tissue culture may also result in accumulation of growth regulators such as ethylene which cause epigenetic changes (KARP, 1994).

BAYLISS (1980) reported that plant growth regulators might preferentially increase the rate of division in those cells already genetically abnormal. D'AMATO (1975) also stated that the genetic composition of a cell population can be influenced by the relative levels of both cytokinins and auxins and cells of normal ploidy are often seen to be at a relative advantage in media where these chemicals are present in low concentrations or totally absent. In cultures of unorganised calli or cell suspension, auxin was found to increase genetic variation by increasing the rate of DNA-Methylation (LoSCHIAVO *et al.*, 1989). The synthetic auxin 2,4-D that is frequently used in callus and cell cultures is often associated with genetic abnormalities such as polyploidy and the stimulation of DNA synthesis that may result in endoreduplication (SWARTZ, 1991). According to SWARTZ (1991) unbalanced concentrations of auxins and cytokinins are most likely to induce polyploidy. OONO 1982, in GEORGE (1993) reported that high levels of BA (30 mg l^{-1}) greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with 2 mg l^{-1} BA.

REUVENI *et al.* (1993) indicated that variation in other medium components used *in vitro* did not directly affect the rate of somaclonal variation in 'Cavendish' bananas. Even with high levels of cytokinins in the medium, which induces the formation of adventitious meristems, the main effect was that of genotype. Despite the recognized importance of hormone levels on shoot regeneration *in vitro* and *ex-vitro*, very few exogenous hormone studies have been reported in banana micropropagation (ZAFFARI *et al.*, 2000). Sub- and supra-optimal levels of plant growth substances in

banana tissue culture media have been associated with somaclonal variation (STOVER, 1987). However, REUVENI & ISRAELI (1990) studied the effect of growth regulators and rate of multiplication and found that these do not affect somaclonal variation in banana. SCOWCROFT (1984) also found that culture media and growth regulators appear not to be mutagenic.

2.3.2.4. Number and time of sub-culturing

SWARTZ (1991) stated that increasing the number of sub-cultures and their duration favours the rate of somaclonal variation. Somaclonal variation is high when a high rate of proliferation is achieved, the period between sub-cultures is short and more sub-cultures are performed in a given time (ISRAELI *et al.*, 1995). REUVENI & ISRAELI (1988) stated that with a relatively constant period of time between sub-cultures, the rate of somaclonal variation increases with generation number. RODRIGUES *et al.* (1998) also showed that somaclonal variants appeared from the fifth sub-culture (1.3%) onwards and increased to 3.8% after 11 sub-cultures. This variation in culture also differs among cultivars. HWANG & KO (1987) for instance reported an overall variation rate of 3% among banana cultures, but the rate of variation for 'Cavendish' banana went as high as 20%, a rather disconcerting development.

In a review LEE & PHILLIPS (1988) explained that plant cells in tissue culture might be especially susceptible to nucleotide pool imbalances (dNTP) because they can be serially transferred from depleted to fresh media almost indefinitely. Intracellular deoxyribonucleotide pools have an important influence on the fidelity of several components of prokaryotic and eukaryotic DNA metabolism; including precursor biosynthesis, replication, DNA repair, recombination, and possibly degradation. Imbalances in the dNTP pools may have serious consequences such as nuclear chloroplast and mitochondrial mutation, mitotic recombination, chromosome (structural) aberrations, aneuploidy, sister chromatid exchange, increased sensitivity to mutagens and encogenic transformation.

2.3.2.5. Effect of stress and genotype

A stress during tissue culture can also induce somaclonal variation. Different genomes however, respond differently to this stress caused variation indicating that somaclonal variation has genotypic components. The differences in stability are related to differences in genetic make-up whereby some components of the plant genome make them unstable during the culture process. This could be better explained by the repetitive DNA sequences, which can differ in quality and quantity between plant species (LEE & PHILLIPS, 1988).

DAMASCO *et al.* (1998b) reported that the inherent instability of a cultivar is another major factor that influences dwarf off-type production in banana tissue culture. They found that cv. New Guinea Cavendish has a higher level of instability *in vitro* than cv. Williams. They further demonstrated that the dwarf off-types were stable *in vitro*, and the tissue culture conditions that induced dwarfism in normal plants did not induce reversion of the dwarf off-type trait. Multiplication of the already existing dwarf off-types on the subsequent sub-cultures along with the irreversible nature of the dwarf off-types *in vitro* plays a significant role in raising the percentage of dwarf population. This situation induces interest to study factors governing or influencing genetic stability *in vitro*.

ETIENNE & BERTRAND (2003) reported that embryogenic cell suspension age and genotype affected the frequency and phenotype of variants produced significantly in *Coffea arabica*. The severity of variation also increased with cell suspension age. MEHATA & ANGRA (2000) indicated that there was variation in disease resistance among regenerants of somaclones of wheat cultivars. In another report POPESCU *et al.* (1997) demonstrated that both genotype and type of explant strongly influenced occurrence of somaclonal variation in callus cultures of strawberry.

2.4. Description and Occurrence of Variants

ISRAELI *et al.* (1995) reviewed the form and percentage of reported main variants of bananas and plantains (Table 2.1). Dwarfism, accounting for 75% (STOVER, 1987) to 80%; (REUVENI & ISRAELI, 1990) of the total variants, is the most common variation

observed in the 'Cavendish' banana sub-group. Variation in stature is a means of characterization for various variants. For example in 'Williams' and 'Grand Naine' the off-types are very similar to 'Dwarf Cavendish', while in the Israeli selection 'Nathan' (derived from 'Dwarf Cavendish') the most common variant is an extra-dwarf type (ISRAELI *et al.*, 1991) which, is an unwanted quality in terms of uniformity of production and yield parameters.

Variation in foliage is another common type of variation, the mosaic type being the most common (REUVENI & ISRAELI, 1990). Thick, rubbery, narrow leaves characterize it, with different degrees of pale-green mottling resembling a virus infection to the inexperienced eye (ISRAELI *et al.*, 1991). There is also variation in colour and morphology of pseudostems such as height, circumference and spacing between petioles. DANIELLS & SMITH (1991) reported an extremely thin pseudostem. ISRAELI *et al.* (1991) and DANIELLS & SMITH (1991) also indicated changes in colour involving various degrees of black, reddish, pale-green and brown associated with colour changes of petioles and midribs. Variation in reproductive organs was also reported. Nipple-like tips, bunches with only male flowers (STOVER, 1987), persistent flowers, split fingers (ISRAELI *et al.*, 1991), changes in bract colour, and in the shape and colour of male buds (STOVER, 1987; DANIELLS & SMITH, 1991) were noticed. There are also unexposed variants some of which are detected for their usefulness. SMITH & DREW (1990b) for example, indicated variants of 'Mons Mari' having extra-long fingers and a dwarf type with no obvious choke-throat problems. HWANG & KO (1987) also screened and selected a somaclonal variant resistant to race 4 of *Fusarium* wilt.

Table 2.1: Incidence and form of somaclonal variation reported for *in vitro* propagated bananas and plantains

Cultivar	Genome	Percentage	Main variant	Reference
Red	AAA	8.6- 16	Green-red	Israeli (1985)
Williams	AAA	0- 8.4	Dwarf	Reuveni *et al.* (1986)
Giant Cavendish	AAA	3	Dwarf	Hwang & Ko (1987)
Grand Nain	AAA	5- 19	Dwarf	Pool & Irrizary (1987)

Red	AAA	10 –20	Green-red	Epp (1987)
Grand Nain	AAA	1.1	Giant	Arias & valverde (1987)
Grand Nain	AAA	25	Dwarf	Stover (1987)
Williams	AAA	2.4- 18.6	Green-red	Israeli *et al.* (1988)
Nathan	AAA	1.4- 12.3	Extra-dwarf	Israeli *et al.* (1988)
Williams	AAA	8- 11	Dwarf	Johns (1988)
Mons Mari	AAA	2- 39	Dwarf	Johns (1988)
Williams	AAA	50	Dwarf	Daniells (1988)
Williams	AAA	4.1- 31.7	Dwarf	Smith (1988)
Grand Nain	AAA	7.2- 29.2	Dwarf	Reuveni & Israeli (1990)
Williams	AAA	1.7- 4.2	Dwarf	Israeli, unpublished
Grand Nain	AAA	2-7. 3	Dwarf	Israeli, unpublished
Grand Nain	AAA	3- 63.3	Dwarf	Carriers (1991)
Figue Sucree	AA	93.4	Male bud degeneration	Carriers (1991)
Moricongo	AAB	21	Tall French plantain	Ramcharan *et al.* (1987)
Dwarf Horn	AAB	38	Dwarf French plantain	Ramcharan *et al.* (1987)
Bobby Tannap	AAB	0	-	Vuylsteke *et al.* (1991)
Ntange 2	AAB	0.5	Plant stature	Vuylsteke *et al.* (1991)
Obino 1'Ewai	AAB	2.1	French reversion	Vuylsteke *et al.* (1991)
Agbagba (4)	AAB	4.4	French reversion	Vuylsteke *et al.* (1991)
Ubok Iba	AAB	12.5	Green petiole	Vuylsteke *et al.* (1991)
Big Ebanga	AAB	35	French reversion	Vuylsteke *et al.* (1991)
Bise Egome 2	AAB	69.1	Green petiole	Vuylsteke *et al.* (1991)
False cuereno (5)	AAB	10.8	Dwarf	Sandoval *et al.* (1991b)
Saba	ABB	0	-	Stover (1987)

References cited in ISRAELI *et al.*, 1995, pp 167

2.5. Detection and/or Characterization of Variants

Present commercial procedures reduce the proportion of off-type plants that reach the farmer to the commercially acceptable rate, below 5%, (HWANG & KO, 1987) even though percentages as high as 29% (REUVENI & ISRAELI, 1988), 80% (RODRIGUES *et al.*, 1998) and 90% (SMITH & DREW, 1990b) were reported. Early detection and elimination of off-types or reducing the rate to below the commercially acceptable rate during the process of *in vitro* production is crucial. Optimising *in vitro* techniques (REUVENI & ISRAELI, 1990), morphological parameters for selecting off-types during the hardening stage, early detection of off-types with molecular markers

(GRILLO *et al.*, 1998) and physiological characterization (DAMASCO *et al.*, 1997) were proposed as means to detect and reduce the rate of variants.

In addition to its use in commercial propagation of tissue cultured plants to remove or minimize off-types before field establishment, understanding the cause and origin of somaclonal variation is important for its realistic potential application to crop impovement.

2.5.1. Morphological detection

In field conditions it is possible to detect dwarf off-types by observing the plant stature and leaf index (leaf length/width) 3 to 4 months after establishment (ISRAELI *et al.*, 1991). However, SMITH & DREW (1990b) indicated that sometimes dwarf off-types could not be detected until the flowering and fruiting stage. Detection at this stage is very costly and too late to replace variants. Therefore, it becomes very important to detect off-types at earlier stages *in vitro* and in the nursery. Dwarf off-types, for example, can be detected by measuring differences in plant height, petiole length and leaf morphology carefully in the nursery (DANIELLS & SMITH, 1991; ISRAELI *et al.*, 1991). ISRAELI *et al.* (1991) indicated that in the nursery dwarf off-types are approximately 5 cm and 10 cm shorter than the normal plant for 'Grand Naine' and 'Williams' respectively. Though it is not convenient for routine detection, SANDOVAL (1994) reported that detection of dwarf and giant off-types of 'Grand Naine' was possible using a combination of leaf characteristics *in vitro*.

2.5.2. Physiological characterization

DAMASCO *et al.* (1998b) demonstrated the application of gibberellic acid (GA_3) and photo-inhibition responses to detect somaclonal variants. The former involves an exogenous application of GA_3 at the *in vitro* or acclimatization stages to enhance morphological differences between dwarf off-types and normal plants. 'Cavendish' dwarf off-types are considered to be GA_3 non-responsive (REUVENI, 1990). SANDOVAL *et al.* (1995) also demonstrated that dwarf off-types have lower levels of endogenous gibberellins compared to normal plants. For effective utilization of this technique it is necessary to determine the GA_3 concentration and timing of application

that gives maximum discrimination between normal and dwarf off-types (DAMASCO *et al.*, 1998b).

DAMASCO *et al.* (1997) indicated that leaf yellowing, due to photo-oxidation of chlorophyll, was more severe in normal plants than in dwarf off-types in the field. The decrease in chlorophyll fluorescence ratio F_v/F_m was also significantly greater in normal plants than dwarf off-types. 'Cavendish' banana leaves are susceptible to photoinhibition under controlled experimental conditions. In both attached and detached leaves, the decrease in F_v/F_m was significantly higher in normal plants than in dwarf off-types (DAMASCO *et al.*, 1997).

2.5.3. Molecular detection

2.5.3.1. Proteins and isozymes

Proteins are the most abundant organic molecules found in cells and have many different functions. The most abundant class of proteins, and those used for electrophoretic analysis, are the enzymes, which catalyse metabolic reactions. These enzymes are highly specific both in the reaction they catalyse and the substrates that they modify (ERASMUS, 2001).

This technique involves analysing clones for protein and enzyme polymorphism (JARRET & LITZ, 1986). It detects naturally occurring genetic variability that had arisen for many enzyme proteins, which can be used to type, or fingerprint, individuals with respect to the isozyme variants present (JARRET & LITZ, 1986; ERASMUS, 2001). Subjecting tissue extracts to various types of gel electrophoresis and subsequently incubating in solutions containing enzyme-specific stains reveals isozymes. A good example of the use of isozyme based characterization of *Musa* clones, as mentioned by JARRET & LIZT (1986), is a resolution for heterozygosity obtained after electrophoresis for isozymes of malate dehydrogenase (MDH), phosphoglucomutase (PGM), glutamate oxaloacetate transmaninase (GOT), shikimate dehydrogenase (SKDH) and peroxidase (PRX). Polymorphism was detected in all 5 enzyme systems.

According to JERRET & GAWEL (1995) total protein and isozyme analysis has also been used in *Musa* for species and cultivar differentiation. But this technique does not reveal differences between normal and dwarf plants and mosaic-like variants in 'Williams'. It has also very limited potential in detecting somaclonal variants in *Musa*. Since isozyme markers represent only a small portion of the genome, there is a very low chance that a mutation event would involve a particular enzyme-coding gene. They are unstable markers (DAMASCO *et al.*, 1998a).

2.5.3.2. RFLP's

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate species (and even strains) from one another (BROWN, 1995).

Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 base pairs in length. Generally, the shorter the recognition sequence, the greater the number of fragments generated. If molecules differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. Restriction enzymes are isolated from a wide variety of bacterial genera and are thought to be part of the cell's defenses against invading bacterial viruses. These enzymes are named by using the first letter of the genus, the first two letters of the species, and the order of discovery (BROWN, 1995).

RFLP analysis has been used in *Musa* species to detect genetic diversity and to differentiate between A and B genomes (JARRET & GAWELL, 1995). Characterization of *Musa* somaclonal variants using this method has not been undertaken due to the following technical limitations (KARP *et al.*, 1996; DAMASCO *et al.*, 1998a):

- A good supply of probes is needed and, if heterologous probes are unavailable, cDNA or genomic DNA probes must be developed;
- The blotting and hybridisation steps are time consuming and difficult to automate; and
- Sufficient quantities of good quality DNA (up to 10 μg per digestion) are required. RFLPs are, thus, not applicable where very limited amounts of source material (such *as in vitro* plants) or preserved tissues are available.

2.5.3.3. The polymerase chain reaction (PCR)

The development of the PCR for amplifying DNA brought an advancement in the applicability of molecular methods and a range of new technologies are developed which can overcome many of the technical limitations of RFLPs. PCR is a technique for amplifying a specific region of DNA, defined by a set of two short oligonucleotides at which DNA synthesis is initiated by a thermo-stable DNA polymerase. Usually, at least a million-fold increase of a specific section of a DNA molecule can be realized and the PCR product can be detected by gel electrophoresis. The most common version of PCR is RAPD (random amplified polymorphic DNA) analysis, in which the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light. The regions amplified are usually between 150-3,000 base pairs in length (BROWN, 1995; KARP *et al.*, 1996).

Polymerase Chain Reaction (PCR)-based techniques offer much potential for detecting somaclonal variants in micropropagated bananas. The advantages of PCR – based genetic markers over other markers such as allozymes and RFLPs include: (MORREL *et al.*, 1995; KARP *et al.*, 1996; DAMASCO *et al.*, 1998a)

- The PCR assay is easy to perform and requires small amounts of DNA (as small as 10 ng);
- For RAPD (random amplified polymorphic DNA), no prior sequence information is required since a number of commercially available arbitrary primers can be used;
- The technique is convenient to use for screening large numbers of plants;

- Since the procedure involves no blotting or hybridising steps, it is quick, simple and automatable. It is absolutely critical, however, to maintain strictly constant PCR reaction conditions in order to achieve reproducible profiles;
- PCR involves fewer steps compared to RFLPs and it is technically straight forward;
- PCR does not require the use of radioactivity;
- At most stages PCR is automated from DNA extraction to data collection and analysis; and
- The vast range of potential primer sequences gives the PCR great diagnostic power.

According to RYBICKI (2001) the following factors affect the effectiveness of PCR conditions; denaturing temperature and time, annealing temperature and primer design, primer length, degenerate primer, elongation temperature and time, reaction buffer and cycle number (see section 2.6).

PEAKALL (1997) defined the basic PCR procedures in three major approaches and stated that the choice of an appropriate method depends on the aim of the study. These approaches include;

1. Sequence-Tagged- Site (STS) PCR;
2. Arbitrary-Primed (AP) PCR; and
3. A combination of the two approaches (AFLP)

1. STS – PCR:

This procedure uses two different specific primers, complimentary to opposite strand of conserved DNA, to amplify the intervening sequence. Micro-satellite or Simple Sequence Repeats (SSRs) are the best example of genetic markers revealed by STS-PCR (PEAKALL, 1997). The prominent advantages of STS-PCR include;

- Produce a co-dominant single locus genetic marker; as a result the DNA profiles are easier to score than multi loci profile and heterozygotes are readily apparent; and
- In the presence of a successful reaction a positive result is always achieved except in the case of null alleles.

The production of false polymorphism during partial failure of the reaction is the limitation of this system, unlike the multi loci method. The importance of high-resolution electrophoresis is another technical complication of SSRs, specially for dinucleotide repeat SSRs where alleles may differ by only two base pairs (PEAKALL, 1997).

2. AP-PCR (RAPDs)

This procedure involves the use of a single short primer of arbitrary (but known) sequence. Under low stringency PCR conditions, multi loci DNA profiles could be produced with polymorphisms revealed as band presence or absence (WILLIAMS *et al.*, 1990; WELSH & McCLELLAND, 1990). RAPD (Random amplified polymorphic DNA) (WILLIAMS *et al.*, 1990), AP-PCR (arbitrary-primed PCR) (WELSH & McCLELLAND, 1990), DAF (DNA amplification fingerprinting) (CAETANO-ANOLLES *et al.*, 1991) and ssRAPDs (silver stained RAPDs) (HUFF & BARA, 1993) are acronyms for AP-PCR techniques. The term RAPDs, however, is widely used as a generic term for AP-PCR (PEAKALL, 1997).

RAPDs differ from other PCR techniques in primer length, primer to template ratio, the gel matrix used and in the visualization procedure. Its advantage over other genetic markers is that DNA sequence knowledge is not required, making it theoretically applicable to any organism (KARP *et al.*, 1996; DAMASCO *et al.*, 1996b; PEAKALL, 1997). Although there are some differences in the type of primer and PCR conditions, the major difference among the PCR procedures is the type of electrophoresis and visualization technique of the DNA fragments (Table 2.2).

Table 2.2: Differences in electrophoresis and visualization techniques of AP-PCR procedures

PCR procedure	Electrophoresis	Visualization technique and staining	Reference
RAPD	Agarose gel	Ethidium bromide stain	WILLIAMS *et al.* (1990)
DAF	Polyacrylamide gel	Silver staining	CAETANO-ANOLLES *et al.* (1991)
ssRAPDs	Polyacrylamide gel	Silver staining	CAETANO-ANOLLES *et al.* (1991)
AP-PCR	Polyacrylamide gel	Radioactive labels	WELSH & McCLELLAND (1990)

CAETANO-ANOLLES *et al.*, (1995), however, explained that these distinctions are becoming less functional due to the many modifications such as fluorescent labelling and high resolution polyacrylamide electrophoresis of RAPDs on automated sequences, and the fluorescent labelling and automated capillary electrophoresis of DAF described in the literature.

Table 2.3: Characteristics of the different MAAP techniques (CAETANO-ANOLLES, 1994)

Characteristics	DAF	AP-PCR	RAPD
Primer length (nt)	5-15	18-32	9-10
Primer concentration (μM)	3-30	1-10	0.3
DNA concentration ($ng\mu l^{-1}$)	0.01-1	0.1-5	1
Primer/template ratio	5-50 000	1-500	<1
Annealing temperature (^{o}C)	10-65	35-50	35-42
Amplification stringency	Low to high	High to low	Low
DNA separation	PAGE	PAGE	Agarose
Visualization	Silver staining	Radiolabeling	EtBr staining
Product resolution	High	Intermediate	Low
Number of products	10-100	3-50	1-10

Note. nt = number of nucleotides; PAGE = polyacrylamide gel electrophoresis

CAETANO-ANOLLES et al., (1992) suggested a new name that encompasses the three techniques (AP-PCR, RAPD and DAF) with an acronym MAAP (multiple arbitrary amplication profiling). CAETANO-ANOLLES (1994) characterised these three MAAP procedures (Table 2.3) to make an important distinction amongst them since

the use of the type of electrophoresis and visualization technique alone for distinction is becoming blurred.

Apart from their advantages MAAP procedures (RAPDs, AP-PCR, and DAF) have the following limitations;

- The markers are dominant and heterozygotes cannot be detected, hence provide less genetic information than co-dominant markers such as allozymes or SSRs;
- In the absence of pedigree analysis, the identity of individual bands in the multi-band profiles is not known and there can be uncertainty in assigning markers to specific loci;
- The presence of a band of apparently identical molecular weight in different individuals cannot be taken as evidence that the two individuals share the same homologous fragment, although this assumption is commonly made; and
- Single bands on the gel can sometimes be comprised of several co-migrating amplification products.

HOWELL *et al.* (1994) reported that RAPD analysis did not reveal variation between normal and off-type plants from micropropagated 'Cavendish' banana cultivar 'Valery'. DAMASCO *et al.* (1996b), however, found a RAPD marker (primer OPJ-04, 5'-CCGAACACGG-3') specific to 'Cavendish' dwarf off-types. GRAJAL-MARTIN *et al.*, (1998) also reported that the primers OPC-15 (5'-GACGGATCAG-3') and OPJ-04 revealed variability in *in vitro* plants. These primers were used for the purpose of this study.

Due to its cost effectiveness and suitability to small projects (RAGOT & HOSINGTON, 1993), RAPD has been used for a number of studies in the assessment of genetic variability, determining varietal purity and assessing germplasm (WEEDEN *et al.*, 1992). Identification and mapping of polymorphism in wheat (WEINING & LANGRIDGE, 1991), somaclonal variation of pear and apple (CABONI *et al.*, 2000), *Populus deltoids* (RANI *et al.*, 1995), strawberry (POPESCU *et al.*, 1997), coffee

(ETEINNE & BERTRAND, 2003), maize (EDWARDS, 2000), turfgrass breeding (PEAKALL, 1997), study of clonality in *Haloragodendron lucasii* (SYDES & PEAKALL, 1998) and disease resistance in wheat (MEHTA & ANGRA, 2000) are among the few examples.

3. AFLP

This is a relatively new addition to the PCR procedures and is termed as amplified fragment length polymorphism (AFLP). It is essentially intermediate between RFLP and PCR. AFLP involves restriction digestion of genomic DNA followed by selective rounds of PCR amplification of the restricted fragments. The amplified products are radioactively or flourescently labelled and separated on sequencing gel. AFLPs appear to be as reproducible as RFLPs, but are technically more demanding and require more DNA (1μg per reaction) than RAPDs. The advantage of this technique over RAPD marker is that because of their large genome coverage on average they give 100 bands per gel compared with 20 for RAPDs. AFLPs are very good for mapping and fingerprinting and genetic distances can be calculated between genotypes. They do, however, share many of the limitations with RAPDs (KARP *et al.*, 1996).

According to KRAUSS & PEAKALL (1998) AFLP involves four steps:

1. Restriction of the DNA; Digestion of the DNA with restriction enzyme;
2. Ligation of adaptors;
3. Preselective amplification by PCR; and
4. Selective amplification by PCR

2.6. Description and Requirements of the PCR and Its Components.

A successful PCR experiment depends on the optimal reaction conditions, the template DNA, and respective primer. There are, of course, parameters that vary according to applications such as magnesium concentration, pH of the reaction buffer, temperature and time of the different cycling steps. In addition the various components (see below) may increase the specificity and yield of some reactions (ROCHE, 2001).

Therefore, it is imperative and is generally recommended to optimise the PCR reaction conditions for each new template primer pair.

Table 2.4: Comparison of concentrations of PCR buffers used in this study with concentrations recommended by INNIS & GELFAND (1990).

Buffer	Recommended concentration	Concentration used in this study
Tris-HCl	10-50 mM	10 mM
KCl	Up to 50 mM	50 mM
$MgCl_2$	0.5-2.5 mM	1.5 mM
Primer	0.2-1 μM	0.2 μM
dNTP	50-200 μM	200 μM
Gelatin or BSA	100 μg ml^{-1}	0.1 mg ml^{-1}
Taq DNA pol.	0.5-1 units	1.5 units
Tween-20	0.05-0.1 % v/v	-
Triton X-100	0.05-0.1 % v/v	-

The number, size and intensity of bands in RAPD's is also affected by several other factors, which includes; PCR buffer, dNTP's, Mg^{2+} concentration, cycling parameters, source of Taq DNA polymerase, conditions and concentrations of DNA and primer type and concentration. The limitations to successful PCR could be overcome by maintaining a constant set of defined reaction conditions and consistency of technical applications (RYBICKI, 2001).

General considerations (RYBICKI, 2001):

- Taq could be inhibited by higher concentration (>50 mM) of KCl or NaCl;
- Taq requires free Mg^{2+} and hence allowance should be made for dNTP's, primers and template;
- Primer annealing, Tm of template, product and primer-template associations, product specificity, enzyme activity and fidelity are affected by Mg^{2+};

- Primer concentration should not go above 1 μM unless there is a high degree of degeneracy and 0.2 μM is usually enough for homologous primers; and
- Although long products may require more, nucleotide concentration need not be above 50 μM each.

2.6.1. Taq DNA polymerase

This enzyme is the recombinant form of the enzyme from the thermophilic eubacterium, *Thermus aquaticus* BM, expressed in *E. coli*. Taq DNA polymerase consists of a single polypeptide chain with a molecular weight of approximately 95 kD. It is highly active 5'-3' DNA polymerase lacking 3'-5' exonuclease activity. This enzyme exhibits its highest activity at a pH of around 9.0 and a temperature around 75°C, though it is stable to prolonged incubation at elevated temperatures of up to 95°C (BROWN, 1995; ROCHE, 2001).

2.6.2. Primer selection

Primers OPC-15 (5'-GACGGATCAG-3') and OPJ-04 (5'-CCGAACACGG-3') were used for the purpose of this study. INNIS & GELFAND (1990) stated that the following points act as the basis for successful primer selection:

1. Primers should be 17-28 bases in length;
2. Base composition should be 50-60% (G+C);
3. Primers should end (3') in a G or C, or CG or GC: this prevents "breathing" of ends and increases efficiency of priming;
4. Tms between 55-80°C are preferred;
5. Runs of three or more Cs or Gs at the 3'-ends of primers may promote mispriming at G or C-rich sequences (because of stability of annealing), and should be avoided;
6. 3'-ends of primers should not be complementary (ie. base pair), as otherwise primer dimers will be synthesized preferentially to any other product; and
7. Primer self-complementarity (ability to form secondary structures such as hairpins) should be avoided.

Primer length is another important consideration to be noted. The optimum length of a primer depends on its (A+T) content and Tm of its partner. The most important consideration in choosing a primer is that it should be complex enough so that the possibility of annealing to sequences other than the chosen target would be very low. If a primer is too long, it is difficult to avoid or prevent mismatch pairing and non-specific priming even with high annealing temperatures (INNIS & GELFAND, 1990).

2.6.3. Denaturing temperature and time

Annealing is the specific complimentary association due to hydrogen bonding of single stranded nucleic acids (NA) (RYBICKI, 2001). To form a stable double stranded hybrid, two complimentary sequences would form hydrogen bonds between their complimentary bases (G to C and A to T or U). Nucleic acids would be made single stranded by heating to a point above the melting temperature (Tm) of the double stranded form for the purpose of annealing, followed by a flush cooling to avoid re-annealing of denatured strands.

Loss of Taq activity (denaturation) is strongly influenced by time at a given temperature. Reducing the time increases the number of possible cycles, irrespective of the temperature. Increase in denaturation temperature and decrease in time could also work; as proposed by INNIS & GELFAND (1990), 96°C for 15 sec in contrary to the normally used denaturation of 94°C for 1min. Generally PCR works with denaturing temperatures of 91-97°C (RYBICKI, 2001).

2.6.4. Annealing temperature and primer design

Primer length and sequence are of critical importance in designing the parameters of a successful amplification. Both the length and increasing (G+C) content increase the Tm (see formula below) of the NA duplex. This implies that the annealing temperature (Ta) chosen for a PCR depends on length and composition of the primer.

$$Tm = 4(G+C) + 2(A+T)\ ^{\circ}C$$

INNIS & GELFAND (1990) recommended the use of Ta about 5°C below the lowest Tm of the pair of primers to be used. RYCHLIK *et al.* (1990) explained that if the Ta is increased by 1°C every other cycle, specificity of amplification and yield of products <1 kb in length are both increased. If the Ta is too low there is a problem of primers annealing to sequences other that the true target or mismatching, which result in reduction of yield of desired products (RYBICKI, 2001). Too high Ta also results in too little product.

2.6.5. Elongation temperature and time

In most commonly used PCR procedures this is between 70-72°C for 0.5-3 min, but 72°C for 5 min in this study gave better amplification. Practically elongation occurs from the moment of annealing, but at about 70°C activity becomes optimal and primer extension occurs up to 100 bases/sec (INNIS & GELFAND, 1990). Generally longer products require longer times and longer times may also be helpful in later cycles when product concentration exceeds enzyme concentration, and when dNTP and/or primer depletion may become limiting (RYBICKI, 2001).

2.6.6. Cycle number

The starting concentration of target DNA largely affects the number of amplification cycles necessary to produce a band visible on a gel. INNIS & GELFAND (1990), for example, recommend from 40-45 cycles to amplify 50 target molecules, and 25-30 to amplify $3x10^5$ molecules to the same concentration. An explanation for this non-proportionality is the plateau effect when product reaches 0.3-1 nM. Factors, which possibly cause this include;

- Degradation of reactants (dNTP, enzyme);
- Reactant depletion (primers, with short products and dNTP's with long products);
- End product inhibition (pyrophosphate formation);
- Competition for reactants by non specific products; and
- Competition for primer binding by re-annealing of concentrated products (INNIS & GELFAND, 1990).

Plateau effect is the attenuation in the exponential rate of product accumulation in late stages of a PCR (RYBICKI, 2001).

2.6.7. DNA Molecular Weight Marker (XIV)

This ladder consists of 15 double stranded DNA fragments ranging from 100-1500 bp and an additional band of 2642 bp. It is prepared by cleavage and restriction

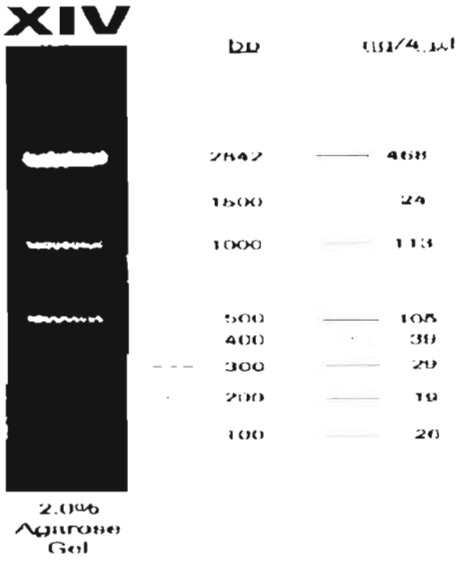


Figure 2.4: Separation of 1 μg Molecular Weight Marker XIV on a 2% agarose gel, ethidiumbromide stain (Roche Diagnostics GMBH, Mannheim, Germany).

digest of a specifically constructed plasmid. The length of the fragments differ by 100 bp and the 500 and 1000 bp banding patterns are two to three times brighter (Figure 2.4) which allows accurate sizing of DNA fragments generated by PCR or restriction digest prepared on agarose gel. It is supplied in a ready – to – use solution in 10 mM Tris-HCl; 1 mM EDTA, pH 8.0. 2 μl was loaded in each lane of the gel.

2.7. Interpreting and Analyzing Data from Molecular Screening Techniques

SOLTIS *et al.*, (1992) stressed the importance of understanding the different ways that data from molecular techniques can be utilized. Before embarking upon a programme of applying any one of the techniques to a diversity and/or variability study, critical selection of an appropriate statistical package should be made for data collection and

analysis. Molecular data are collected in two fundamentally different ways, namely, as discrete characters or as continues characters.

The data derived from PCR have their strength in distinguishing individuals, cultivars or accessions. The difficulty of achieving a robust profile in these types of data, particularly in RAPDs, makes the reliability for typing questionable. The presence or absence of bands, however, can be scored and data is converted to similarity matrices for calculation of genetic distance between populations (ELLSWORTH *et al.*, 1993) or the presence or absence of bands could be scored and compared to a standard Molecular Weight Marker as employed in this study.

2.8. Aims of The Study

The aims of the project were:

1. To determine the effect of auxins and cytokinins on somaclonal variation of 'Cavendish' banana;
2. To determine the interaction effect of different concentrations of cytokinins and sub-culturing; and
3. To assess the effectiveness of molecular detection of somaclonal variants, specifically RAPD markers.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Tissue Culture

3.1.1. Plant material

Tissue cultured 'Cavendish' banana (*Musa* AAA cv. 'Zelig') obtained from African Biotechnologies Pty. Ltd., South Africa, were used in all the experiments. All experimental plants were of the same age and genetically uniform. The plants received had been sub-cultured for three cycles (20-weeks-old in culture), at intervals of four weeks.

3.1.2. Tissue culture media

MURASHIGE & SKOOG (1962) macro and microelements (Appendix 1) supplemented with sodium dihydrogen (0.38 g l^{-1}), ascorbic acid (0.18 g l^{-1}), adenine sulphate (0.2 g l^{-1}), sucrose (30 g l^{-1}), Gelrite (2 g l^{-1}), and growth regulators, as stated in Sections 3.1.4.1 and 3.1.4.2 were used. After adjusting the pH to 5.8 the media were autoclaved at 121°C and 103 kpa for 20 min. Filter sterilized ascorbic acid was added when the media were just above the solidification temperature (about 50°C). Cultures were then incubated in a growth room having 16h light/8h dark conditions and irradiance of 43 μmol m^{-2} s^{-1} at a temperature of 26±1°C.

3.1.3. Explant selection and preparation

This procedure generally followed the protocol outlined by KRIKORIAN & CRONAUER (1984) with some modification. Aseptically established *in vitro* plantlets were cut transversely to separate leaves and produce a section of pseudostem approximately 1 cm in length including an intact vegetative bud. The lower part of the pseudostem was trimmed to remove darkened or necrotic tissues and the sheath removed carefully by peeling. The explants were then cut in half longitudinally. Each half was transferred to a culture tube with a screw-cap. Sub-cultures were made at seven-week intervals.

3.1.4. Design of experiments

Two separate experiments were conducted; one dealing with the type of plant growth regulators used during culture and the other with hormone concentrations. Plant material was sub-cultured ten times for both experiments. Plants from the fifth sub-culture were used as a control for both experiments.

3.1.4.1. Effect of the type of plant growth regulators (auxins and cytokinins) on somaclonal variation.

To determine the effect of different types of auxins and cytokinins on somaclonal variation of 'Cavendish' banana, a combination of auxins and cytokinins that are frequently used for *in vitro* multiplication were tested. Growth regulators tested included; auxins (IAA, IBA and NAA) and cytokinins (BA and TDZ). A total of 6 (2x3) treatments (Table1) were considered and 10 explants per treatment were used.

Table 3.1: Experimental set-up to study the effect of auxins and cytokinins on somaclonal variation

Treatments	Auxins (2 mgl^{-1})			Cytokinins	
	IAA	IBA	NAA	BA (5 mg l^{-1})	TDZ (3 mg l^{-1})
1	+	-	-	+	-
2	+	-	-	-	+
3	-	+	-	+	-
4	-	+	-	-	+
5	-	-	+	+	-
6	-	-	+	-	+

Note: + Indicates presence

- Indicates absence

3.1.4.2. Combined effect of sub-culturing and cytokinin (BA) concentration on somaclonal variation.

To determine the combined effect of hormones and sub-culturing, the interaction of sub-culturing and growth regulators were investigated. Plants were sub-cultured 10 times on media with cytokinin concentrations of 2.5, 5 or 7.5 mg l^{-1} BA and an IAA concentration of 2 mg l^{-1}. Sub-cultures were made every seven weeks from the fifth

to the tenth sub-culture. There were a total of 18 (3x6) treatments (Table 3.2) each having 10 replicates. Therefore, a total of 180 explants were used at the initial stage of the experiment.

Table 3.2: Effect of sub-culturing and cytokinin concentration on somaclonal variation detected.

Treatments	IAA (mg l^{-1})	BA (mg l^{-1})			Number of Sub-cultures					
	2.0	2.5	5.0	7.5	5	6	7	8	9	10
1	+	+	-	-	T-1					
2	+	-	+	-	T-2					
3	+	-	-	+	T-3					
4	+	+	-	-		T-4				
5	+	-	+	-		T-5				
6	+	-	-	+		T-6				
7	+	+	-	-			T-7			
8	+	-	+	-			T-8			
9	+	-	-	+			T-9			
10	+	+	-	-				T-10		
11	+	-	+	-				T-11		
12	+	-	-	+				T-12		
13	+	+	-	-					T-13	
14	+	-	+	-					T-14	
15	+	-	-	+					T-15	
16	+	+	-	-						T-16
17	+	-	+	-						T-17
18	+	-	-	+						T-18

Note: + Indicates presence

- Indicates absence

3.2. RAPD Analysis

3.2.1. DNA extraction

The protocols described by RICHARDS (1997) and (HILLS & VAN STADEN, 2002) with slight modifications were employed to extract total genomic DNA from *in vitro* grown plantlets.

1. Reagents were prepared according to RICHARDS (1997). These reagents include: CTAB extraction solution, CTAB/NaCl solution, CTAB precipitation solution, high salt TE buffer, TE buffer, 80% ethanol and 24:1 Chloroform:Iso-amyl alcohol (see Appendix 2).
2. Ethanol (100%), Iso-propanol, Polyvinylpolypyrolindone (PVPP) insoluble, 2-Mercaptoethanol (2-ME) and liquid nitrogen were used directly.
3. Sterile mortar and pestle, 1.5 ml Eppendorf, pipette tips (1.5 ml and 100 µl) were prepared.
4. CTAB extraction solution (500 µl) heated to 65°C were added to 3% PVPP (0.015 g) in a 1.5 ml Eppendorf tube. 2-ME (10µl) was added to the mixture to give a final concentration of 2% (v/v) immediately before the start of extraction.
5. Leaf tissue obtained from *in vitro* plantlets (0.25 g) was frozen in liquid nitrogen (-196°C) in a mortar and ground to a fine powder.
6. The ground and frozen tissue was then added to a warm (65°C) PVPP/2-ME/ CTAB extraction solution. The mixture was then incubated for 30 min at 65°C in a water bath, with occasional mixing.
7. The homogenate was then mixed with an equal volume of 24:1 Chloroform:Iso-amyl alcohol by inversion followed by centrifugation at 10,000 rpm (7500 x *g*) for 5 min in a microcentrifuge (HERSTELLER SPINTRON Ltd, Wehingen, Germany . Model, Z 160 M) (Figure 3.1). The upper aqueous part was then recovered.
8. CTAB/NaCl solution (1/10 volume at 65°C) were added to the recovered aqueous phase and mixed well by inversion.
9. The mixture was then extracted with an equal volume of Chloroform/Iso-amyl alcohol. After mixing and centrifuging for 5 min at 10,000 rpm (7500 x *g*) the upper aqueous layer was recovered.
10. One volume (1 ml) of 65°C CTAB precipitation solution was added to the recovered aqueous phase. In most cases the precipitate was visible at this stage but in a situation where it was not visible an additional incubation period of 30 min at 65°C was applied.

11. The mixture was centrifuged for 5 min at 2700 rpm (500 x *g*) and the pellet was recovered by removing the supernatant. The pellet was then dissolved and suspended in high-salt TE buffer (2 ml per gram of starting material).
12. The DNA was subsequently precipitated by adding 0.6 volumes (1.2 $ml g^{-1}$ of starting material) of ice-cold Iso-propanol followed by mixing and centrifuging for 15 min at 10,000 rpm (7500 x *g*).
13. The supernatant was decanted and the pellet first washed with 80% ethanol followed by 100% ethanol. The dried pellet was then suspended in a minimal volume of TE buffer (0.2 ml per gram of starting material). The DNA was then stored at –20°C until further use.

Figure 3.1: The microcentrifuge used during DNA extraction.

3.2.2. DNA quantification

Quantification of DNA was done for each sample using spectrophotometry (Figure 3.2) at A_{260}, A_{280} and A_{320} nm absorbance using a 250 x dilution (4 µl of DNA stock and 996 µl of TE buffer as blank). Ratio, purity and concentration were then calculated using the following formulae:

Ratio = A_{260}/A_{280}

Purity (%) = Ratio/1.8 X 100

Concentration in (μg μl^{-1}) = (Corrected A_{260} X ΔE X dilution)/1000 Where: corrected $A_{260} = A_{260} - A_{320}$

ΔE = 50 (extinction coefficient for genomic DNA); and dilution = 250 x

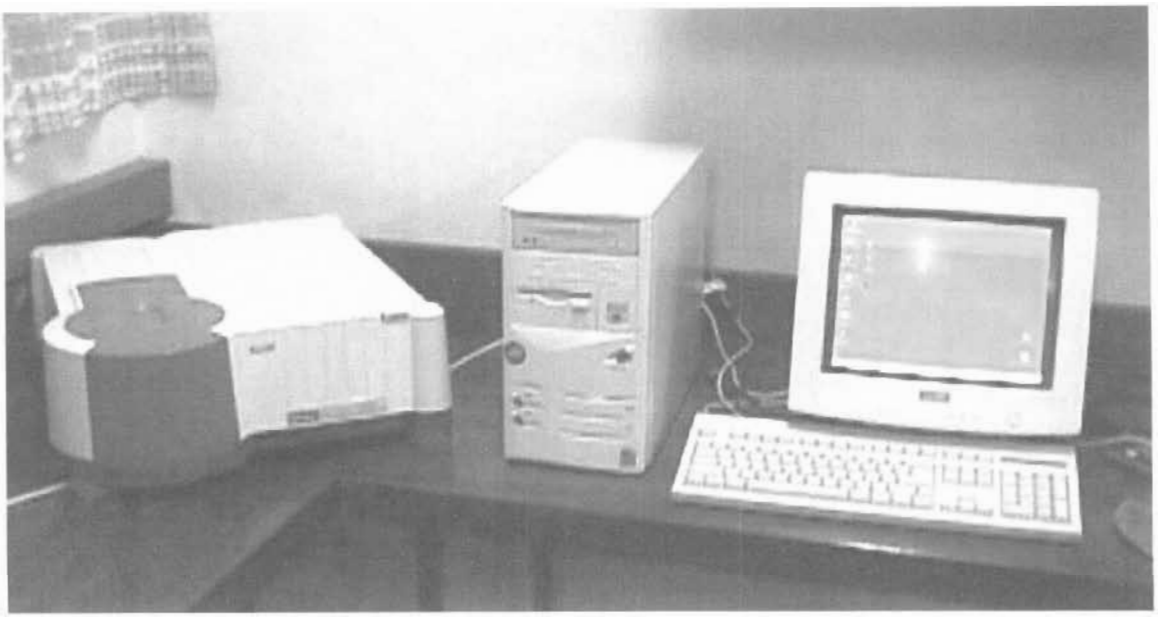


Figure 3.2: UV-Visible spectrophotometer (VARIAN AUSTRALIA Ltd) used for DNA quantification and purity analysis.

3.2.3. DNA dilution

DNA (2 μg) were diluted in 300 μl of TE buffer to make a final concentration of 0.006 μgμl^{-1} of DNA. Diluted DNA solution (9 μl) was then used to add 54 ng of DNA to the 25 μl of PCR reaction mixture.

3.2.4. DNA amplification and electrophoresis

DNA amplification reactions were performed in volumes of 25 μl containing reaction mixture as described by DAMASCO *et al.* (1996b) with slight modifications. These included 2.5 μl PCR Manufacturer's Reaction Buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50mM KCl, 0.1 mg ml^{-1} gelatine, pH 8.3; Roche Diagnostics GmbH, Mannheim, Germany), 0.2 mM each of dATP, dCTP, dGTP and dTTP (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 μM of OPC-15 and OPJ-04 random decamer primer (Operon Technologies, Alameda, CA, USA), 54 ng of banana genomic DNA and 1.5

units of Taq DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany). Sterile HPLC grade water was used to adjust the final volume to 25 µl and overlaid with 50 µl of paraffin oil (Table 3.3).

Amplification was performed in a Hybaid Thermal Reactor (HYBAID Ltd., UK , HYBAID 1991 Model)) (Figure 3.3). The amplification reaction was performed at an initial denaturation temperature of 95°C for 1 min, 36°C for 20 sec and 72°C 2 min (1 cycle) followed by 45 cycles of each 95°C for 10 sec, 36°C for 20 sec and 72°C for 2 min with a final elongation step (1 cycle) of 72°C for 5 min and 35°C for 1 min.

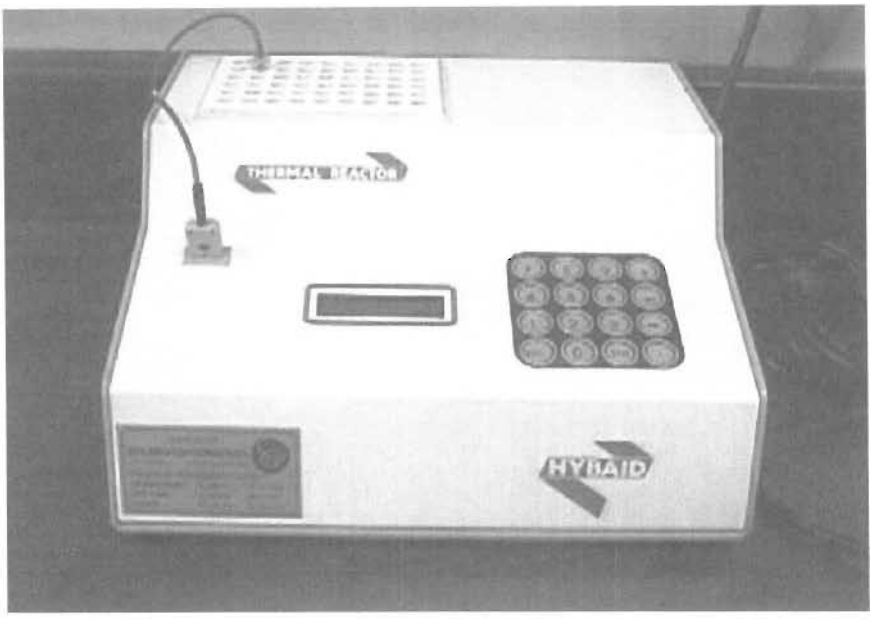


Figure 3.3: The HYBAID Thermal Reactor used for DNA amplification (RAPD-PCR).

The amplification products were analysed by gel electrophoresis (Figure 3.4) in 1.5% agarose (HISPANAGAR, Burgos, Spain) containing 0.25 µg ml^{-1} of ethidium bromide. After thermocycling, 3 µl of gel loading buffer were mixed with the 25 µl reaction mixture, and 15 µl were loaded onto a 1.5% agarose gel. The gel was run in 1 x TAE (running) buffer at 50V (5 V/cm) for 3.5 h (HOEFER SCIENTIFIC INSTRUMENTS, USA). DNA molecular weight marker XIV (Roche Diagnostics GmbH, Mannheim, Germany) was included on each gel. Visualization of the amplification product was

made under UV light, 6x8 W – 312 nm tube, (UVItec Limited, Cambridge, UK , BTS-20. M, model) (Figure 3.5). The image was then saved for statistical analysis.

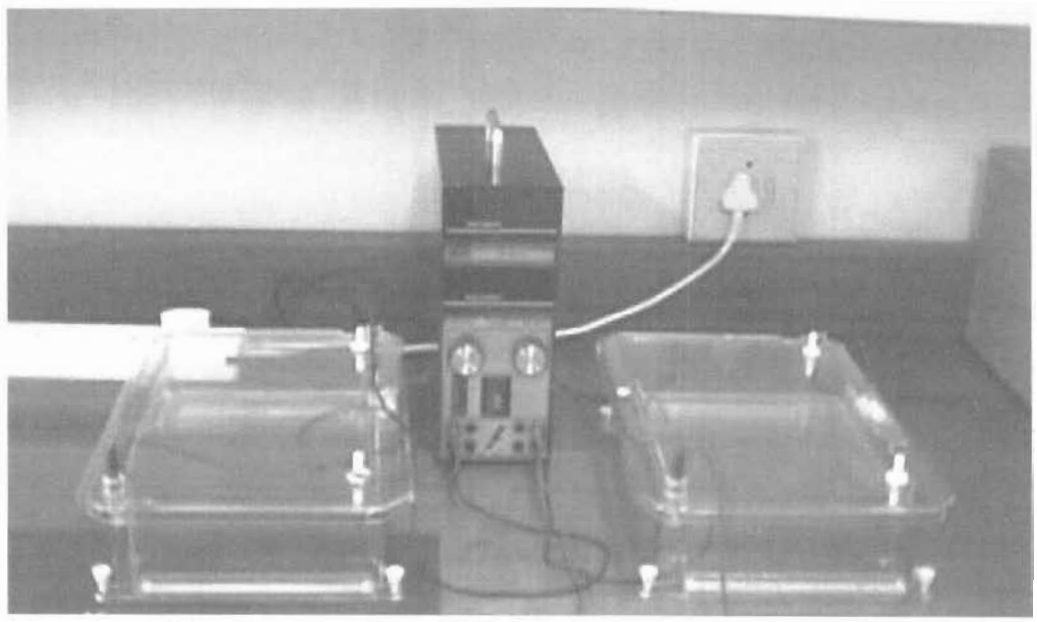


Figure 3.4: Apparatus for gel electrophoresis.

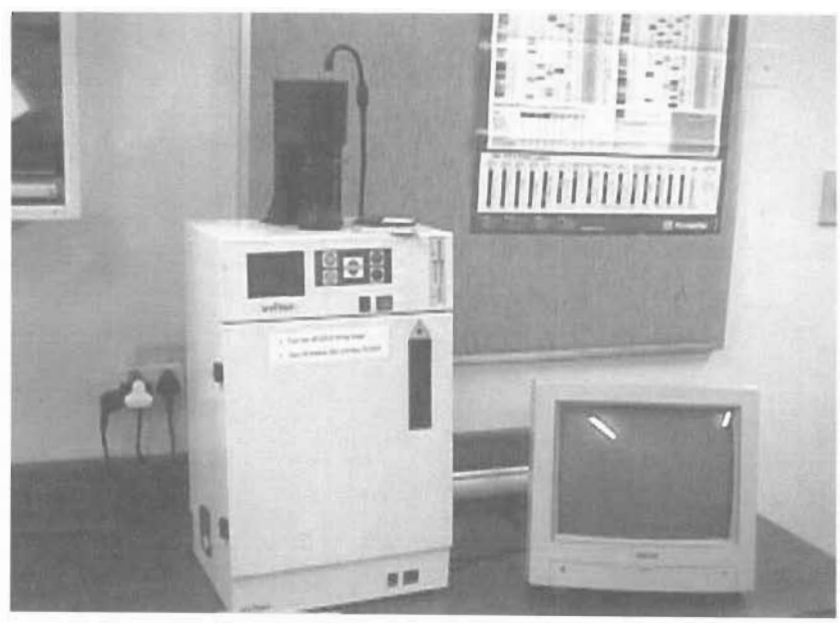


Figure 3.5: UV apparatus (Uvitec) used to capture the image after agarose gel electrophoresis of PCR products.

3.3. Statistical Analysis of Results

DNA fragments (bands) detected after separation by electrophoresis were scored for the presence (1) or absence (0) with the Molecular Weight Marker XIV ladder on the corresponding fragment size (Appendix 4). Data was then analysed using single linkage cluster analysis (GenStat). A dendrogram was created using the "nearest neighbours" method and samples clustered according to their similarities as indicated by the similarity axis.

3.3.1. Sampling and data collection

Random sampling of jars, with all the plantlets in the randomly selected jars, were used both for tissue culture and collection of leaf material for DNA extraction. For the first two sub-cultures (sub-cultures 5 and 6) all available culture were used. Randomly selected plantlets (20 per treatment) were used for the analysis of experiment one and DNA analysis was done for randomly selected plantlets: 10 for sub-cultures 5 and 6; 15 for sub-culture 8; 20 for sub-culture 9; and 25 for sub-culture 10 of experiment two. There is a missing value for sub-culture 7 of experiment two due to technical problems.

3.3.2. Cluster analysis

Techniques for cluster analysis seek to separate a set of data into groups or clusters. There are different types of clustering techniques, which differ in application and type of data analysed, namely;

- Hierarchical techniques: - In which the classes themselves are classified into groups, the process is repeated at different levels to form a tree;
- Optimisation techniques: - In which the clusters are formed by optimisation of the clustering criterion. The classes are mutually exclusive, forming a partition of the set of entities;
- Density or mode-seeking techniques: - In which searching regions containing relatively dense concentration of entities forms clusters;
- Clumping techniques:- In which the classes or clumps can overlap; and
- Others: - Methods, which do not fall clearly into any of the four previous groups.

The hierarchical clustering technique was employed for the purpose of this study. The technique involves partitioning data into classes in multiple steps. First data separated into a few broad classes, each of which is further divided in to smaller classes, and each of these further partitioned, and so on until terminal classes are generated which are not further subdivided. These techniques could be of two types (EVERITT, 1980):

1. Agglomerative: - Methods, which proceed by a series of successive fusion of the N entities into groups; and
2. Divisive: - Methods, which partition the set of N entities successively into further partitions

The agglomerative hierarchical clustering technique was used for this study. There are different algorithms of agglomerative hierarchical clustering. The basic procedure with all these algorithms is that they begin with the computation of a similarity or distance matrix between the entities and end with a dendrogramme showing the successive fusion of individuals, which culminate at the stage where all the individuals or groups of individuals are in one group. The difference between these methods is the way distance or similarity between individuals is defined (EVERITT, 1980). This method includes the following algorithms:

1. The nearest neighbours or single linkage method: - Used for both similarity and distance measures. The distance between groups is defined as the distance between their closest members;
2. The furthest neighbours or complete linkage method: - In this method the distance between groups is defined as the distance between their most remote pair of individuals. Also used with similarity and distance measures;
3. Centroid cluster analysis: - Here groups are depicted to lie in Euclidean spaces, and are replaced on formation by the co-ordinates of their centroid. The distance between groups is defined as the distance between the group centroids;
4. Median cluster analysis: - This tackles the limitations of the centroid method of comparing two groups of very different size by assuming the groups to be fused are equal. Its limitation is that interpretation of geometrical sense is lost;

5. Group average method: - This method defines distance as the average of the distance between all pairs of individuals in the two groups; and
6. Ward's method, McQuitty's method and Lance and Williams's flexible methods are other algorithms of the agglomerative group.

Nearest neighbours or single linkage cluster analysis was utilised for the purpose of this study (GenStat). The distance measure between populations was taken as the Euclidean distance function given by;

$$d_{ij} = \sqrt{\left\{\sum_{k=1}^{p}\left(X_{ik} - X_{jk}\right)^2\right\}}$$

Where:- X_{ik} is value of variable k for individual i

X_{jk} is value of variable k for individual j

p is value of variables $X_1, X_2, \ldots X_p$ for n objects

3.3.3. Correlation analysis

The significance of the relationship between multiplication rate and percent of somaclonal variation was tested using correlation analysis. The null hypothesis for this significance test was that there is no linear relationship at all between the X and Y variables where X is multiplication rate and Y is somaclonal variation.

To test the hypothesis the product moment correlation coefficient (r) was calculated using the formula:

$$r = \frac{\sum (X - \bar{X}) * (Y - \bar{Y})}{\frac{S_x}{N-1}\quad\frac{S_y}{}}$$

Where S_x is the sample standard deviation of x data; S_y is the sample standard deviation of y data; and N-1 is unbiased estimate of variance.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1. Results

4.1.1. Effect of type of plant growth regulators on somaclonal variation.

Polymorphic bands produced by primer OPC-15 revealed variability as high as 55% (Table 4.1). Percentage and types of variant individuals produced by *in vitro* culture varied considerably amongst the treatments. Treatments with a high multiplication rate showed greater variation compared to those with a lower multiplication rate (Table 4.1).

A correlation analysis was performed to assess the statistical significance of the relationship between multiplication rate and percentage somaclonal variation. There was a strong correlation ($r = 0.725$; $n = 6$) between multiplication rate and somaclonal variation.

Dwarfism was the most common type of variant, accounting for 87.7% of the total variation. Other DNA polymorphisms observed were not associated with dwarf off-type traits. This could be due to other changes involving different variant traits. It was also observed that all dwarf off-types have the same banding pattern.

A particular band of approximately 1500 bp was consistently present in all the normal plants but was absent in all the dwarf off-types (Figure 4.1). Primers OPC-15 and OPJ-04 were used to determine whether the fragment / band was similar to that reported by DAMASCO *et al.* (1996b). The band isolated in this study, $OPC\text{-}15_{1500}$, is, in fact, similar in size to the $OPJ\text{-}04_{1500}$ fragment identified by

Table 4.1: Percentage variation at the tenth level of subculture, average number of normal plants produced *in vitro* per explant, mean similarity and number of clusters for Cavandish banana cv. 'Zelig' grown in various combinations of auxins (IAA, IBA, NAA at 2 mg l^{-1}) and cytokinins (BA at 5 mg l^{-1} and TDZ at 3 mg l^{-1}).

Treatment No.	Treatments	Similarity levels	Mean similarities	Variation (%)	No. of clusters	Most typical members	Normal plants per explant
0	CONTROL[1]	-	-	3.3	-	-	-
1	IAA X BA	100, 90	96.8	40	4	10/20	3.5
2	IAA X TDZ	100, 90	98.1	20	2	15/20	1.5
3	IBA X BA	100, 90	92.6	55	4	9/20	3.25
4	IBA X TDZ	100,90,80	95.9	30	4	14/20	1.25
5	NAA X BA	100, 90	96.1	30	3	13/7	1.8
6	NAA X TDZ	100, 90	95.0	15	3	17/20	1

[1] The control plants were at the fourth level of subculture

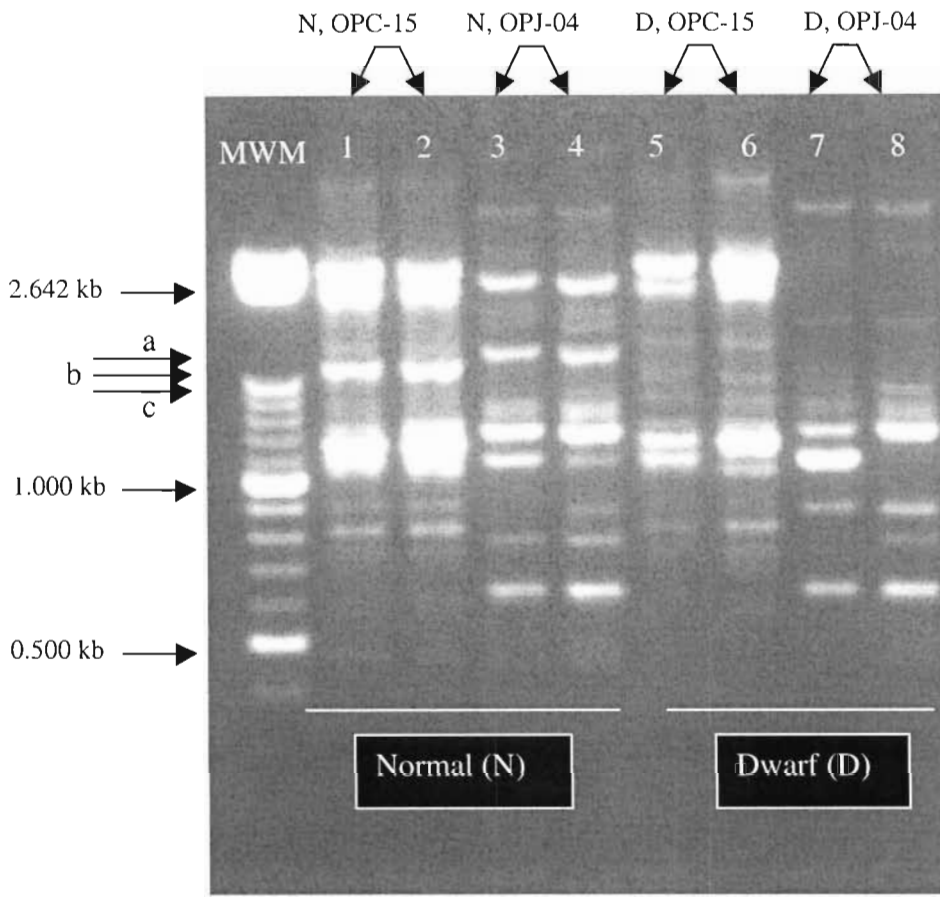


Figure 4.1: Comparisons of RAPD profiles: amplified by primers OPC-15 (lanes 1, 2, 5 and 6) and OPJ-04 (lanes 3, 4, 7 and 8). Lanes 1-4 are from one sample of normal plant (N) while lanes 5-8 represent a single dwarf type (D). Arrow 'a' indicates (lanes 3 & 4) the fragments OPJ-04_{1500} as named by DAMASCO *et al.* (1996b). Arrow 'b' indicates the fragment OPC-15_{1500} consistently produced (lanes 1 & 2) by primer OPC-15 in all normal plants but absent in all the dwarfs in this study called OPC-15_{1500}. Arrow 'c' indicates the brighter 1500 bp fragment of the MWM XIV ladder on 1.5% agarose gel electrophoresis. Other arrows indicate the 2-3 times brighter fragments of the MWM XIV that make the use of this marker more convenient for appropriate sizing of DNA fingerprints.

DAMASCO *et al.* (1996b). Both primers resulted in the amplification of a band that was present in normal plants but not in the dwarf varieties. The fragment obtained in this investigation referred to, hereafter, as OPC-15_{1500} was similar in size as OPJ-04_{1500}.

4.1.2. Combined effects of cytokinin (BA) concentration and sub-culturing on somaclonal variation

The RAPD technique revealed polymorphisms among the population of cultivar 'Zelig'. The size of the scorable bands produced ranged from approximately 2500 bp to 350 bp and the number of bands ranged between 7 to 11. In general, the amount of variation differed between the treatments. As the concentration of BA and level of sub-culture increased, so did the amount of variation (Figure 4.2). The Multiplication rate increased the longer the plants were left in culture (Figure 4.3). It was also observed during the study that Hyperhydric shoots that developed, treatments with higher level of cytokinins, in the initial stages of the culture period and produced healthy shoots later on (Figure 4.3). This later development of shoots could be adventitious and hence have higher chance of variation occurring.

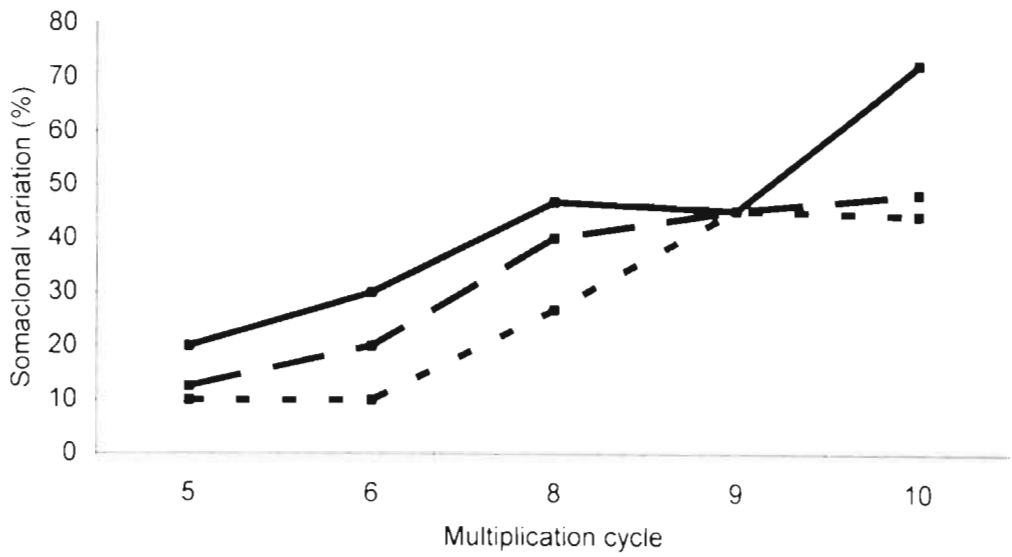


Figure 4.2: Rate of somaclonal variation as affected by BA concentration and duration in culture. Note: ..... indicates 2.5 mg l^{-1} BA, _ _ _ _ _ indicates 5.0 mg l^{-1} BA and _____ indicates 7.5 mg l^{-1} BA.

Mean similarity among populations (Table 4.2) was negatively correlated with percentage variation (r = -0.729; n = 15). This means that higher mean similarities are associated with lower variation rates. The ratio of normal to total plants ('most typical members') decreased as the type of variants increased. There were between 1 and 3 types of variants (Table 4.2). Number of clusters increased with an increase in variation rates and multiplication cycle. Numbers of clusters are also indicative of the number of variant types. A treatment having four cluster groups, for example, has three variant types.

Table 4.2: Summary of dendrograms created using the hierarchical single linkage cluster analysis after scoring bands for presence or absence in a particular locus. Please refer to appendix 4 for description of treatment code and appendix 5 for format of scoring sheet.

Treatment code[1]	Similarity levels	Mean similarities	Variation in percent	Number of clusters	Most typical members
215[1]	100, 90	98.9	10	2	9/10
225	100, 90	98.7	10	2	9/10
235	100, 90	98.0	20	2	8/10
216	100, 90	98.9	10	3	9/10
226	100, 90	94.7	30	4	7/10
236	100, 90	94.3	30	2	7/10
217	-	-	-	-	-
227	-	-	-	-	-
237	-	-	-	-	-
218	100, 90	96.9	26.67	4	11/15
228	100, 90	96.2	40	3	9/15
238	100, 90	94.7	46.67	3	8/15
219	100, 90	95.5	45	3	11/20
229	100, 90	97.1	45	2	11/20
239	100, 90	96.4	45	3	11/20
210	100, 90	96.3	44	4	15/25
220	100, 90	95.6	48	4	13/25
230	100, 90	95.3	72	5	7/25

[1]In the treatment code the first number implies experiment number, the second number treatment and the third number stands for number of sub-culture (Example 215 means experiment two treatment one sub-culture five). Treatment one 2.5 mg l^{-1} BA, treatment two 5.0 mg l^{-1} BA and treatment three 7.5 mg l^{-1} BA (refer Appendix 4).

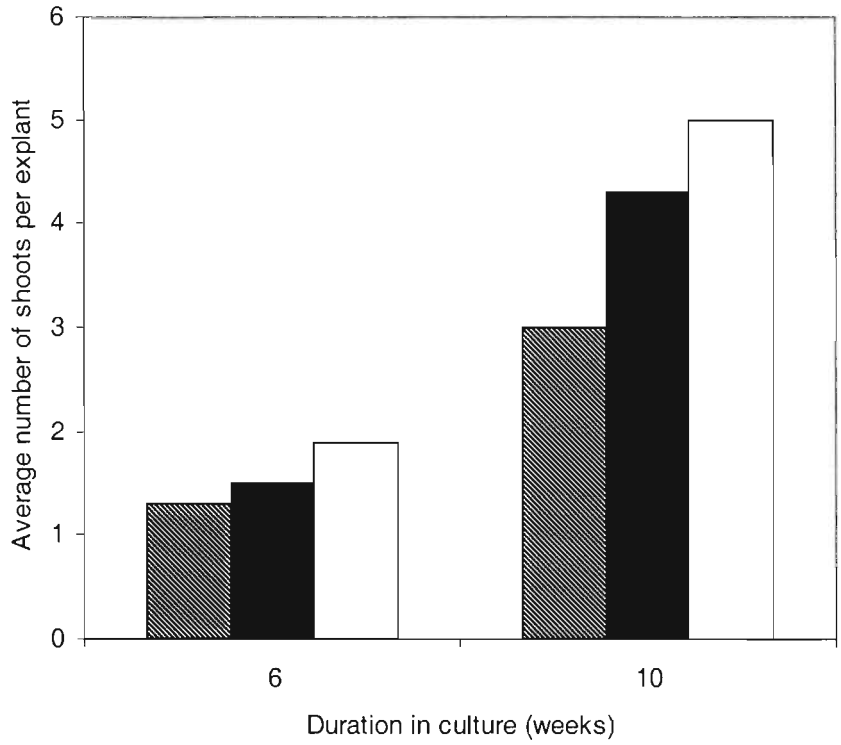
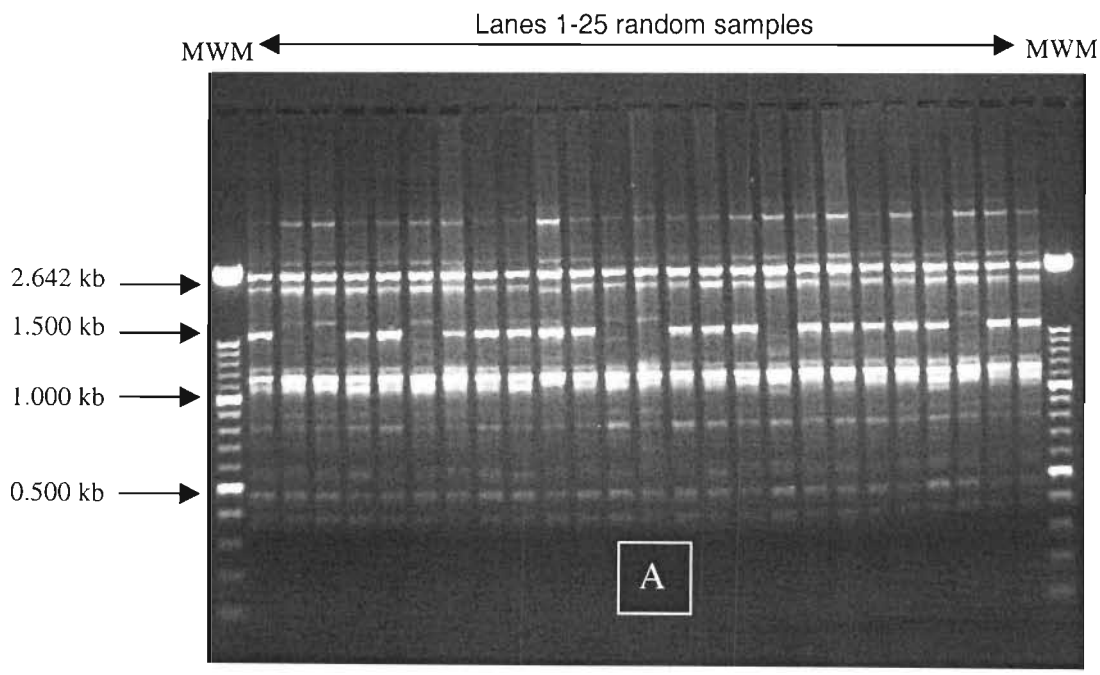
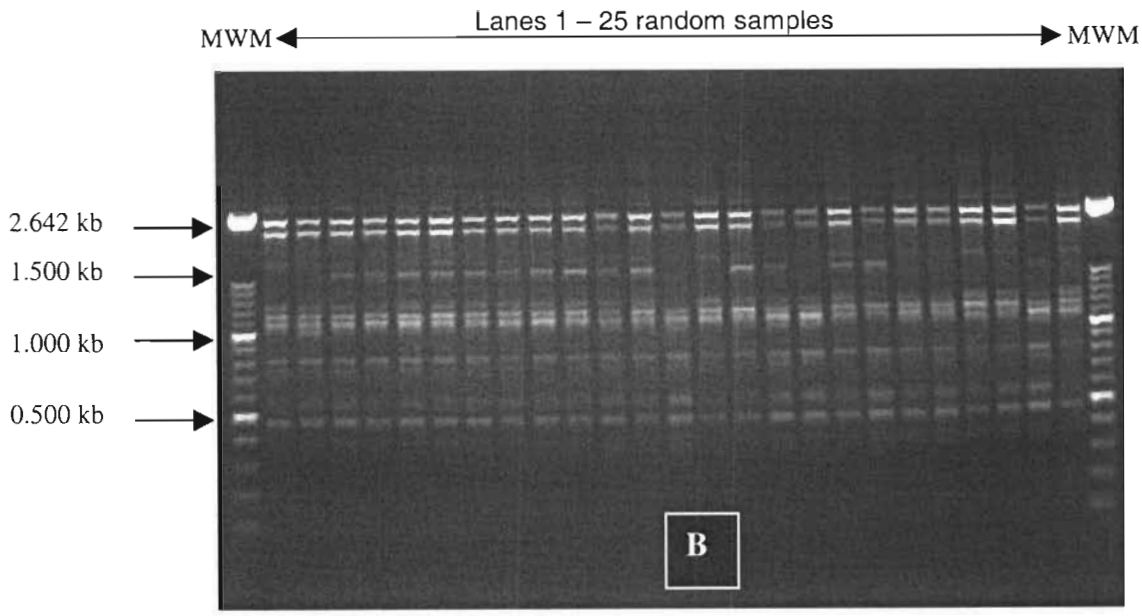


Figure 4.3: Shoot multiplication as affected by BA concentration and duration in culture. Note the stripe bar indicates 2.5 mg l^{-1} BA, the black bar indicates 5.0 mg l^{-1} BA and the white bar indicates 7.5 mg l^{-1} BA.





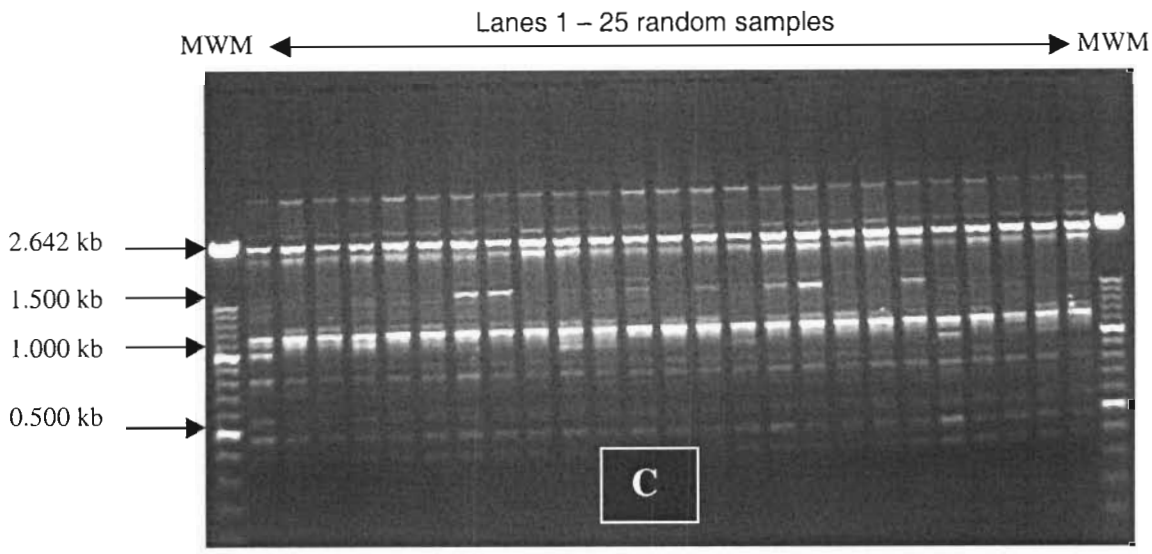


Figure 4.4: RAPD profiles of randomly selected plantlets at the 10th level of multiplication cycle. 'A' represents populations grown on media supplemented with 2.5 mg l^{-1} BA; 'B' with 5.0 mg l^{-1} BA and 'C' 7.5 mg l^{-1} BA. Note the fragment $OPC\text{-}15_{1500}$ that corresponds with the 1500 bp band of the MWM ladder. Arrows indicate the brighter fragments of the ladder.

4.2. Discussion

The primer OPC-15 was used throughout the study (Figure 4.4) as it is used to identify sources of variation *in vitro* (GRAJAL-MARTIN *et al.*, 1998). Primer OPJ-04 was only used to detect the presence of the dwarf off-type marker, $OPJ\text{-}04_{1500}$, reported by DAMASCO *et al.* (1996b). The presence of the dwarf specific marker was confirmed for cv. 'Zelig', which could be used to detect dwarfs *in vitro* (Figure 4.1). The presence of the band $OPC\text{-}15_{1500}$, a fragment similar in size with $OPJ\text{-}04_{1500}$, could explain the presence of only one type of dwarf mutation in dessert banana, as reported by DAMASCO *et al.* (1996b). Although both fragments are similar in size, it is necessary to sequence both fragments to determine their base composition and so identify similarities and differences.

REUVENI *et al.* (1993) reported that the rate of variation in Cavendish banana was not affected by both the medium composition and rate of multiplication. The rate of multiplication and variation were, however, strongly correlated in this study ($r = 0.725$; $n = 6$). The correlation between variation rate and cytokinin concentration could possibly be due to the accumulation of endogenous cytokinin during the culture period. ZAFFARI *et al.* (2000) demonstrated the progressive accumulation of endogenous levels of cytokinin in the basal portion of the plant with a significant decrease in the apical portions. Since both the basal and apical meristematic parts of the plantlets are used for propagation, it is possible that cytokinins accumulate with each sub-culture. This could swing the culture conditions towards the development of adventitious shoots. Since increased duration in culture decreases the auxin/cytokinin ratio (ZAFFARI *et al.*, 2000), a phenomenon that favours shoot development, it could also have contributed towards shoot development. This in turn contributes to excessive tissue proliferation due to the influence of cytokinin.

In this study different types of variants were obtained. However, only the dwarf off-types were identified on the basis of the dwarf specific marker from the work of DAMASCO *et al.* (1996b). Of the total variation, dwarfism accounted for 87.7%. This result is very similar to reports using other Cavendish cultivars (DANIELLS & SMITH,

1991; ISRAELI *et al.*, 1991). It is very difficult to detect the dwarf off-types during multiplication. This contributes to the significant increase in the dwarf off-types in subsequent generations. ZAFFARI *et al.* (2000) compared the levels of cytokinin and IAA in the proliferation medium. Although endogenous levels of cytokinin increased in the basal portion of the explant, the IAA concentration did not vary significantly. One could infer that the cytokinin level plays a more important role. They also indicated that the IAA/cytokinin ratio was higher during the early period of the culture and decreased thereafter leading to a smaller ratio, which favours shoot proliferation. Results presented on Figure 4.3 would support this explanation.

Plant growth regulators affect the rate of somaclonal variation indirectly. This is in agreement with the work of DAMASCO *et al.* (1998a). They indicated that the concentration of cytokinin used in the multiplication medium influenced the production of dwarf off-types. The relatively higher variation rate at 7.5 mgl^{-1} of BA in this study could be due to increased adventitious shoot proliferation as a consequence of including BA in the medium (Figure 4.2).

The high variation (72%) can also be explained by the large number of sub-culture cycles (10 generation times), which is far more than the number of cycles used for commercial multiplication '5-6 sub-cultures' (African Biotechnologies Ltd. South Africa, personal communication). Large numbers of multiplication cycles promote the further multiplication of the variants already produced in the previous cycles. Therefore, the rapid increase in variation during the later stages of multiplication could be due to extended duration in culture (DAMASCO *et al.* 1998a) or the multiplication of the variants produced in previous stages, or both. The stable nature of some variants, such as the dwarf off-types, and failure of the tissue culture condition to reverse the dwarf off-type trait (DAMASCO *et al.*, 1998a) could have contributed to the high occurrence of variants within the total population.

Polymorphism among *in vitro* plants of cv. 'Zelig' might be due to pre-existing recessive traits, transposable elements or propagation through tissue culture. The

presence and stability of dwarf off-types in culture may be due to some resistance or stability factor(s) associated with this trait. DAMASCO *et al.* (1998a) showed that after 30 months of continuous multiplication the dwarf off-types maintained 100% stability.

In this study variation started to appear as early as the 4th cycle (3.3%) (Table 4.1). REUVENI & ISRAELI (1988) also detected variation for the non-stable 'families' in the first generation of multiplication. DAMASCO *et al.* (1998a) also detected variation at the 5th cycle. The amount of variation increased with an increase in the number of sub-cultures. REUVENI & ISRAELI (1990) demonstrated that the frequency of variation was genotype dependent. They found some groups of plants with no variants independent of the medium, rate of multiplication and type of plantlet produced in culture. DAMASCO *et al.* (1998a) also reported similar results. SMITH & DREW (1990) undertook field trials to assess the growth and yield characteristics of dwarf off-types. They found that the dwarf characteristics were maintained for five generations tested, which indicates that the dwarf off-types are stable in nature. These results explain the importance of genotype stability and warrant a detailed investigation of the factors governing genetic stability.

With a large number of multiplication cycles, if the number of initial explant is small, the final rate of variants in the population will be very high. REUVENI & ISRAELI (1993) found that plantlets obtained from a single explant showed 80% variation, whereas those derived from 21 different explants showed only 8.4% variability. Since only five initial explants per treatment were sub-cultured 10 times, the number of multiplication cycles may have contributed to the high variation rates.

CHAPTER FIVE

CONCLUSIONS

From the results of this investigation it can be concluded that plant growth regulators affect somaclonal variation indirectly by increasing multiplication rate. Speed and large multiplication rates are very important in commercial banana tissue culture. The association of larger multiplication rates with increased variation rates, therefore, seeks an alternative mechanism of fighting the anomalies to maintain high multiplication and low variation rates. Reducing the concentration of cytokinins in the subsequent subcultures after culture establishment could possibly reduce the rate of somaclonal variation.

It was found in this study that dwarfism was the most common variant type. The irreversible and highly abundant nature of dwarf off-types warrants the need for further research to trace the genetic factors that govern or control this trait. This knowledge could lead to the understanding and control of the genetic stability and instability factors *in vitro*.

The relatively high variation rate signals the need for future studies to try and get stable mother plants to reduce the rate of variation. There is also a need for a more detailed study about the interaction and mode of action of plant growth regulators during the culture process in relation to their impact on genetic stability of plants.

The RAPD technique revealed variability *in vitro*. This is a confirmation of its successful application to detect off-types. Thorough characterization and development of specific genetic markers for all types of variant types is of importance. This could lead to the understanding of the nature of variants in relation to the mother plant, which is important in the application of molecular detection of variants in commercial laboratories.

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APPENDICES

Appendix – 1

Tissue culture media MS stocks and additives

M-S basal medium full strength (MURASHIGE & SKOOG, 1962)

Stocks	Nutrients	Mass (g) per volume of media		Volume used per liter
		500 ml	1000 ml	
1	NH_3NO_3	82.5	165.0	10
2	KNO_3	47.5	95.0	20
3	$CaCl_2.2H_2O$	22.0	44.0	10
4	$MgSO_4.7H_2O$	18.5	37.0	10
5	NaFe EDTA	2.0	4.0	10
6	KH_2PO_4	8.5	17.0	10
7a	H_3BO_4	0.31	0.62	10
	$ZnSO_4.7H_2O$	0.430	0.860	
	KI	0.0415	0.083	
7b	$MnSO_4.4H_2O$	1.115	2.230	10
8	$NaMoO_4.2H_2O$	0.0125	0.025	10
	$CuSO_4.5H_2O$	0.00125	0.0025	
	$CoCl_2.6H_2O$	0.00125	0.0025	
9	Thiamin HCl (B_1 / Aneurine	0.005	0.01	10
	Niacine (Nicotinic Acid)	0.025	0.05	
	Pyridoxine HCl (B_6)	0.025	0.05	
	Glycine	0.1	0.2	

Additives to the tissue culture media used

Component	Concentration
Sucrose	30 g l^{-1}
Gelrite	2 g l^{-1}
Adenine Sulphate	0.2 g l^{-1}
Sodium-dihydrogen	0.38 g l^{-1}
Ascorbic acid	0.18 g l^{-1}

Plant growth regulators used

Name	Type	Concentration
IAA	Auxin	2 mg l^{-1}
NAA	Auxin	2 mg l^{-1}
IBA	Auxin	2 mg l^{-1}
BA	Cytokinin	See experiments
TDZ	Cytokinin	3 mg l^{-1}

Appendix – 2

DNA extraction recipes

CTAB – extraction solution: (2% (w/v) CTAB; 100 mM Tris.Cl pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NACl)

CTAB/NaCl solution: (10% CTAB; 0.7 M NaCl mixed while heating (65°C) and stirring)

CTAB – precipitation solution: (1% w/v CTAB; 50 mM Tris.Cl, pH 8.0; 10 mM EDTA)

High- salt TE buffer: (10 mM Tris.Cl, pH 8.0; 0.1 mM EDTA, pH8.0; 1 M NaCl)

TE buffer: (10 mM Tris.Cl, pH 7.4, 7.5 or 8.0; 1 mM EDTA, pH 8.0)

Chloroform: Iso-amyl alcohol (24:1 ratio)

Appendix – 3

Description of treatment combinations for experiment two

Treatment code	Description
215	Experiment 2 treatment 1 subculture 5
225	Experiment 2 treatment 2 subculture 5
235	Experiment 2 treatment 3 subculture 5
216	Experiment 2 treatment 1 subculture 6
226	Experiment 2 treatment 2 subculture 6
236	Experiment 2 treatment 3 subculture 6
217	Experiment 2 treatment 1 subculture 7
227	Experiment 2 treatment 2 subculture 7
237	Experiment 2 treatment 3 subculture 7
218	Experiment 2 treatment 1 subculture 8
228	Experiment 2 treatment 2 subculture 8
238	Experiment 2 treatment 3 subculture 8
219	Experiment 2 treatment 1 subculture 9
229	Experiment 2 treatment 2 subculture 9
239	Experiment 2 treatment 3 subculture 9
210	Experiment 2 treatment 1 subculture 10
220	Experiment 2 treatment 2 subculture 10
230	Experiment 2 treatment 3 subculture 10

Note: Experiment two indicates treatments with IAA x BA x subcultures (5-10). Treatment one indicates 2.5 mg l^{-1} BA, treatment two 5.0 mg l^{-1} BA and treatment three 7.5 mg l^{-1} BA.

Appendix – 4

Scoring sheet for PCR products

Data sheet

Experiment _____

Treatment _____

Code _____

Date _____

Sam	MWM XIV Fragment sizes at 2% agarose gel																	
No.	350	400	450	500	550	600	700	800	900	1000	1100	1200	1300	1400	1500	1600	2400	2500
1																		
2																		
3																		
4																		
5																		
6																		
7																		
8																		
9																		
10																		
11																		
12																		
13																		