SCAR Marker Analysis of Somaclonal Variation on Different Abnormal Morphological Characteristics at Post-Planting Stage in *Musa accuminata* cv. Berangan

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Agrotechnology) with Honours

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DECLARATION

I hereby declare that the work embodied in this thesis is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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I certify that the thesis of this final year project entitled "SCAR Marker Analysis of Somaclonal Variation on Different Abnormal Morphological Characteristics at Post-Planting Stage in *Musa accuminata* cv. Berangan" by Nursyamimiatikah Binti Abdul Manan, Matric Number F15A0176 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Agrotechnology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFLP	Amplified fragment length polymorphism
BAP	6 – Benzylaminopurine
cv.	Cultivar
CTAB	Cetyl trimethylammonium bromide
dNTP	Nucleoside triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture
FOR	Forward
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrolidone
REV	Reverse
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease
SCAR	Sequence characterized amplified region
TBE buffer	Tris/Borate/EDTA
TE buffer	Tris EDTA
Tris – HCl	Tris hydrochloride

SCAR Marker Analysis of Somaclonal Variation on Different Abnormal Morphological Characteristics at Post-Planting Stage in *Musa accuminata* cv. Berangan

ABSTRACT

The regeneration of various horticultural species in vitro as tissue culture protocols make it potential for commercial scale multiplication which are available for any crops due to the improvements made in tissue culture techniques. Clonal propagation and preservation of elite genotypes which are being selected for their superior characteristics requires high degree of genetic uniformity amongst the regenerated plants. However, plant tissue culture can generate genetic variability which leads to the somaclonal variations. Morphological changes to the explant were observed in parallel with the increased BAP concentration used during growth in tissue culture stages of banana (*Musa accuminata* cv. Berangan) seedling. Previously, samples of banana tissue culture were treated with different BAP concentration (0 mg/l, 5 mg/l, 10 mg/l and 15 mg/l) to study the effect of somaclonal variation by plant growth regulator. Polymorphisms was observed among the plantlet by using RAPD analysis which were later developed to SCAR marker. In this study, the SCAR markers were used to analyse the genome of somaclonal variation on different abnormal morphological characteristics at post planting stage in banana. Fifty-one samples of somaclonal variation clone of bananas were collected at the field for each treatment with the knowledge of their plant height. The plantlets were screened using SCAR marker which were UMK-01,02; OPU06-01,02; OPU06-03,04 and OPJ13-03,04. After optimization of PCR, annealing temperature of 55 °C and template DNA volume of 4 µl were selected for all primers as the condition provide the most specific amplification. All of the SCAR markers showed monomorphic banding pattern except for OPJ13-03,04 where sample 35 showed a missing band. The variation however was unique only to this sample and not consistent thorough the samples of the same treatment nor the same plant height average. This indicated that the genetic variant that occur to the sample is random and that any genetic change induced by the BAP was not uniform for all sample within the same treatment. Most importantly, the markers used could not differentiate the sample based on their group plant height, which were significantly different among the treated samples. Further analysis of the somaclonal variants of banana need to be done using other type DNA marker that were able to explain the variance among the treatment, notably its plant height. This information is crucial for early identification of stunted clones which are usually uneconomic for commercial planting.

Keywords: RAPD, SCAR, PCR, polymorphism, somaclonal variation

Analisis Variasi Somaklonal Pada Ciri-Ciri Morfologi Yang Tidak Normal Pada Peringkat Mula Penanaman Dalam Pisang (*Musa accuminata* cv. Berangan) Menggunakan Penanda SCAR

ABSTRAK

Pertumbuhan semula pelbagai spesies hortikultur secara *in vitro* atau kaedah tabung uji sebagai protokol kultur tisu menjadikannya berpotensi untuk peningkatan skala komersial yang tersedia untuk sebarang tanaman disebabkan peningkatan yang dibuat dalam teknik kultur tisu. Penyebaran klon dan pemeliharaan genotip elit yang dipilih untuk ciri-ciri unggulnya memerlukan tahap keseragaman genetik yang tinggi di antara tumbuhan yang ditumbuh semula. Walau bagaimanapun, tumbuhan kultur tisu berkemungkinan dapat menjana kebolehubahan genetik yang membawa kepada variasi somaklonal. Perubahan morfologi terhadap eksplan diperhatikan selari dengan peningkatan kepekatan BAP yang digunakan semasa pertumbuhan dalam peringkat kultur tisu pisang (*Musa accuminata* cv. Berangan). Sebelumnya, sampel kultur tisu pisang dirawat dengan kepekatan BAP yang berlainan (0 mg/l, 5 mg/l, 10 mg/l dan 15 mg/l) untuk mengkaji kesan variasi somaklonal oleh pengawal atur pertumbuhan. Polimorfisme diperhatikan di antara tanaman dengan menggunakan analisis RAPD yang kemudiannya ditukar kepada SCAR. Dalam kajian ini, penanda SCAR digunakan untuk menganalisisi genom variasi somaklonal pada ciri-ciri morfologi yang tidak normal pada peringkat mula penanaman dalam pisang. 51 sampel klon variasi somaklonal pisang dikumpulkan daripada ladang untuk setiap rawatan dengan pengetahuan ketinggian tumbuhan pisang. Anak tumbuhan telah ditapis menggunakan penanda SCAR iaitu UMK-01,02; OPU06-01,02; OPU06-03,04 and OPJ13-03,04. Selepas pengoptimuman PCR, suhu 55 ° C dan jisim templat DNA 4 µl dipilih untuk semua primer SCAR kerana keadaan ini menunjukkan amplifikasi lebih spesifik. Semua penanda SCAR menunjukkan corak monomorf kecuali OPJ13-03,04 di mana sampel 35 menunjukkan jalur yang hilang. Variasi bagaimanapun adalah unik hanya untuk sampel ini dan tidak konsisten kepada keseluruhan sampel rawatan yang sama atau purata ketinggian tumbuhan yang sama. Ini menunjukkan bahawa variasi genetik yang berlaku kepada sampel adalah secara rawak dan sebarang perubahan genetik yang disebabkan oleh BAP yang tidak seragam untuk semua sampel rawatan yang sama. Paling penting, penanda yang digunakan tidak dapat membezakan sampel berdasarkan ketinggian tumbuhan kumpulan mereka yang sangat berbeza di antara sampel yang dirawat. Analisis lanjut mengenai variasi somaklonal pisang perlu dilakukan menggunakan jenis penanda DNA yang lain yang dapat menjelaskan variasi di antara rawatan, terutama ketinggian tanaman. Maklumat ini penting untuk pengenalan awal klon terbantut yang biasanya tidak ekonomik untuk penanaman komersial.

Kata kunci: RAPD, SCAR, PCR, polimorfisme, variasi somaklonal

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Banana is generally well-known as a grown fruit crop in tropical areas. It is considered as important horticultural crops in food and agricultural industry because of its great economic value and good dietary sources. Banana is likewise called as “poor man’s apple” because of its cheap price compared to other fruits in the country and considering its high nutritive value of banana.

In 1981, Larkin and Scowcroft proposed that the term of somaclonal variation comes from the variation among plants that are regenerated from any form of cell culture as cited in Deepthi, 2016). A problem in any *in vitro* propagation to produce true to type plant material can occur due to the occurrence of somaclonal variation (Hare, Mahdi, Dhurendra, Udayvir, Nitesh, Maliheh, Radha, 2015). Somaclonal variation has become alternative tools to the plant breeders in obtaining genetic variability rapidly and without efficient technology in horticultural crops, the breeding processes become limited and lengthy. The variation that occurred can also be unstable or no heritable. Somaclonal with undesired features, for example, reduction of fertility, poor rate of growth and overall

performance of plants that obtained from somaclonal variation may lose the power for the plant to regenerate.

There are two sources of somaclonal variation which are pre-existing variation known as variants and tissue culture induced variation known as mutants. Pre-existing variation (variants) is defined as the mutated cells arise spontaneously. Regeneration of plants of such cells lead to creation of somaclones. Meanwhile, tissue culture induced variation (mutants) is defined as the tissue culture environment which acts as a mutagenic agent and alters normal control of cell division and chromosome leading to the generation of somaclonal variation. There are some factors that contribute to somaclonal variation which were the effect of explant, the culture age, the plant growth regulators effect in vitro, genotype fidelity, flexibility of genotype, ploidy level effect, role of transposable elements and karyotype changes.

The polymerase chain reaction (PCR) is a technique that have been established to allow the selective in vitro DNA amplification and used for molecular marker studies. A PCR-based marker is used to detect polymorphism where the techniques of PCR are more reliable. The DNA-based markers are functioned powerfully in identifying polymorphism and used widely due to the potential large number of polymorphisms that can be detected, and it allows analysis and identification of either coding or non-coding regions for the entire genome. The marker is also independent of development of the plant and seldom influenced by environment. DNA-based markers such as RFLP, AFLP, RAPD, SSR etc. are widely used to detect somaclonal variation.

In this study, SCAR marker used was designed from the previously cloned RAPD fragments that linked to the interest trait. SCAR markers do not have a problem regarding the low reproducibility as encountered with the RAPD. SCAR marker is a codominant

marker that may have advantage in converting RAPD to SCAR even though SCAR marker might exhibit the dominance when the primers partially overlap at the site of the sequence variation. The polymorphisms can be detected by the gel electrophoresis. The main advantage of using SCAR marker is its ease of use. In addition, SCAR marker produced high reproducibility compared to the RAPD marker. Similar to RAPD, the SCAR marker also used minute amount of DNA for analysis and this is advantageous to the screening process where the amount of sample can be limited. However, SCAR markers also have its weakness which are the need for data sequencing in order to design the PCR primers with the linked to the trait of interest. Thus, SCAR markers have been applied in many studies such as gene mapping and marker assisted selection in molecular biology.

1.2 Problem Statement

Tissue culture techniques of banana propagation produced the genetic variants which is may be genetic or epigenetic variation. Previously, during the tissue cultures stage, benzylaminopurine (BAP) was used to induce somaclonal variation among the plantlet of banana. To study the effect of BAP, various amounts of BAP concentrations were tested during the *in vitro* tissue culture growth of the banana seedling.

From the treatment, morphological changes to the explant were observed in parallel with the increased BAP concentration used during growth in tissue culture stages. Plantlets that were exposed to high BAP developed into scalp morphology, however when the BAP treatment were removed, the plantlet revert back to normal with formation of roots and shoots. In addition, polymorphism of bananas with varied morphologies during tissue culture stages could be detected by using the molecular marker RAPD, which

implies the genetic changes that occurred in parallel with the various morphologies observed. However, the testing was done by bulking ten samples with the same morphology and treatment due to its limited size for DNA extraction.

Early investigation of the polymorphism observed between the plantlets with different morphologies showed that the genetic changes observed were related to the repetitive element of the genome. In term of morphology, the plantlet can revert to normal when the BAP treatment was removed, however at post-planting the individuals showed significant phenotypic changes (data not published). This study focuses on the assessment of the genetic polymorphism between the individuals that were treated with different BAP concentration and showing significant phenotypic changes at post-planting stages by using SCAR marker.

1.3 Objective of Study

The objectives of this study were:

a) To optimize PCR conditions of SCAR markers developed from previous RAPD marker that showed polymorphism on banana tissue culture clones.

b) To identify DNA polymorphism among the *Musa accuminata* cv. Berangan clones of tissue culture at post-planting stages using SCAR marker analysis.

1.4 Hypothesis

H_o = There is no DNA polymorphism that can be detected between the clones of *Musa accuminata* cv. Berangan at post-planting stages by using SCAR marker.

H_A = There is DNA polymorphism that can be detected between the clones of *Musa accuminata* cv. Berangan at post-planting stages by using SCAR marker.

1.5 Scope of Study

In this study, *Musa accuminata* cv. Berangan was used which have been planted at the field in the Agropark, UMK. In order to understand occurrence of somaclonal variation in banana during the tissue cultures stage, benzylaminopurine (BAP) was used to induce somaclonal variation among the plantlet of banana and during the treatment. Four different treatments of BAP concentrations (0.0 mg/l, 5.0 mg/l, 10.0 mg/l and 15.0 mg/l) were used during tissue culture stages to induce variations in *Musa accuminata* cv. Berangan. These clones have been acclimatized and transferred on field. In this study, for each treatment of BAP concentration, ten individual clones will be assessed for genetic variation at post planting stages. Genetic polymorphisms assessment was performed among the individual banana with different BAP treatment regardless of its possible phenotype variability. SCAR markers were developed from the previous RAPD marker analysis that could detect polymorphisms between the plantlets with different treatment of BAP at tissue culture stages. The SCAR markers were designed based on the sequence of RAPD product that have shown polymorphism. Two different primers were designed for each RAPD product, especially if the products are above 1000 bp in size. The primers target the start and the end of the sequences with product size range at 150 bp to 200 bp.

Four primers of SCAR marker have been used which were UMK-01,02; OPU06-01,02; OPU06-03,04 and OPJ13-03,04.

1.6 Significance of Study

Musa spp. is considered as one of the most widely grown horticulture fruit crops in Malaysia. There are some farmers that already used the modern technology in Biotechnology industries instead of using the traditional techniques. However, there were some farmers who prefer to purchase suckers and plant it on the field rather than using plantlet originated from tissue culture. This is due to the problem of non-uniformity of the plantlet derived from the tissue culture technology. It was said that the plantlet from tissue culture were not uniform and were less robust compared to plantlets from the suckers. However, this has not yet been confirmed but one of the known problems in tissue culture is somaclonal variation among the clones. The induction of somaclonal variation may come from the stress during tissue culture stages.

Previous studies have detected parallel genotypic changes in parallel with the phenotypic changes observed among the clones of tissue culture. This showed that there is genetic basis behind the incidence of somaclonal variation. The difference in stability related to different in genetic make-up whereby some component of plant genomes is unstable during tissue culture stages. In this study, different concentration of BAP has been used to induce somaclonal variation during tissue culture growth. The somaclonal variation that have been induced will be used for assessment of the stability of the genetic changes observed during tissue culture up until they are being transplanted on field at post planting stages.

The research data acts as guidance for tissue culture researchers especially plant breeders and industries as to make the tissue culture propagation more uniform. In addition, the research data also important as references for the researchers in characterizing and identifying the somaclonal variants during tissue culture for production and research. This research data of the study can be used to prevent the somaclonal variants from occurring in tissue culture stage.

CHAPTER 2

LITERATURE REVIEW

2.1 Banana

2.1.1 Banana trait

Musa spp. is an important crops plants in tropical areas. *Musa accuminata* known as AA genome while *Musa balbisiana* known as BB genome. Both AA and BB genome with 2n = 22 chromosomes will represent the two main ancestors of cultivated banana varieties. According to Simmonds and Shephered (1955), the genomic groups which are (AA), (BB), (AB), (AAA), (AAB), (ABB), (AAAA) and (ABBB) have been identified in order to classify the edible clones of bananas.

2.1.2 Banana morphology

Bananas comes from the family of *Musaceae* and belong to *Eumusa* of the genus *Musa* and order of *Zingiberales* (Gill, 1988). Bananas are monocotyledonous plant. The root system has been replaced to the adventitious root system as the bananas at the primary seedling root dies early. Roots are 5 - 8mm thick, white and fleshy if healthy, but later become corky depend on the state of health of the plant. In addition, the roots usually

developed in the group of fours at the surface of the central cylinder of the corm. These roots produce fibrous lateral roots that are responsible for water and mineral uptake. The rhizome system of the banana is sympodial.

2.1.3 Banana distribution

Bananas are the fourth most important crop in developing countries. Some of the poorest country of the world have been provided with the banana as a starch staple, while dessert bananas are a noteworthy economical crop in numerous nation (Food and Agriculture Organization, 2007). According to Pollefeys, Sharrock, and Arnaud (2004), the cultivated bananas mostly are derived from interspecific and intraspecific crosses between two diploid (2n = 2x = 22) wild species which are *Musa accuminata* and *Musa balbisiana* (Simmonds & Shephered, 1955). In terms of the chromosome sets, it has been designated as having the genome constitution AA (*M. accuminata*) or BB (*M. balbisiana*). With genome constitutions of AAA, AAB or ABB, it has been proved to be triploid, 2n = 3x = 33 as for domesticated bananas. Meanwhile, genome constitutions of AAAA, AAAB, AABB and ABBB are tetraploids (2n = 4x = 44) as for seedless cultivated AA and AB diploids.

2.1.4 Status of cultivated banana industry in Malaysia

According to the Third National Agricultural Policy (1998-2010), bananas have been chosen as one of fifteen fruit types for commercial horticulture (Nik Mohd. Masdek, 2002). Major producers of banana industry are Johor, Pahang, and Sarawak with perennial industrial crops (Mokhtaruddin & Willian, 2011). Berangan and Cavendish

cultivars were cultivated about half of the 31,000 hectares for local food consumption and exportation while the Mas, Rastali, Nangka, Raja, Awak, Abu, and Tanduk were cultivated for the local market (Nik Mohd. Masdek, 2002). In this study, the cultivars of banana which is Berangan will be used.

2.2 Tissue Culture in Banana

There are many assortments in tissue culture stages that can be expected which are aseptic techniques growth of cells, *in vitro* regeneration of plants, transferring gene techniques, genome characterization and recombinant DNA methods (Brown & Thorpe, 1995). In *Musa* spp., propagation often has been done via vegetative due to its status of being the most noticeable sterile crop of the world (Khatri, Khan, Ahmad & Siddiqui, 1997). Micropropagation will produce plantlets that established fast, healthy, strong with smaller production cycles and produce high productions. Plant hormones are used to control the aspect development of plants. For examples of plant hormones are auxin, cytokinin, abscisic acid, gibberellin and ethylene (Hay et. al., 2004). However, cytokinin are commonly used for banana micropropagation such as Benzylaminopurine (BAP).

Some study has been done on tissue cultures of banana cultivars “Berangan Intan” (AAA), “Berangan” (AAA), “Rastali” (AAB) and plaintain cultivars “Baka Baling” (AAB) and “Nangka” (AAB). The study showed the relationship that the use of high BAP concentrations can increase the number of shoots but at the same time can lead to a higher abnormality index and reduced shoot regeneration.

2.3 Somaclonal Variation in Bananas

Somaclonal variation is also known as the genetic defects that usually occur in tissue culture using shoot tips (Bairu et.al., 2006). In other terms, somaclonal variation also known as protoclonal, gametoclonal, and mericlonal variation to describe variants from protoplast, anther, and meristem cultures (Bairu, Aremu, & Van Staden, 2011).

Several factors are known to contribute to somaclonal variation which were the effect of explant, the culture age effect or number of subculture cycles, the plant growth regulators effect *in vitro*, genotype fidelity, flexibility of genotype, and effect of ploidy level, karyotype changes and role of transposable elements. For the effect of explant, the explants with pre-existing meristem will produce less variation compared to highly differentiated tissues. Culture age will enhance the variability within the regenerated plants. Genetic stability may be influenced by the tissue culture conditions and rapid multiplication of a tissue which may lead to somaclonal variation. Similarly, it also may attribute by the longer duration of the culture and increased number of subcultures.

For the effect of plant growth regulators *in vitro*, the first event that may activate tissue cultured variability caused by exogenously application of chemicals is cell cycle disturbance. For example, cytokinin such as benzylaminopurine (BAP) and kinetin are functioned in inducing the genetic variability in banana. For genotype fidelity, all *Musa* spp. derived from *Musa accuminata* (genome A) and *Musa balbisiana* (genome B). The differences response *in vitro* generation may attribute to the influence of microenvironment on cellular behaviour. For flexibility of genotype, arrangement and mutation rate will be higher when the genome is susceptible to the stress during tissue culture stages. For the effect of ploidy level, the variability in plants regenerated *in vitro* is low among the ploidy and lower chromosome number in explant donor species

compared to the high polyploids and higher chromosome number. Genetic stability of the somaclonal variants in karyotype change is important. The selected traits can be useful if the variations are stable.

Detection of variations in bananas tissue culture will consume time, laborious and expensive. Although there is a probability of early detection, however, it needs individual inspection and an optimal uniform growth condition for all the plants (Bairu, Fennell, & Van Staden, 2006).

2.4 Molecular Marker

The molecular markers were selected based on the natural occurrence polymorphisms in DNA sequences. There are various kinds of molecular markers which are biochemical markers or isozymes and DNA-based markers. Biochemical markers are the molecular markers which reveal polymorphisms at protein level, while DNA-based markers will reveal polymorphisms at DNA levels. Biochemical markers also known as isozymes based on the multiple forms of enzyme which might differ in electrophoretic mobility.

DNA-based markers are a powerful tool in detecting polymorphisms and are used extensively because of its advantages. DNA-based markers potentially detect a large number of polymorphisms and allow investigation of both coding and non-coding regions of the whole genome. In addition, DNA-based markers are independent of growth and development of the plants. Since the markers can directly reveal the genetic variability through the DNA analysis, the method can bring significantly improvement in breeding approaches (Staub, Sequen, & Gupta, 1996).

2.4.1 PCR-based genetic markers

Polymerase Chain Reaction (PCR) is a revolutionary new technology that has been launched by the development of the programmable thermocycler. There are three processes involved in the PCR which are the separation of the strands known as denaturation, annealing the primer to the template and the synthesis of new strands known as extension or elongation. Each piece of DNA from the samples was copied by the end of the cycle. The cycle is then repeated for 30 times or more. For each newly synthesized DNA, it can act as a new template. PCR techniques are used widely in medical sector and in biological research labs for many applications. Example of DNA-based markers are RFLP, AFLP, RAPD, SCAR, and SSR.

a) Random Amplified Polymorphic DNA (RAPD)

RAPD can be a powerful tool in identifying of genetic variation in plants (Welsh et al., 1990). RAPD products are amplified from the total genomic DNA by using non-specific primers. The advantages of RAPD markers are being simple, easy and quick to handle which only requires small amount of DNA. However, RAPD also has its disadvantages where it suffers from low reproducibility. Initially, in previous research several tissue culture clones that were treated with different concentration of BAP developed into different morphologies as shown in Table 2.1. This samples were later profiled using RAPD primers OPA01, OPA02, OPA06, OPA15, OPA19, OPA21, OPA24, OPA25, OPC03, OPH09, OPJ04, OPJ10, OPJ13 and OPU06 as shown in Figure 2.1. Two different marker showed significant polymorphism between the sample treated with BAP and the negative control (no BAP). The two primers were OPU06 and OPA19, which both were able to consistently differentiate the negative control and the BAP

treated tissue culture. Based on Figure 2.1, OPJ13 did not showed any significant polymorphism among the sample of different treatment and morphology. However, OPJ13 was selected because in further analyses OPJ13 showed variation at the nursery stage (data not shown). In addition, it is also important to note that in previous RAPD analysis, the screening was performed by bulking the tissue culture sample in same treatment and harboured the same morphology. Thus, the RAPD profile obtained for each treatment and morphology were the representation of ten clones.

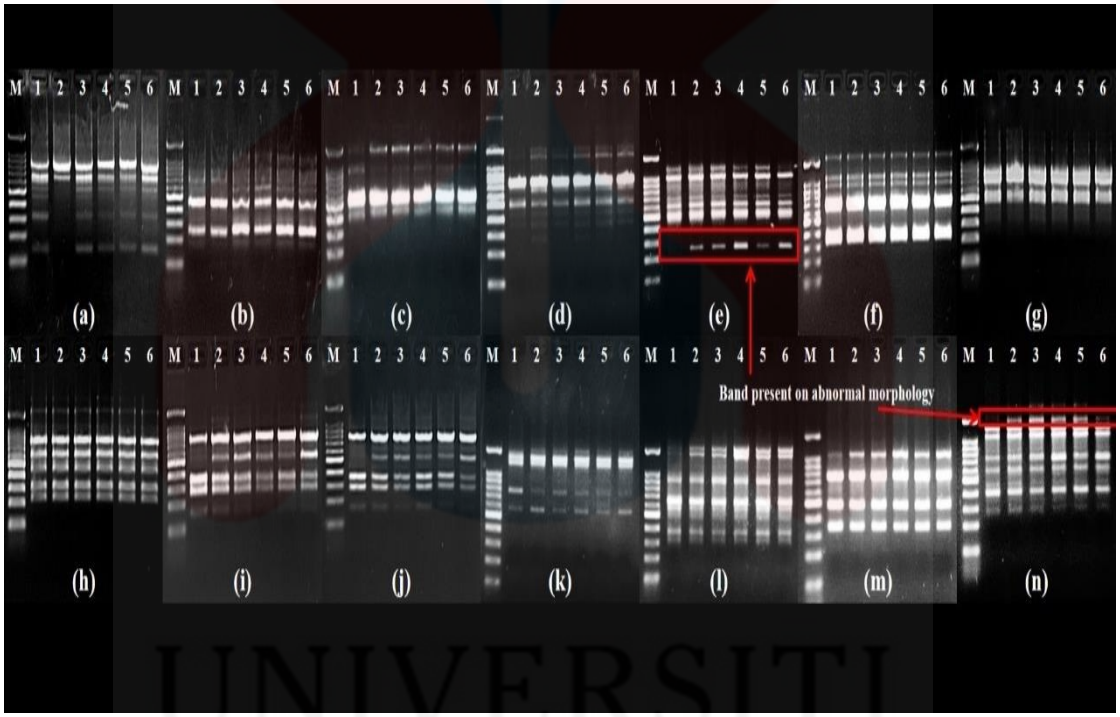


Figure 2.1**:** RAPD profile of banana cv. Berangan obtained with primers (a) OPA01, (b) OPA02, (c) OPA06, (d) OPA15, (e) OPA19, (f) OPA21, (g) OPA24, (h) OPA25, (i) OPC03, (j) OPH09, (k) OPJ04, (l) OPJ10, (m) OPJ13 and (n) OPU06; RAPD profiles at 20th subculture based on different morphology of banana cv. Berangan. The arrows are shown as missing band. The lanes are labelled as in Table 2.1. M = 100 bp ladder marker.

Table 2.1: Detailed list of the different morphological characters produced at the 20th subculture

No.	Concentration of BAP (mg/l)	Morphology
1	0	Normal shoot
2	5	Rosette-like structure
3	5	Scalp
4	10	Rosette-like structure
5	10	Scalp
6	15	Scalp

b) Sequence Characterized Amplified Regions (SCAR)

SCAR marker was established to overcome the problem of reproducibility which associated with the RAPD technique (Paran & Michelmore, 1993). SCAR markers characterize genomic DNA fragments at defined loci which can be determined by using a sequence specific primer. SCAR markers arise previously from RAPD analyses. A SCAR marker can detect by using the SCAR primers previously developed based on RAPD marker through PCR amplification. Locus specific band can be detected after PCR amplification followed by the gel electrophoresis (Bhat, et al., 2010).

CHAPTER 3

METHODOLOGY

3.1 Plant Materials

The fifty-one samples of leaves from *Musa accuminata* cv. "Berangan" that originated from tissue culture and then transplanted to post-planting field in AgroTechno Park, UMK was collected. Ten samples were collected as replicate for each sample treatment, with the exception of sample T3 which had eleven samples [Table 3.1]. All samples were assessed individually. As indicated in Table 3.1, the sample in each treatment had a different average height. This is included in this study as reference to the different somaclonal variants that have emerged due to the exposure of BAP during its tissue culture stages. The measurement however was done in this study. Only young leaves or shoots collected and these the entire sample were chosen to represent the wide range of genomic groups. Most molecular studies prefer DNA extraction from leaves.

Table 3.1: Sample treatment with height measured

Sample Treatment	Number of samples	BAP concentration (mg/l)	Mean height measured (cm)
T0	10	0	94.88
T1	10	5	196.60
T2	10	10	118.22
T3	11	15	73.68
T4	10	Commercial seedling	296.85

3.2 Methods

3.2.1 DNA extraction: Hexadecyltrimethylammonium (CTAB) DNA extraction

DNA extraction was performed by using modified CTAB method to detect DNA-based on protocol of Murray and Thompson (1980). Fresh and young leaves were picked. The fresh and young leaves were dried and grinded until it turns to powder in nitrogen (liquid) by using mortar and pestle. Then, a small amount of the grind powder was transferred immediately into a sterile micro-centrifuge tube. 500 µl of warm (60 °C) extraction buffer containing 2% × CTAB (1.4 M NaCl, 20 mM EDTA; 100 mM Tris-HCl with pH 8.0) was added into tube to prevent degradation of DNA by cellular enzymes. Clumps were suspended by using a spatula and were incubated in a water bath at 65 °C for 30 minutes.

The content was cooled briefly and 750 µl of chloroform-isoamyl alcohol (24:1) was added and gently mix for 1 minute to prevent DNA degradation by using a shaker. The tube was centrifuged at approximately 10, 000 rpm for 5 minutes (4 °C). The clear

upper aqueous phase was pipette out carefully and was transferred into a new sterile micro-centrifuge tube that contain 2/3 volume of ice-cooled isopropanol and gently mix by inverting the tube several times. The content was mixed gently by a few inversions. The evident was identified which are the white threads of DNA. The samples were placed in a - 20 °C freeze for 30 minutes or longer. At this stage, the extractions can be kept in the freezer overnight.

The content was centrifuged for 5 minutes at approximately 10, 000 rpm by inverting the tube to pellet the DNA. The supernatant was discarded gently by pouring away and the pellet not be discarded. After that, 500 µl of 70% ethanol was added to wash the DNA. The tube was tapped gently and allows to stand for a few minutes. The content was centrifuged to re-pellet the DNA at approximately 10, 000 rpm for 5 minutes. The supernatant was discarded gently by pouring away and then, the remaining excess liquid was drained away by inverting the tubes using a clean paper towel. 100 µl of 1 × TE buffer was added and allow the DNA to re-suspend in buffer and tapping them occasionally aids in re-suspension. The DNA was stored at 4 °C. The DNA quality was checked on 1% agarose gels by using the same techniques as for the PCR product visualization. The DNA concentration against standards of 10 ng/µl, 20 ng/µl and 100 ng/µl lambdas DNA was compared.

3.2.2 DNA Quantification and Qualification

a) Quantification of DNA

The determination of DNA concentration in a solution was measured by measuring the absorbance of DNA solution at 260 nm and 280 nm using an ultraviolet spectrometer. The Nanodrop 2000 spectrometer (Thermo Scientific) was used to determine the absorption spectrum of solution of DNA which functioned to determine their purities and DNA concentration based on simple absorbance ratio determination. The 260 nm reading is indication of DNA concentration and 280 nm reading shows the protein contamination. The $A_{260/280}$ ratio of pure DNA should read appropriately 1.8 ± 1 accepted as "pure" for DNA but the reading of the samples in the range of 1.6 to 2.0 considers to be sufficient purity. Meanwhile, $A_{260/280}$ of <1.6 shows the presence of protein contamination. The readings were taken against a blank of TE buffer at 260 nm. To eliminate the DNA contamination, contamination samples were treated with high purity PCR template preparation kit.

b) Determination of DNA quality

DNA quality was determined by using agarose gel electrophoresis which has been stained with 1 µl FloroSafe DNA which non-carcinogenic alternative of ethidium bromide. The DNA extract was electrophoresed in 1.5% agarose gels and was run at 100 V for 30 minutes. Generated bands were observed under ultraviolet light. Large molecular weight DNA appear as a sharp band meanwhile DNA band forms a long smear from a large smear band towards the small fragments which at the bottom at the gel was

contaminated with RNA. These samples again treated with RNAse to eliminate the contaminant.

3.2.3 Optimization of PCR condition

The standardized PCR mastermix of 100 ng/µl DNA template, 1.5 mM/ml $MgCl_2$, 10 mM dNTP, 100 mM PCR buffer, 1.25 U *Taq* polymerase and 1 µM primer was prepared. The PCR program was run by the initial denaturation at 94 °C for 4 minutes. Then, 30 cycles of denaturation process were followed at 54 °C for 15 seconds, gradient annealing temperature ranging from 34 °C to 40 °C for 40 seconds and cycles the step for many primer combinations. At the last step in PCR techniques, the temperature was set at 72 °C for 90 seconds for the extension process and was terminated by final elongation 72 °C and was incubated at 4 °C.

The PCR products were stored at - 20 °C for long term storage. Then, the annealing temperature was optimized by gradient PCR technique, followed by the optimization of concentration of DNA. Five standardized PCR mastermix was prepared where the variables of concentration of DNA of 1 µl, 2 µl, 3 µl, 4 µl and 5 µl. SCAR primers were used for the optimization purposes.

3.2.4 PCR amplification

The standardized PCR mastermix of 100 ng/µl DNA template, 1.5 mM/ml $MgCl_2$, 10 mM dNTP, 100 mM PCR buffer, 1.25 U *Taq* polymerase and 1 µM primer was prepared. PCR consist of the repeated cycling through three steps which are denaturation

of double-stranded DNA, annealing of primer to single-stranded target sequences and DNA polymerase-catalyzed primer extension using dNTP as substrate. First step, double-stranded DNA was separated into single strands by heating to 94 °C for 4 minutes in order to make them accessible to primer binding. Second step, annealing reduces the reaction temperature from that used for denaturing double-stranded DNA to range temperature from 34 °C to 40 °C for 40 seconds before proceeding to the extension process. Third step, extension process involves increasing the reaction temperature from that used for annealing to 72 °C for 90 seconds, the optimum temperature for enzymes such as *Taq* polymerase to function well.

Table 3.2: Set cycles of Polymerase Chain Reaction (PCR)

1 cycle	94 °C for 4 min	Initial denaturation
30 cycles	54 °C for 15 seconds	Denaturation
	34 °C to 40 °C for 40 seconds	Annealing
	72 °C for 90 seconds	Extension
1 cycle	60 °C for 30 min	Final extension
1 cycle	10 °C indefinite	Keeping

3.2.5 Visualizing DNA: Agarose gel electrophoresis and FloroSafe staining

Gel electrophoresis was used in order to inspect the DNA during the process. The quantity of agarose was made by 1.5% depends on the size of DNA fragments inspected. The agarose gel was weighed out into a conical flask for the concentration and volume of the gel. The agarose powder was mixed with 1 × TE buffer in weight or volume ratio

depends on the desired concentration and swirl the contents. Before the gel is casted, the mixture of agarose and 1 × TE buffer was heated in the microwave until the solution become clear, and then, the flask was swirled.

The agarose which have been cooled (approximately 55 °C, takes a few minutes) was poured into a previous prepared gel tray until 5 mm in depth. Any bubbles in the gel was pushed out by using a disposable pipette tip. The previous preparation of the tray was sealed with tape at each end. The gel was allowed to solidify for ~ 30 minutes and the tape was removed and then, was placed in an electrophoresis tank below liquid level which containing 0.5 × TBE. The tank was top up with 0.5 × TBE until the gel fully covered. The prepared samples were loaded carefully by using a pipette into a well and was mixed with half of the volume of 6 × agarose gel loading dye.

The voltage (approximately 50 to 200 V) used depends on the size of the gel, for example, if the gels are bigger in size, it was run at higher voltages. The gels were run normally until the first dye markers have travelled approximately three-quarters of the length of the gel when resolving PCR products. Samples was run either be shorter or longer distances. The power was turned off; the gel was removed and stained by using FloroSafe DNA for 45 minutes. The stain was added with 50 µl of stock solution to a liter of 1 × TBE. The gels were viewed on a UV tans-illuminator and the results was captured and recorded by using a camera and dark background.

3.2.6 SCAR marker analysis

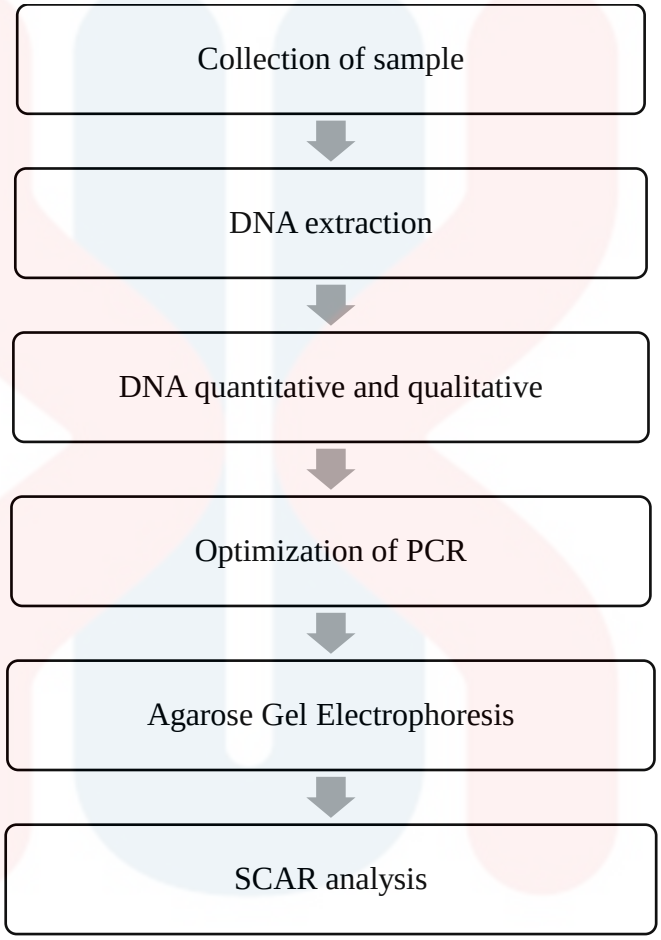
Based on Damasco et. al., (1996) and as highlighted by Ramage et. al., (2004) and Suprasanna et. al., (2008), polymorphisms specific primers were synthesized and characterized. According to Agarwal et. al., (2008), polymorphisms was recognized either with a presence or absence of the amplified band or it can appear as the length polymorphisms convert dominant arbitrary primed marker loci into a codominant SCAR marker. SCAR marker was used as genetic markers or as physical maps in the genome. The use of codominant SCAR markers provided information for genetic mapping compared to the dominant arbitrary-primed markers as the SCAR markers were used in order to screen genomic libraries for physical mapping, to define specificity of locus and acts as comparative mapping for homology studies among related plant species. Two specific SCAR markers were designed from the previous RAPD study as in Table 3.3. However, OPJ13 was selected because in further analyses OPJ13 showed variation at the nursery stage (data not shown).

Table 3.3: Nucleotide sequence of two specific primers

SCAR markers	Nucleotide sequence
UMK-01,02	Derived from OPA19 from previous RAPD study
OPU06-01,02	OPU06-01 FOR 5' - TCG GAT CCT AGA GCT CGG TA – 3' OPU06-02 REV 5' – ATT GAC TCG TGG ACC AGA CC – 3'
OPU06-03,04	OPU06-03 FOR 5' – AGC TGT GCC CTA TCA TCC TC – 3' OPU06-03 FOR 5' – AGC TGT GCC CTA TCA TCC TC – 3'
OPJ13-03,04	OPJ13-03 FOR 5' – TGT TGG AAG CAC CAG TTT CA – 3' OPJ13-04 REV 5' – GTC CCT CGC ATT CCG TAT TA – 3'

3.3 Research Activities and Flow Chart

Figure 3.1: Flow chart of whole process



CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of DNA

By using the protocol of Murray and Thompson (1980) with modification, sufficient amount of genomic DNA has been successfully extracted. The experiment was conducted in Post-Graduate Laboratory of University Malaysia Kelantan, Jeli Campus. The method used have a minor modification such as the ethanol concentration in order to purify the DNA. Large amount of genomic DNA was isolated from the fresh and young leaves. The DNAs obtained were then quantified by using the Nanodrop 2000 spectrometer (Thermo Scientific) in 1 µl drops. The good quality of extracted DNAs and $A_{260/280}$ shown the values of 1.6 to 2.0 which considered to be of sufficient purity for further analyses. A working stock solution of each sample of banana was diluted into 50 ng/µl and kept in – 20 °C for further use.

It is preferable to extract the DNA from the young and fresh leaves as Jobes et al. (1995) had proposed, as it contains less quantities of phenolics and terpenoid compounds (Deshmukh, Thakare, Chaudhari, & Gawande, 2007). However, to treat the high

secondary metabolites, β-mercapethanol in the extraction buffer have been used. In general, cell wall of plants mostly was very hard to be broken. Therefore, it is recommended to incubate the grinded leaves in water bath at 65 °C for 30 minutes.

For greater yield, the leaves were grinded until it turns to fine powder by using the liquid nitrogen. The use of ethylenediaminetetraacetic acid (EDTA) in DNA extraction as a chelating agent of divalent cations was to remove DNAse from the environment after the nuclear membrane was completely broken down. Chloroform-isoamyl alcohol was used in order to break down the bonds between the proteins and polysaccharides from the nucleic acids in the cell. The extracted DNAs were then purified in order to reduce the amounts of contaminant.

4.2 DNA Quantification

The concentration of extracted DNA samples of *Musa accuminata* cv. Berangan from the nursery were measured by using the Nanodrop 2000 Spectrometer (Thermo Scientific). All the readings showed a good quality purification which showing $A_{260/280}$ ranging from 1.6 to 2.0 as shown in Table 4.1. The amount of DNA obtained shows high concentration which range from 200 ng/µl to 2500 ng/µl. Based on the readings, the best purification of DNA ranges from 1.8 to 2.0. The readings which shows $A_{260/280}$ of <1.8 have the possibility to contaminate with protein. Meanwhile, the readings that shows $A_{260/280}$ of >2.0 might contaminated with RNA. To avoid contamination of RNA and protein, the DNA extracted were treated with RNAse and Proteinase K.

Table 4.1: DNA purification and concentration of all the extracted *Musa accuminata* cv. Berangan

Sample Number	Sample Name	260/280	Concentration
1	T4R2	1.85	1412.939
2	T4R4	1.83	1776.141
3	T4R14	1.80	1519.728
4	T4R7	1.80	1663.56
5	T4R18	1.89	1156.288
6	T4R1	1.87	895.64
7	T4R12	1.99	594.275
8	T4R24	2.00	1447.684
9	T4R30	1.81	1181.426
10	T4R26	1.84	2439.039
11	T0R8	1.85	1626.159
12	T0R30	1.89	2440.938
13	T0R3	1.80	2037.21
14	T0R5	1.85	1038.973
15	T0R6	1.80	1971.743
16	T0R1	1.90	666.167
17	T0R22	2.00	1809.84
18	T0R17	1.99	550.046
19	T0R121	1.89	1852.491
20	T0R2	1.91	1313.047
21	T1R7	1.88	1814.703
22	T1R11	1.99	1880.098

Table 4.1**:** continued

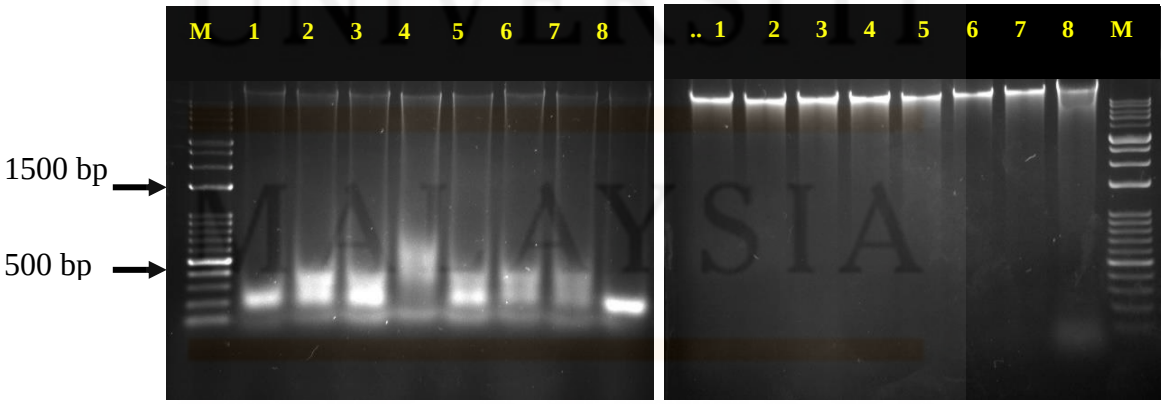
23	T1R16	2.00	1505.587
24	T1R121	1.98	634.571
25	T1R10	1.95	2144.156
26	T1R26	1.83	762.208
27	T1R29	1.87	1227.486
28	T1R6	1.85	2092.237
29	T1R3	1.81	1747.459
30	T1R28	1.83	1707.029
31	T2R17	1.95	2037.616
32	T2R9	2.00	914.616
33	T2R5	1.97	2168.814
34	T2R16	1.85	1279.01
35	T2R21	1.89	1797.277
36	T2R24	1.87	2130.525
37	T2R1	2.00	1483.117
38	T2R18	1.82	252.387
39	T2R20	2.00	787.571
40	T2R8	1.95	2049.868
41	T3R22	1.99	1003.854
42	T3R23	1.96	875.511
43	T3R6	1.93	2308.808
44	T3R21	1.93	2210.795
45	T3R1	1.98	2246.973

Table 4.1**:** continued

46	T3R18	1.86	1867.003
47	T3R16	1.91	1301.883
48	T3R5	1.82	1477.739
49	T3R10	1.90	1293.265
50	T3R25	1.80	659.19
51	T3R28	1.85	1744.227

4.3 DNA Qualification

DNA quality from the extraction was determined by using electrophoresis in 1.5% agarose gel with FloroSafe DNA stain. The gels were observed under the ultraviolet radiation by using UV transilluminator which functioned to visualize the DNA bands in the gel. Large molecular weight DNA appears as a sharp band while the partially degraded DNA forms a long smear consist of various fragments size. The samples with the contamination of RNA that appear near the end of the gel were treated with RNAse.



(a) DNA qualification before treate with RNAse

(b) DNA qualification after treated with RNAse

Figure 4.1: DNA qualification of *Musa accuminata* cv. Berangan before (a) and after treated (b) with RNAse. M = 100bp DNA ladder

4.4 Optimization of The PCR Conditions for Scar Analysis

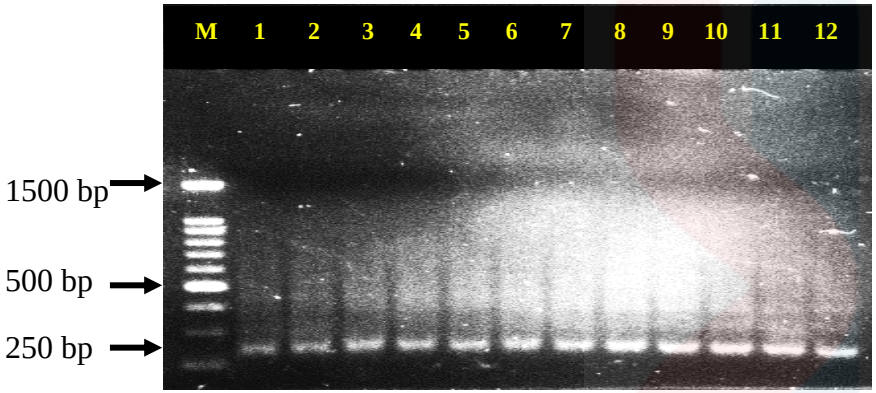
The extracted DNAs of *Musa accuminata* cv. Berangan were continued for PCR optimization for SCAR analysis. The PCR products were run by using gel electrophoresis on 1.5% agarose gel at 80 V for 1 hour with TE buffer. The results showed a monomorphic band observed among the individual selected bananas for SCAR primers. The PCR products firstly undergoes optimization of annealing temperature by gradient (34 °C to 40 °C) using each primer followed by the optimization of DNA concentration (1 µl, 2 µl, 3 µl, 4 µl and 5 µl). For SCAR analysis, the annealing temperature is higher than the PCR reaction average which range from 50 °C to 60 °C in order to have a single and strong amplified band of the expected size (Shiou, Jean, & Rozi, 2011).

4.4.1 Optimization of the annealing temperature

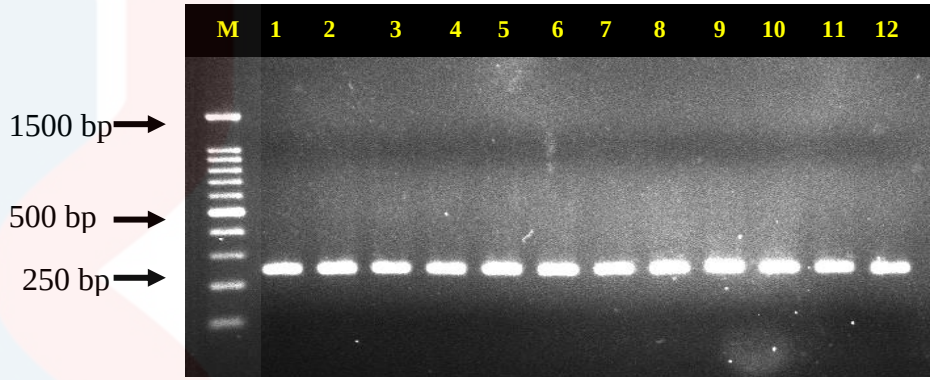
The amplified bands of each primer range from 150 bp to 250 bp. All of the amplified SCAR marker produced monomorphic band in all conditions tested. The different were only on the intensity of the band. The optimized annealing temperature for all the marker were as in Table 4.2. The temperature was selected based on the band that produced the brightest and the thickest band which indicates its effective amplification of the PCR product. In addition, the produced bands from the PCR amplification all showed a single band. This indicated its specificity despite of the various annealing temperature attempted.

Table 4.2: Suggested annealing temperature for *Musa accuminata* cv. Berangan

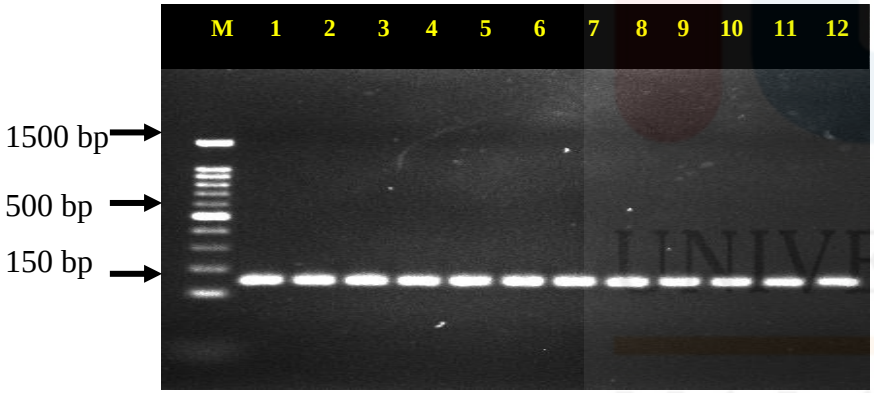
Primers	Suggested annealing temperature by gradient
UMK – 01,02	55 °C
OPU06 – 01,02	55 °C
OPU06 – 03,04	55 °C
OPJ13 – 03,04	55 °C



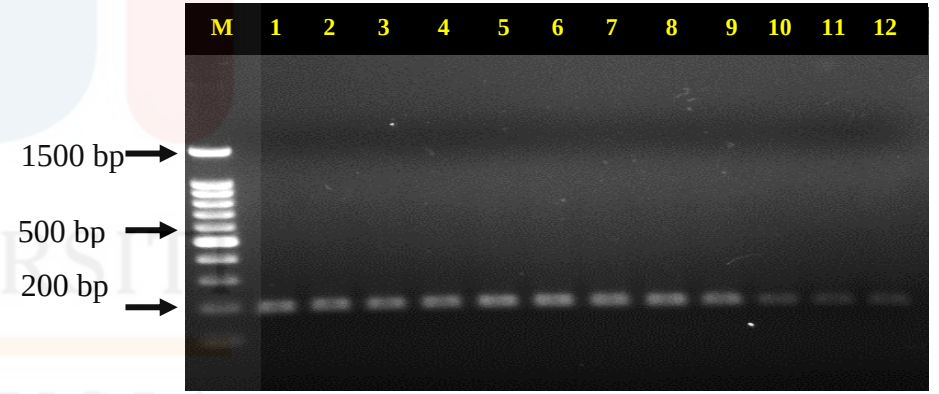
(a) Optimization annealing temperature of UMK-01,02



(b) Optimization annealing temperature of OPU06-01,02



(c) Optimization annealing temperature of OPU06-03,04



(d) Optimization annealing temperature of OPJ13-03,04

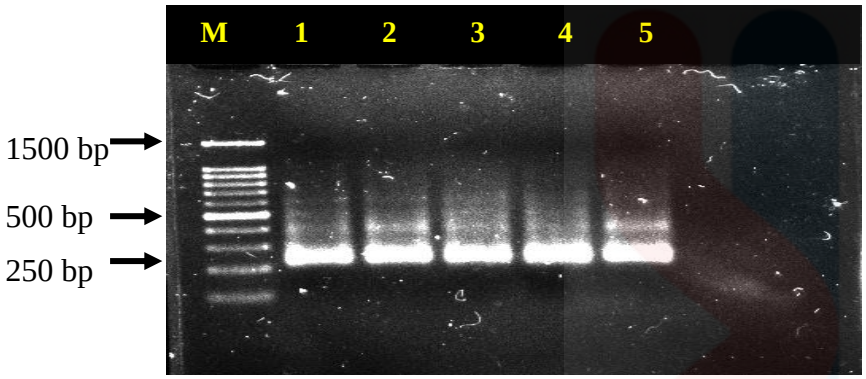
Figure 4.2: A gel visualization of optimization annealing temperature of *Musa accuminata* cv. Berangan of each SCAR marker as in (a), (b), (c) and (d). M=100 bp DNA ladder, Lane 1= 50.0 °C, Lane 2= 50.3 °C, Lane 3= 50.9 °C, Lane 4= 51.8 °C, Lane 5= 53.1 °C, Lane 6= 54.4 °C, Lane 7= 55.6 °C, Lane 8= 56.9 °C, Lane 9= 58.2 °C, Lane 10= 59.1 °C, Lane 11=59.7 °C, Lane 12= 60.0 °C

4.4.2 Optimization of the DNA concentration

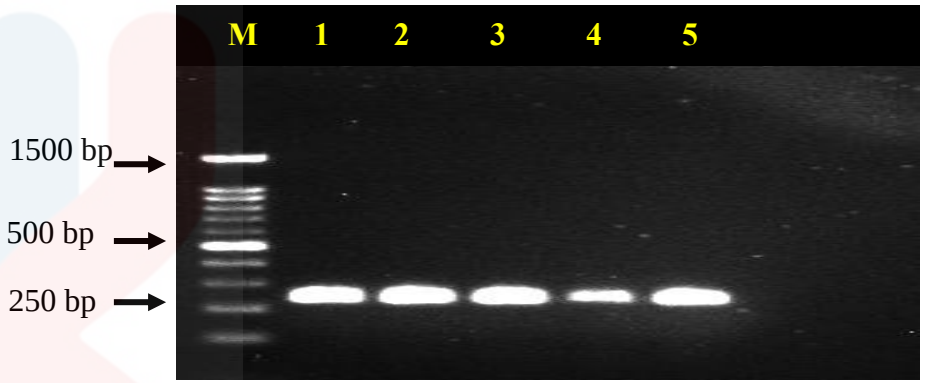
All of the amplified SCAR marker produced monomorphic banding pattern in all concentrations tested. The generation of the amplified bands range from 150 bp to 250 bp as shown in Figure 4.3. All the five concentration of DNA showed a slight difference between each other in the amplified bands. For primer UMK01,02 various amount of DNA has caused the PCR to produce more than one band, which indicate its unspecificity. Only with 4 µl of DNA the PCR amplification using the primer produced single specific band as expected. For the rest of other primer tested, all of PCR product produced only a single intact band. However, with 4 µl of DNA template the band produced are sharper and brighter as compared to 1 µl, 2 µl, 3 µl and 5 µl. Therefore, it is suggested to use the optimized DNA concentration for all the marker as in Table 4.3. Sample for primer OPJ13-03,04 produced faint band as observed in the gel picture due to lower amount of PCR product loaded into the gel in comparison to the DNA ladder. Thus, the PCR products showed faint band as compared to the DNA marker in its first lane.

Table 4.3: Suggested DNA concentration of *Musa accuminata* cv. Berangan

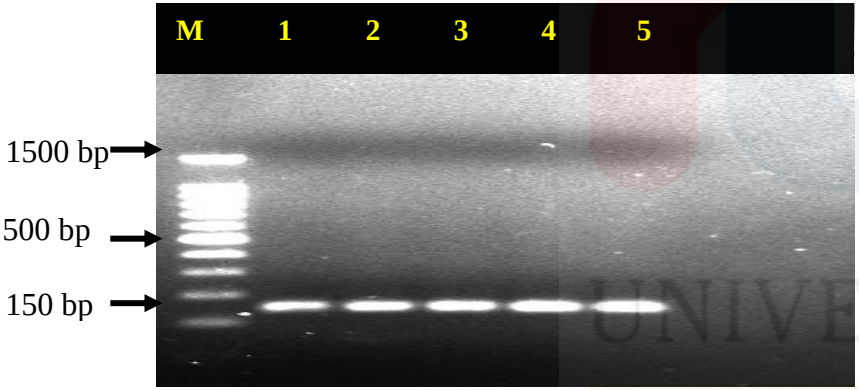
Primer	Suggested DNA concentration
UMK – 01,02	4 µl
OPU06 – 01,02	4 µl
OPU06 – 03,04	4 µl
OPJ13 – 03,04	4 µl



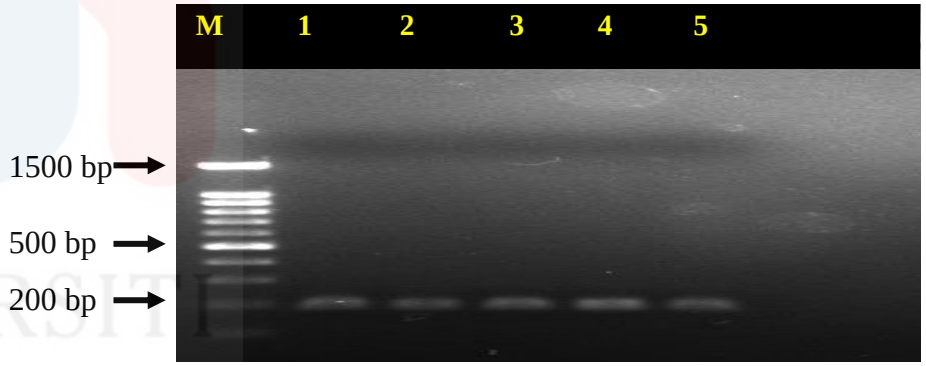
(a) Optimization of DNA concentration of UMK-01,02



(b) Optimization of DNA concentration of OPU06-01,02



(c) Optimization of DNA concentration of OPU06-03,04



(d) Optimization of DNA concentration of OPJ13-03,04

Figure 4.3: A gel visualization of *Musa accuminata* cv. Berangan optimisation of DNA concentration of each SCAR marker as in (a), (b), (c) and (d). M= 100 bp DNA ladder, Lane 1= 1 µl, Lane 2= 2 µl, Lane 3= 3 µl, Lane 4= 4 µl, Lane 5= 5 µl

4.5 Scar Marker Analysis

In the previous study, four SCAR markers were developed based on the sequence analysis of RAPD marker that show polymorphism among soma-variant clones of banana. The SCAR marker has not been tested on the tissue culture clones. Nevertheless, the primer targets the regions in the genome of banana that were polymorphic as assessed previously during the tissue culture stages. The design of the primer is not part of this research. Four SCAR markers (UMK-01,02; OPU06-01,02; OPU06-03,04 and OPJ13-03,04) used to assess the polymorphism of somaclonal variants of banana at post-planting stages. PCR amplification for all four primers were able to produce a single band at the targeted size.

4.5.1 Analysis of SCAR Marker UMK 01,02

From previous RAPD study, SCAR primers UMK-01,02 was derived from OPA19. OPA19 was used due to its high number of polymorphic bands. In the experiment, generated band of UMK – 01,02 shows a monomorphic banding pattern which were sharp and clear at 250 bp. However, this SCAR marker could not identify any polymorphism occur among the individual bananas of *Musa accuminata* cv. Berangan. The results can be seen as Figure 4.4.

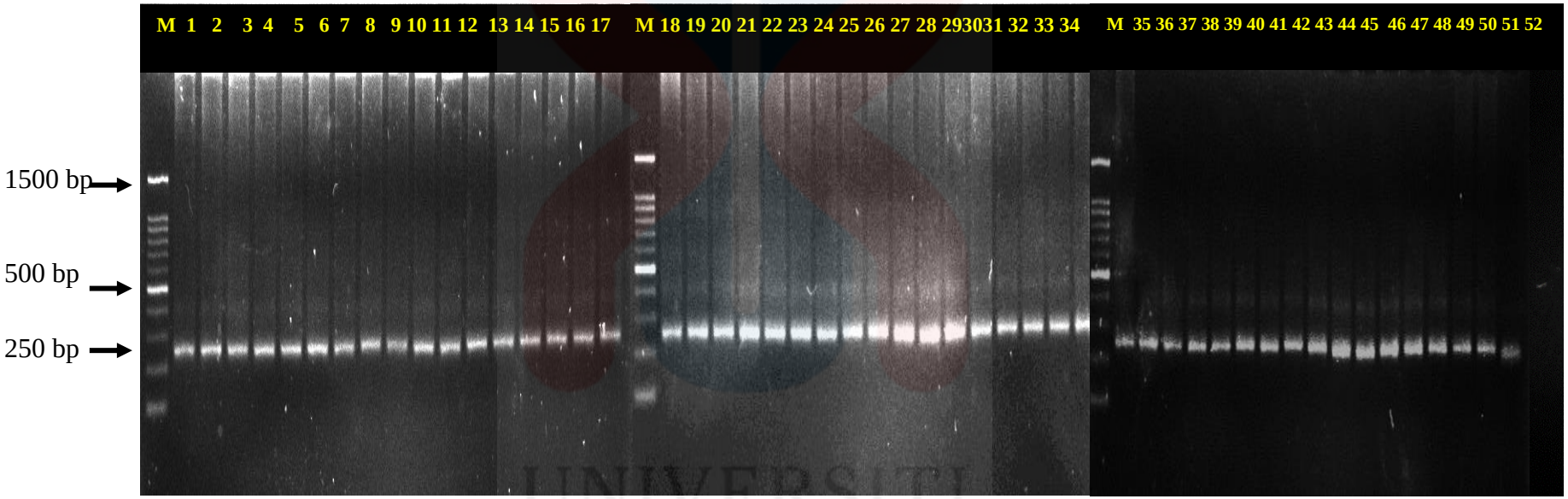


Figure 4.4: A gel visualization of *Musa accuminata* cv. Berangan using primer UMK-01,02. M=100 bp DNA ladder, Lane 1 – Lane 10 (commercial seedling, T4), Lane 11 – Lane 20 (0 mg/l BAP, T0), Lane 21 – Lane 30 (5 mg/l BAP, T1), Lane 31 – Lane 40 (10 mg/l BAP, T2), Lane 41 – Lane 51 (15 mg/l BAP, T3), Lane 52 (negative control)

4.5.2 Analysis of SCAR Marker OPU 06 – 01,02

Based on the characterized sequence, two specific primers were designed from the previous RAPD fragments. Based on the result in Figure 4.5, the generated band showed a single monomorphic banding pattern of 250 bp. However, this SCAR marker could not identify any polymorphism occur among the individual bananas of *Musa accuminata* cv. Berangan.

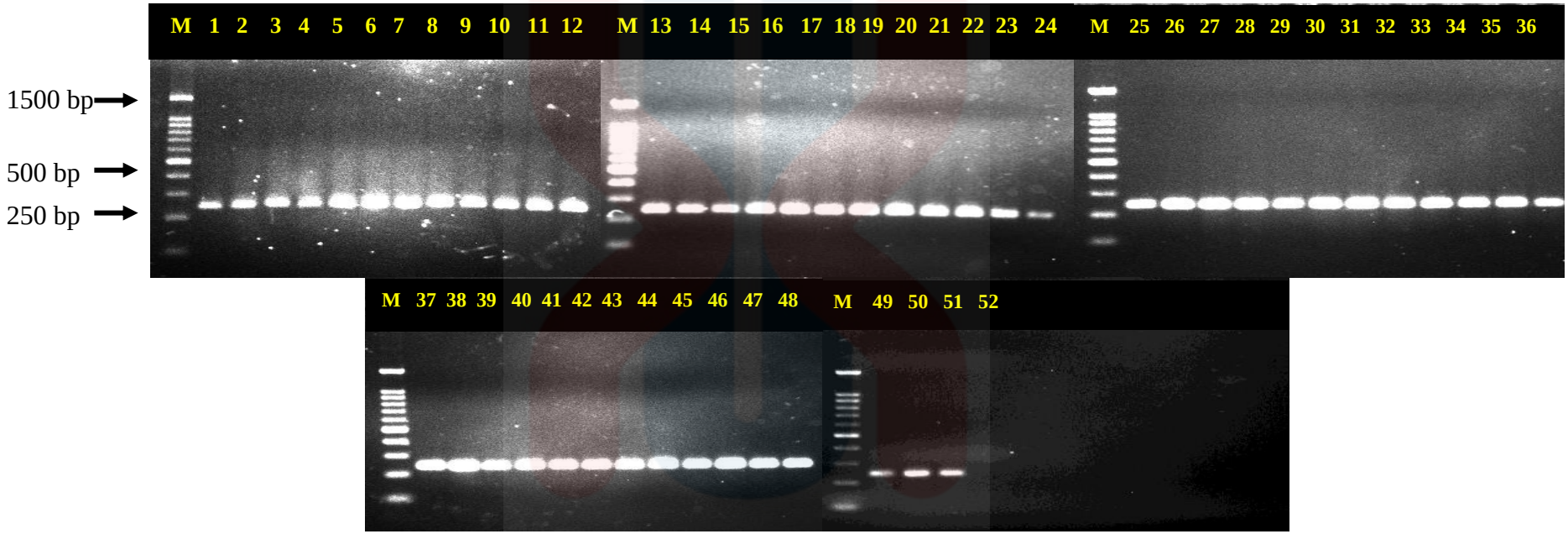


Figure 4.5: A gel visualization of *Musa accuminata* cv. Berangan using primer OPU06-01,02. M=100 bp DNA ladder, Lane 1 – Lane 10 (commercial seedling, T4), Lane 11 – Lane 20 (0 mg/l BAP, T0), Lane 21 – Lane 30 (5 mg/l BAP, T1), Lane 31 – Lane 40 (10 mg/l BAP, T2), Lane 41 – Lane 51 (15 mg/l BAP, T3), Lane 52 (negative control)

4.5.3 Analysis of SCAR Marker OPU 06 – 03,04

Based on the characterized sequence, two specific primers were designed from the previous RAPD fragments. Based on the result in Figure 4.6, the generated band showed a single monomorphic banding pattern of 150 bp. However, this SCAR marker could not identify any polymorphism occur among the individual bananas of *Musa accuminata* cv. Berangan. Even though there were some bands (i.e. sample 39) that showed lower intensity compared to other, that criteria are not sufficient for scoring polymorphism among the banana clones.

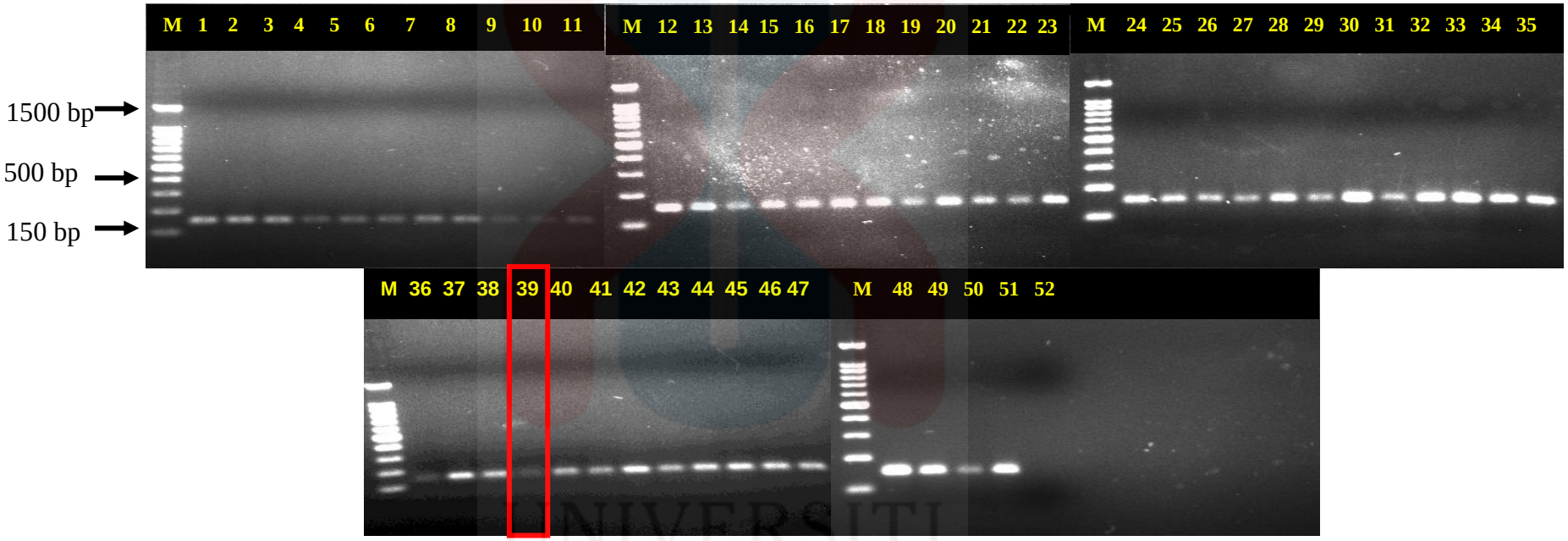


Figure 4.6: A gel visualization of *Musa accuminata* cv. Berangan using primer OPU06-03,04. M=100 bp DNA ladder, Lane 1 – Lane 10 (commercial seedling, T4), Lane 11 – Lane 20 (0 mg/l BAP, T0), Lane 21– Lane 30 (5 mg/l BAP, T1), Lane 31 – Lane 40 (10 mg/l BAP, T2), Lane 41 – Lane 51 (15 mg/l BAP, T3), Lane 52 (negative control)

4.5.4 Analysis of SCAR Marker OPJ 13 – 03, 04

Based on the result in Figure 4.7, the generated band shows a single monomorphic banding pattern of 200 bp. The result showed that, there was a missing band for sample 35 (Figure 4.7) which the morphological of the banana sample was stunted.

It is important to note that the polymorphisms detected was not exclusive to the plantlet that were in the same treatment nor the one that has similar plant height. The polymorphisms detected was unique only to the sample 35. This indicate that the genetic variant occurred may be random and was not uniform across all samples in the same treatment. Thus, even though the marker has shown polymorphisms, it could not explain the phenotype by the clone that have shown various group of plant height.

According to the previous RAPD study, OPJ13 did not showed significant polymorphism as the normal sample still showed visible band even though at lower intensity. However, OPJ13 was selected because in further analyses OPJ13 showed variation at the nursery stage (data not shown).

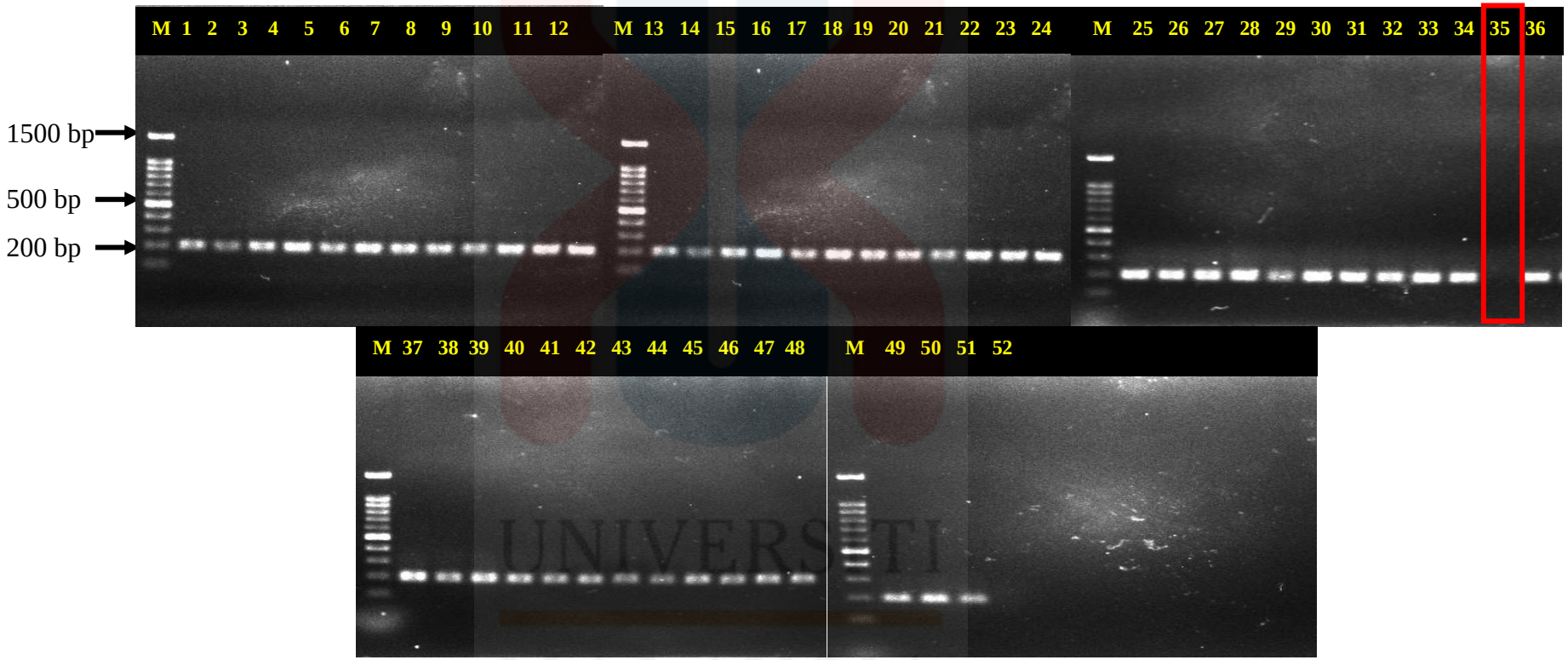


Figure 4.7: A gel visualization of *Musa accuminata* cv. Berangan using primer OPJ13-03,04. M=100 bp DNA ladder, Lane 1 – Lane 10 (commercial seedling, T4), Lane 11– Lane 20 (0 mg/l BAP, T0), Lane 21 – Lane 30 (5 mg/l BAP, T1), Lane 31– Lane 40 (10 mg/l BAP, T2), Lane 41 – Lane 51 (15 mg/l BAP, T3), Lane 52 (negative control)

In this study, various BAP concentrations used during tissue culture stage have shown a various variation in morphology of the regenerants. According to Bairu et. al (2006), the plant growth regulators could increase the multiplication rate, thus inducing the adventitious shoots of the explants (Arifurrahman, 2012). Bairu et.al (2006) also proposed that the possibility of mutation will be increased if adventitious shoot formation had occurred due to the use of plant growth regulators (Arifurrahman, 2012). It was shown that increased of BAP concentration lead to the increased of stunted regenerants. Shirani et.al (2009) have observed that high rate of abnormal growth can be obtained in banana cultivars such as "Berangan Intan", "Berangan", "Rastali", "Nangka" and "Baka Baling" due to high concentration of BAP used. A major problem in micropropagation was the dwarf somaclonal variant which arises from the genetic changes that occurred during the tissue culture process. However, the study was uncertain that the stunted regenerants would either become dwarfs or not at the field.

During tissue culture stages, the plantlet of bananas was treated with different BAP concentration which were 0 mg/l, 5 mg/l, 10 mg/l and 15 mg/l. The results proven that concentration of BAP during tissue culture can increase the possibility of somaclonal variation to occur. According to the findings of Bairu et. al. (2006) and Shirani et. al. (2009) lower concentration of BAP exhibited fewer variations of micro-propagated cultures. The results showed a morphology change between scalp, rosette and normal as shown in Table 2.1.

For RAPD analysis, ten individuals were bulked for every treatment due to the small size of sample as in Table 3.1. This mean that the analysis done was not individual but represented by ten different samples in the same treatment. On contrary, in this study genetic profiling of the clones were performed individually. When it comes to somaclonal

variant, the genetic changes that occur may be random and not consistent across all samples, despite of exposing to the same treatment. Thus, it is incorrect to assume that sample in the same treatment will show the same genetic profile. This is because the changes that happened to each clone may differ, even though they were exposed to the same treatment. That is why, polymorphism detected is not consistent for all sample in the same treatment. Nevertheless, the polymorphism detected with OPJ13-03,04 also could not explain the various plant height obtained among the clones (Figure 4.7).

The RAPD markers that showed polymorphism were chosen to design SCAR markers. During tissue culture, it can be assumed that the genetic changes at the tissue culture stages maintained due to its stability. At post-planting stages, DNA analysis was done by individual which result in possibility whether present or absence of genetic changes. The DNA analysis were carried out by using SCAR markers where the abnormal individual clones have the same banding pattern as the normal clones. Three SCAR markers (UMK-01,02; OPU06-01,02 and OPU06-03,04) that being used in this study showed monomorphic banding pattern of DNA that present in all individuals except for OPJ13-03,04 which showed polymorphic banding pattern where band that present in an individual but absent in another individual as seen in Figure 4.7 sample 35. The morphology of the individual was stunted. The missing band in sample 35 Figure 4.7 shown the BAP treatment 10.0 mg/l for treatment two (T2) which the sample name was T2R21 based on Table 4.1.

Therefore, SCAR markers have been successfully used for detecting polymorphisms of *Musa accuminata* cv. Berangan and have high sensitivity of PCR. PCR protocol applied in this study was applicable as the 100 ng/ml of template DNA were easily extracted from the pure leaves of *Musa accuminata* cv. Berangan and produced

sharp and almost clear bands after PCR. Thus, this technique can be applicable in providing possible solution for designing a specific primer for related species of plants.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Application of high concentration of BAP during tissue culture have been shown to increase the possibility of somaclonal variation to occur. In this study, BAP concentrations of 0.0 mg/l, 5.0 mg/l, 10.0 mg/l and 15.0 mg/l have induced the morphological aberrations (somaclonal variation) among the plantlets of *Musa accuminata* cv. Berangan.

In modern era, there have been developed new molecular tools to enable the researchers in detecting somaclonal variation that occur in early stage of tissue culture. Sequence characterized amplified repeat (SCAR) marker is a conversion of RAPD analysis to detect polymorphisms. However, SCAR marker only showed monomorphic bands from the targeted sequence on previous RAPD analysis. This technique was applied to study the potential of the *Musa accuminata* cv. Berangan to have any genetic changes that lead to due absence of bands. This study also focuses on the PCR optimization by testing different gradient of annealing temperature and concentration of DNA.

Results for the optimization of annealing temperature gradient showed that most of the primers used in this study gave the optimum temperature around 55.0 °C. As the optimum DNA concentration being tested which were 1 µl, 2 µl, 3 µl, 4 µl and 5 µl, It suggested that 4 µl was the best to apply for all the four primers tested (UMK-01,02; OPU06 – 01,02; OPU06 – 03,04 and OPJ13 – 03,04) due to its sharpness and clearest band.The second objectives were achievable which need to identify DNA polymorphism among the *Musa accuminata* cv. Berangan clones of tissue culture at post-planting stages using SCAR marker analysis. All of three SCAR markers (UMK-01,02; OPU06-01,02 and OPU06-03,04) have showed monomorphic banding pattern of DNA that present in all individuals except for OPJ13-03,04 which showed polymorphic banding pattern. The polymorphisms detected was unique only to the sample 35 which was T2R21 and the morphology was stunted as shown in Figure 4.7. This indicated that the genetic variant occurred may be random and was not uniform across all samples in the same treatment. Therefore, for the detection of polymorphism can only be seen in primer OPJ13 – 03,04.

5.2 Recommendation

i. It is suggested that the DNA purification is performed prior to SCAR marker analysis, as impurities in DNA template may interfere with the amplification process.
ii. It is suggested to further assess the clone (T2R21) for other phenotypic variability which may explain the genetic variability detected by the SCAR marker.
iii. Other DNA based markers need to be used to explain the phenotypic variability of plant height observed at the field.

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APPENDICES

Figure 1: Water bath

Figure 2: PCR machine (Brand Eppendorf)

Figure 3: The microcentrifuge used during DNA extraction

Figure 4: Centrifuge machine

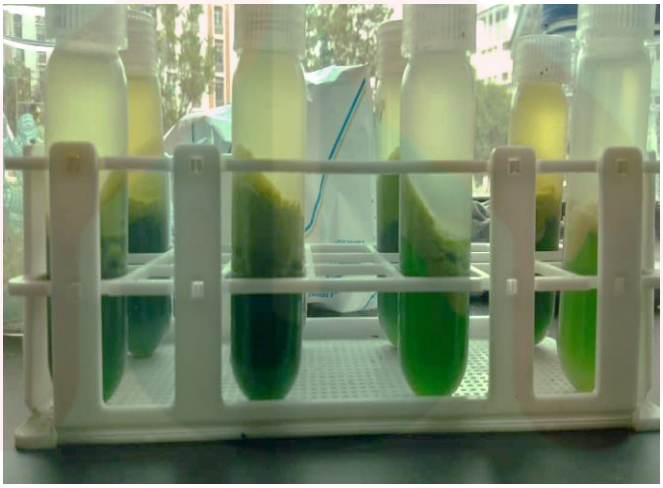

Figure 5: Supernatant of extracted DNA

Figure 6: Pellet of DNA