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ANALYSIS OF GENETIC DIVERSITY AND SOMACLONAL VARIATION IN BANANA USING MOLECULAR MARKERS

Tesis que presenta

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RECONOCIMIENTO

Por medio de la presente, hago constar que el trabajo de tesis titulado "Analysis of Genetic Diversity and Somaclonal Variation in Banana Using Molecular Markers" fue realizado en los laboratorios de la Unidad de Bioquímica y Biología Molecular de Plantas del Centro de Investigación Científica de Yucatán, A.C. bajo la dirección de los Drs. Rosa Maria Escobedo Gracia-Medrano y Andrew C. James, dentro de la Opción Bioquímica y Biología Molecular de Plantas, perteneciente al Programa de Posgrado en Ciencias Biológicas de este Centro.

Atentamente,

Dr. Oscar Alberto Moreno Valenzuela

Director Académico

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DECLARACIÓN DE PROPIEDAD

Declaro que la información contenida en la sección de materiales y métodos experimentales, los resultados y discusión de este documento proviene de las actividades de experimentación realizadas durante el período que se me asignó, para desarrollar mi trabajo de tesis, en las Unidades y Laboratorios del Centro de Investigación Científica de Yucatán, A. C., y que dicha información le pertenece en términos de la Ley de la Propiedad Industrial, por lo que no me reservo ningún derecho sobre ello.

Merida, Yucatán octubre 2010

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List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
ORF	Open Reading Frame
SRAP	Sequence Related Amplified Polymorphism
UPGMA	Unweighted Pair-Grouping Method with Arithmetic Average
PCO	Principal Co-Ordinate Analysis
MS	Murashige and Skoog
BAP	6-Benzylaminopurine
IAA	Indole-3-acetis acid
2,4-D	2,4-dichlorophenoxyacetic acid
SE	Somatic Embryogenesis
IMFs	Immature Male Flowers
SCV	Settled Cell Volume
ECS	Embryogenic Cell Suspension
MM	Maturation Medium
GM	Germination Medium
ABA	Abscisic acid
RCBD	Random Complete Block Design

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تحليل التنوع الوراثي والإختلافات الوراثية الناتجة من زراعة الأنسجة فى الموز باستخدام الواسمات الجزيئية

الملخص العربي:

يعتبر الموز من الفواكه الهامة جداً، كما يمثل مصدر غذاء رئيسي ودخل لملاين من سكان العالم. فى هذا البحث، تم دراسة عاملين من أهم العوامل التى تؤثر في تحسين الموز وهما التنوع الوراثي وتكوين الأجنة الخضرية.

تساعد دراسات التنوع الوراثي فى الموز علي ايضاح النقاط ذات الصلة بالعلاقات الوراثية والتقسيمية على مستوى الجنس والعائلة بالإضافة الى تحسين الموز. كما تعتبر دراسة هذا التنوع والحفاظ عليه كعنصر أساسي و مصدر للجينات التى تستخدم فى تحسين الأصناف التجارية عن طريق التقنيات الحيوية التى تستخدم عملية تكوين الأجنة الخضرية. في هذه الدراسة، تم تحليل أربعين مجموعة نباتية تشتمل على أصناف تجارية وطرز برية من أقسام الموز المختلفة وذلك لدراسة التنوع الوراثى باستخدام اثنين من الواسمات الجزيئية (AFLP و SRAP). أوضحت النتائج أنه باستخدام كلا الواسمين الجزيئيين، أمكن بنجاح التفرقة بين النباتات المختلفة. أظهر الواسم الجزيئي SRAP فاعلية أكثر حيث أعطي تقريباً نسبة 37% و تقدر بثلاث أضعاف الحزم الوحيدة الخاصة ببعض النباتات مقارنة بالواسم الجزيئي AFLP حيث كانت نسبة الحزم 13% من مجموع الحزم الكلية المتضاعف. كما أسهمت هذه الدراسة في وضع القواعد الأساسية لانشاء وتنظيم بنك الأصول الوراثية للموز، الكائن فى المزرعة البحثية بمنطقة أوشمال بولاية يوكاتان بالمكسيك.

العامل الآخر الذي تم دراسته هو مدى استجابة صنفين من أصناف الموز الهامة تجارياً (ويليامز وجراند ناين) لعملية تكوين الأجنة الخضرية، وذلك باستخدام طريقة الأزهار المذكرة غير الناضجة حيث تم تقييم النسبة المئوية للاستجابة لتكوين الأجنة الخضرية فى كلا الصنفين، كما تم أيضاً دراسة تأثير كلاً من التركيب الوراثي للصنف المستخدم و المرحلة التطورية للأجزاء النباتية المستخدمة. أوضحت النتائج وجود اختلافات معنوية جداً فى درجة الاستجابة لتكوين الأجنة الخضرية، و اعتمد ذلك على التركيب الوراثى وعمر البرعم الذكرى المستخدم كمصدر للأجزاء النباتية المستخدمة. وتوضح هذه النتائج امكانية الوصول الى الدرجة المثلى من نظم تكوين الأجنة الخضرية فى الموز. بالإضافة لذلك، تم مقارنة النباتات الناتجة من الأجنة الخضرية بالنباتات الأم وذلك باستخدام الواسم الجزيئي AFLP لكشف أي اختلافات ممكنة ناتجة من نظام زراعة الأنسجة المستخدم. أظهر الواسم الجزيئي AFLP قدرة عالية على تحديد الاختلافات الوراثية الموجودة فى النباتات المتكشفة من الأجنة الخضرية. وكانت نسبة الاختلافات الوراثية بين النباتات الناتجة من الأجنة الخضرية والنباتات الأم 1.6 و 1.4% فى ويليامز وجران ناين على التوالي. وعلى الرغم من ذلك فتعتبر هذه الاختلافات أقل معنوياً من الاختلافات التى سبق نشرها والخاصة بتقنية الاكثار الخضري الدقيق فى الموز.

Resumen:

Los bananos y plátanos son frutales de gran importancia como fuente principal de alimento e ingreso familiar para millones de personas en el mundo. En este trabajo se investigó sobre dos de los factores que más influyen en el mejoramiento del banano y plátano; la diversidad genética y la embriogénesis somática (ES). Los estudios de diversidad en musáseas auxilian a esclarecer problemas afines a las relaciones genéticas y taxónomicas del género y la familia. Además, en cuanto al mejoramiento genético de banano y plátano, el estudio y conservación de la diversidad es esencial como fuente de genes para mejorar los cultivares comericales mediante la herramientas biotecnológicas que hacen uso de la ES. En este trabajo, se investigó la diversidad genética en *Musa* mediante el análisis de cuarenta accesiones que incluyen cultivares comerciales y especies silvestres de diferentes secciones del género. Se utilizaron dos sistemas de marcadores moleculares. Estos fueron el Polimorfismos en la Longitud de Fragmentos Amplificados (AFLP) y el sistema de Polimorfismo Amplificado de Secuencias Relacionadas (SRAP). Los resultados mostraron que ambos marcadores discriminaron entre las diferentes accesiones. Sin embargo, en comparación con el marcador AFLP, el marcador SRAP fue más informativo mostrando cerca de tres veces más información a manera de bandas únicas y específicas en comparación con el sistema de AFLP, 37 y 13% de las bandas totales amplificadas, respectivamente. Como contribución del trabajo, se sentaron las bases para un mejor ordenamiento del banco de germoplasma de banano y plátano del CICY ubicado en el Campo Experimental del INIFAP-Uxmal,Yucatán.

El segundo aspecto que se investigó fue la respuesta de dos genotpos AAA, 'Williams' y 'Enano Gigante', al proceso de ES *in vitro*. El método de ES utilizado fue el que emplea flores masculinos jóvenes como explante inicial, y se evaluó la capacidad de cada genotipo para formar callo embriogénico, así como la relación que tiene la etapa fisiológica de desarrollo del tejido donador de explante, sobre la respuesta embriogénica encontrada. Las evidencias mostraron diferencias altamente significativas, dependiendo del genotipo y de la etapa de desarrollo de los explantes. Dichos resultados tienen implicaciones importantes en la optimización de los protocolos de embriogénesis. Conjuntamente, con el fin de revelar posibles variantes somaclonales resultado del sistema de ES, mediante el marcador AFLP se analizaron las plantas regeneradas de ambos genotipos y se les comparo con plantas de sus respectivos progenitores. El

sistema AFLP demostró ser una buena herramienta para la detección de la variación genética en plantas derivadas de la embriogénesis somática. Además, se observó 1.6 y 1.4 % de polimorfismo entre las plantas regeneradas y sus plantas parentales en 'Williams' y 'Enano Gigante', respectivamente. Sin embargo, estas variaciones son menos significativamente de lo reportado para la organogénesis en el banano.

Abstract:

Bananas and plantains are very important fruits and considered as a main food source and household income for millions of people worldwide. In this study two of the most influential aspects related to banana improvement were investigated, i.e. genetic diversity and somatic embryogenesis (SE). The first aspect was to study the genetic diversity in *Musa* which assist to clarify issues related to genetic and taxonomic relationships in the genus and the family as well as the genetic improvement of banana and plantain. The study and conservation of diversity is essential as a source of genes for the genetic improvement of commercial cultivars through the biotechnological tools that use SE. The genetic diversity was assessed by analyzing forty accessions including commercial cultivars and wild species of different *Musa* sections. Two molecular marker systems were used; amplified fragments length polymorphism (AFLP) and sequence related amplified polymorphism (SRAP). Results showed that both markers successfully discriminated amongst the different accessions however, SRAP was more informative and exhibited approximately threefold more unique and specific bands than AFLP, with 37 and 13% of the total amplified bands, respectively. In addition, the work put the keystone and bases for better management of the germplasm collection of banana and plantain of CICY located in the experimental field, INIFAP-Uxmal, Yucatán

The second investigated aspect was the response of two genotypes (AAA) 'Williams' and 'Grand Naine', to the SE. The SE method was achieved using immature male flower tissue as an initial explant; the response of embryogenic callus induction for each genotype was evaluated as well as the effect of explant developmental stage. Highly significant differences were found in the embryogenic response, depending on the genotype and the developmental stage. These results gave important implications for the optimization of SE protocols. Additionally, to uncover possible somaclonal variants, regenerated plants from SE were analyzed by comparing them to their parental plants using AFLP molecular marker. AFLP was a good tool for detection of genetic variation in somatic embryogenesis derived plants, and showed 1.6 and 1.4% polymorphism amongst the regenerated plants and their parental plants, in 'Williams' and 'Grand Naine', respectivaly. However, these variations are significantly less than what have been reported for organogenesis in banana.

Introduction:

The center of origin of the genus *Musa* L. (Musaceae) is southern and south eastern Asia[1]. A great number of important plants are found in the genus; however, those that bear edible fruit are the most significant. In addition to fruit, bananas and plantains provide many cultures with medicines, beverages, fibers, edible floral parts, dyes, fuel, steam for cooking, cordage, wrapping materials, etc. With few exceptions, the familiar dessert and cooking bananas are naturally occurring hybrids amongst the various subspecies of *M. acuminata* Colla and interspecific hybrids between *M. acuminata* and *M. balbisiana* Colla.

Musa comprises about 35 species, related to different sections; Eumusa which contains *Musa acuminata* (genome A) and *M. balbisiana* (genome B), ancestors of edible banana and plantain and *M. schizocarpa* N.W. Simmonds (genome S)[2]. Section Australimusa includes *M. textilis* Nee (genome T) which is particularly important in Philippine culture, and to a certain extent in some traditional islands of Micronesia, as a source of fiber. The other two sections are of ornamental importance; Rhodochlamys eg. *M. laterita* Cheesman and Callimusa eg. *M. coccinea* Andrews[1]. *Musa* taxonomy is confused by several factors including the sterility, ancient domestication, and hybrid origins of the cultivated varieties (Chapter I, Fig. 1.1).

The properties of the molecular marker technique known as sequence related amplified polymorphism (SRAP)[3], have been exploited by others in the characterization of genetic diversity. For example, Ferriol et al.[4], made use of (amplified fragment length polymorphism) AFLP's and SRAP's markers for analyzing the molecular variability in accessions of *Cucurbita pepo* L., and found that the values of similarity and genetic diversity varied between the two systems of molecular marker. Accordingly, the data obtained with SRAP's, unlike those of AFLP's, were more consistent with the morphological variability and history of the morphotypes. Ude et al.[5,6] found that AFLP is a very useful tool in determining taxonomic relationships in banana, however, the same authors recognized that the relations amongst *Musa* genotypes are far from been resolved, furthermore, they recommended that other primers and different enzyme combinations be used to screen for polymorphism.

Developing and utilizing basic biotechnological tools to assist germplasm improvement is important for edible *Musa* (seedless banana), especially in the context of the use of *Musa* genomic diversity. Particularly, plant regeneration through somatic embryogenesis, in species such as the edible cultivars of *Musa* spp., have compromised and reduced their sexual reproduction owing to sterility and polyploidy problems. An alternative system to classic improvement is the use of biotechnological techniques such as the generation of new varieties by genetic engineering, *in vitro* mutagenesis, protoplast fusion, and somaclonal selection.

The basic problem for the production of embryogenic cells in edible banana is the requirement to be derived from differentiated tissues. There is a low response to form ideal callus for somatic embryogenesis (SE) in only between 0.1 to 6.2 % of explants produce ideal callus. Therefore, large numbers of explants have to be induced from either immature floral meristems[7-9], or "scalps" which are derived from proliferating meristems[10-11]. Evidence from field evaluations indicate that the rate of somaclonal variation in plants derived from SE cell suspensions depends on the genotype. This can be as low as 2% in diploid *M. acuminata* IRFA 90 3, or as high as 99% in Williams line E4000[12]. This variation has often been related to genetic instability and epigenetic variation of the cells during culture. In fact, chromosome number variation has been detected in *Musa* embryogenic cell suspensions and regenerated plants, abnormal ploidy levels coincide with poor regeneration ability[13]. Moreover, AFLP and methylation-sensitive amplification polymorphism (MSAP) have been used to detect somaclonal variation in several plants including banana[14-17].

Genetic diversity and somatic embryogenesis are important aspects involving in plant improvement. Assessment of the genetic variation and understanding the relationships amongst different sections, species and genotypes in *Musa* could be helpful to optain a valid classification, which is required to assist selection of proper genotypes for breeding and/or genetic transformation. In addition, somatic embryogenesis technique is a valuable tool either for germplasm .reservation or as a source of plant material for biotechnological applications as well as a source of genetic variation. Studying factors affecting the formation of somatic embryos could improve SE capacity. Subsequently, this study could present useful information which might be helpful in further research for *Musa* improvement.

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Chapter I

GENERAL ANTECEDENTS

1.1. Banana (*Musa* spp.):

Bananas are cultivated in more than 120 countries in tropical and subtropical zones on five continents. Banana products represent an essential food resource and have an important socioeconomic and ecological role[1]. Worldwide, over 1,000 banana cultivars or landraces are recognized[2]. The genus name '*Musa*' is thought to be derived from the Arabic name for the plant (*mouz* موز) which, in turn, may have been applied in honour of Antonius Musa (63 – 14 BC), physician to Octavius Augustus, first emperor of Rome[3]. The name 'banana' is derived from the Arabic *banan* (بنان) which means finger[4] and was thought to be used in Guinea (West Africa) concomitant, the name then spread to the New World[5].

1.1.1. Banana botany:

Banana, plantains and cooking banana are giant herbaceous and monocarpic perennial plants that grow to a height of from two to fifteen meters. The underground stem (corm, rhizome) has short internodes and is able to produce suckers by which the plant can reproduce vegetatively. Each pseudostem produces a single inflorescence, the female flowers of which give rise (either parthenocarpically or following fertilization) to the banana fruits. The inflorescence develops at specific stages usually after about 25 to 50 leaves have been produced approximately one month later the inflorescence has emerged from the leaf crown. The inflorescence, which can be vertical, pendant or sub-horizontal, is complex and made up of an ear of cymes. Cymes are inserted spirally on the floral stem and are composed of one spathe and single or double rows of flowers at its axils[1]. These are the first ranks of flowers, usually called 'hands', from which the fruit bunches develop. In the first five to 15 clusters the flowers are functionally female and give rise to fruit, while subsequent flowers are hermaphrodite and eventually male. One by one the bracts rise to expose the flowers; they usually fall after one or two days, but in some cultivars are retained and dry on the stalk. At the lower end they form a bulbous "male bud". The banana fruit is a berry, contains many ovules, but in cultivated varieties the fruit has no

seeds and develops by means of parthenocarpy, i.e. without fertilization. Whereas the bunch grows downwards, the fruits curve up. A fruit cluster is generally called a "hand" and a single fruit a "finger." Fingers differ from cultivar to cultivar in characteristics such as shape, size, color of skin, and flavor. A good bunch of a commercial dessert banana consists of eight hands of 15 fingers; each finger has an average weight of 150 g, so that the entire bunch weighs 18-20 kg. Traditional banana cultivars and plantains tend to have much smaller bunches[6].

1.1.2. Economic importance:

Banana is one of the most important food crops in the world. World production of bananas reaches approximately 106 million tonnes per year, of which 57.1% are dessert bananas and 42.9% plantains and cooking bananas[7]. Around 120 countries produce bananas throughout the tropical and sub-tropical regions with approximately one-third being produced in each of the African, Asian-Pacific, and Latin American-Caribbean regions. Around 87% of all the bananas grown worldwide are produced by small-scale farmers for home consumption or for sale in local and regional markets, whilst the remaining 13%, mainly dessert bananas, are traded internationally. Bananas provide a staple food for millions of people, particularly in Africa, an area where the green revolution has had little influence. Bananas are an important food security crop, providing a cheap and easily produced source of energy. These perennial plants often survive where conflict or natural disasters have adversely affected the production of annual, arable crops. In addition, bananas are rich in certain minerals and in vitamins A, C, and B6. Bananas have also been proposed as a useful mean to deliver edible vaccines: the fruit can be eaten uncooked, is sterile until peeled, and is often the first solid food eaten by babies[6].

1.1.3. *Musa* genome size:

The genome size of *Musa* has been determined using flow cytometry of cell nuclei of wild bananas, which appears to be smaller than many other angiosperms[8]. The nuclear genome is relatively small, e.g. 1C ~ 610 Mbp for *M. acuminata*[9,10]. The nuclear DNA content of *M. balbisiana* is significantly lower (average genome size of 537 Mbp) than that of *M. acuminata* subspecies and cultivars[10,11]. The genome of diploid *Musa* is thus three fold that of *Arabidopsis thaliana*. Larger variation in genome size (8.8%) was found

amongst triploid *Musa* cultivars (ranging from 559 to 613 Mbp) with 2C DNA values ranging from 1.61 to 2.23 pg. Similarly, the genome size (2C) of tetraploid cultivars range from 1.94 to 2.37 pg. The genomic base composition of *Musa* taxa examined has a median value of 40.8 ± 0.4 % GC[10]. In excess of 50% of the banana genome may include repetitive and non-coding DNA sequences.

1.1.4. *Musa* Taxonomy:

The family Musaceae contains *Musa* and *Ensete*, which are clearly distinguishable by their distinct morphology and through research with genetic markers. A third genus, *Musella*, with a single species, was recently added to this family. The genus *Musa* consists of five sections, viz.: Eumusa, Rhodochlamys, Callimusa, Australimusa and Ingentimusa, distinguished by their specific chromosome numbers and morphological characters. Two sections have a chromosome number of 2n = 20 (Callimusa and Australimusa) and another two sections (Eumusa and Rhodochlamys) have a basic chromosome number of 11 (2n = 22) with the exception of *M. beccarii* (x=9)[12,13]. The species in the sections Callimusa and Rhodochlamys are of ornamental interest only, as the characteristic of fruit parthenocarpy is absent. The section Australimusa includes *M. textilis* which is source of Manila hemp. Within this section the edible Fe'i bananas, which are found mainly in the Pacific islands[14]. The fifth section includes *M. ingens* (2n = 14), which has yet to be assigned for *M. lasiocarpa* and *M. boman*[15]. There are four known genomes in *Musa*: A, B, S, and T that belong to *M. acuminata, M. balbisiana* and *M. schizocarpa* species of section Eumusa, and the Australimusa section, respectively[16]. The Eumusa section is geographically the most widely distributed and contains the two major species, *M. acuminata* and *M. balbisiana,* which are the origin of the great majority of the edible bananas. *M. acuminata* has been divided into eight subspecies (*banksii, burmannica, burmannicoides, malaccensis, microcarpa, zebrina, siamea, truncata*)[6]. On the other hand, *M. balbisiana* appears to have a relatively lower genetic diversity than *M. acuminata*[17]. However, to date, this species is under-represented in collections and in the samples which have been studied. Geographical structuring in *M. balbisiana* wild populations from south China has been reported using highly polymorphic SSR markers[18]. Figures 1.1 and 1.2 showed a diagram of *Musa* taxonomy and sections distribution, respectively.

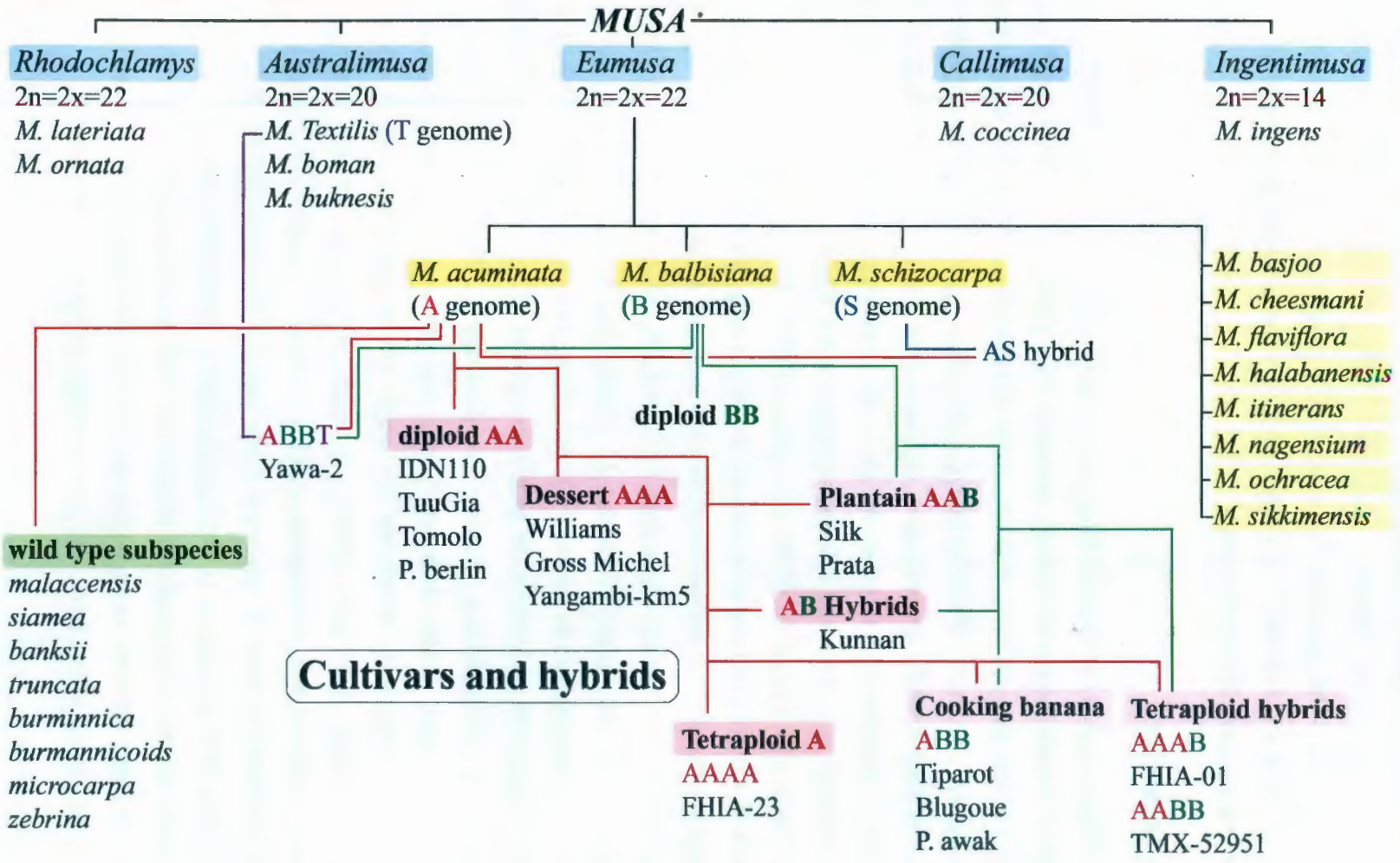

Fig. 1.1: A diagram showing *Musa* taxonomy and genome distribution

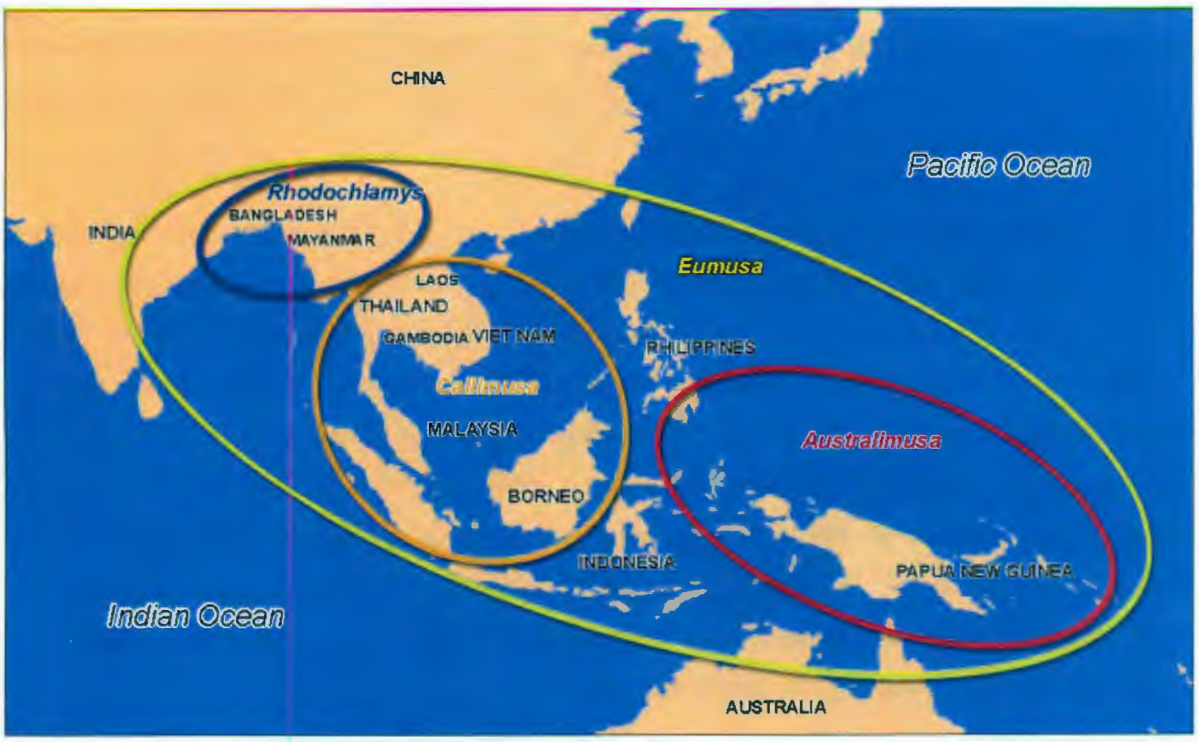

Fig. 1.2: Distribution of *Musa* sections

1.2. Somatic embryogenesis:

Somatic embryogenesis is the formation of embryogenic callus and obtaining somatic embryos by dedifferentiation of tissues using growth regulators. It was first reported in 1958, using carrot tissue cultures[19,20]. Somatic embryos are formed from plant cells that are not normally involved in the development of embryos, i.e. somatic plant tissue. No endosperm or seed coat is formed around a somatic embryo. Cells derived from a competent mother tissue are cultured to form an undifferentiated mass of cells called a callus. Plant growth regulators in the tissue culture medium can be manipulated to induce embryogenic callus formation and subsequently changed for induction of somatic embryos. The ratio of different plant growth regulators required for callus induction or embryo formation varies with the type of plant, genotype and explant source. Asymmetrical cell division appears to be important in the development of somatic embryos.

Somatic embryogenesis produces new, perfect plantlets that have both shoots and roots. Therefore, somatic embryogenesis tissue-culture systems are very useful for plant regeneration and transformation. Applications of this process include: clonal propagation

of genetically uniform plant material; elimination of viruses; provision of source tissue for genetic transformation; generation of whole plants from single cells called protoplasts; development of synthetic seed technology.

Various conditions that may affect somatic embryo induction have been examined include genotype, explant type, stage of development, phase change (in woody plants), phytohormones, osmotic stress, temperature and nitrogen sources[21,22]. Tissue culture systems for somatic embryo induction have been established for many plant species. Morphologically and physiologically, somatic embryo development closely resembles that of zygotic embryos (Fig. 1.3); therefore, somatic embryogenesis is used extensively as an experimental system to examine physiological, biochemical and morphological events during embryogenesis[23]. Increasingly, somatic embryogenesis tissue-culture systems are used as model systems to examine the mechanisms regulating gene expression and other molecular events during zygotic embryogenesis.

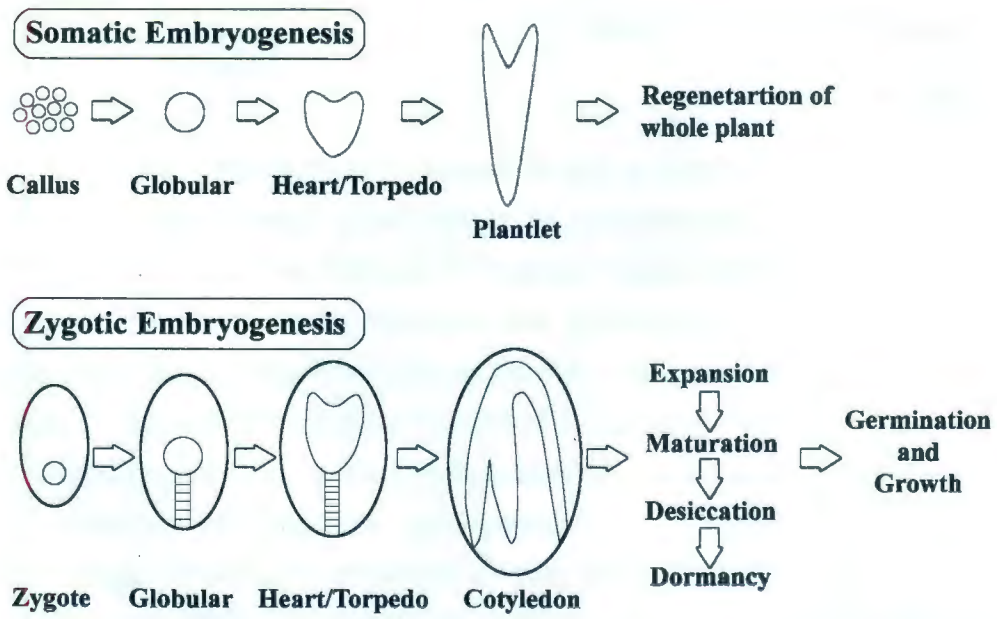

Fig. 1.3: A comparison of somatic and zygotic embryogenesis (After Zimmerman[23]).

A multitude of parallel signals (Fig. 1.4), including auxin (either exogenously supplied or endogenously altered), evoke a wide cellular response including reorganizations at the levels of cell structure, physiology, chromatin and gene expression. As a result, the dedifferentiated cells become competent for embryogenesis. Competent cells will indeed be embryogenic if external and cellular conditions allow the expression of the embryogenic programme that is, in most cases, preceded by or parallel to, cell divisions. Further cell divisions together with polarity establishment and pattern formation result in the development of the embryo. The central role of chromatin remodelling can be hypothesised in all phases, including dedifferentiation, embryogenic reprogramming and embryo differentiation. They are all associated with the parallel activation/inactivation of a large number of genes[24].

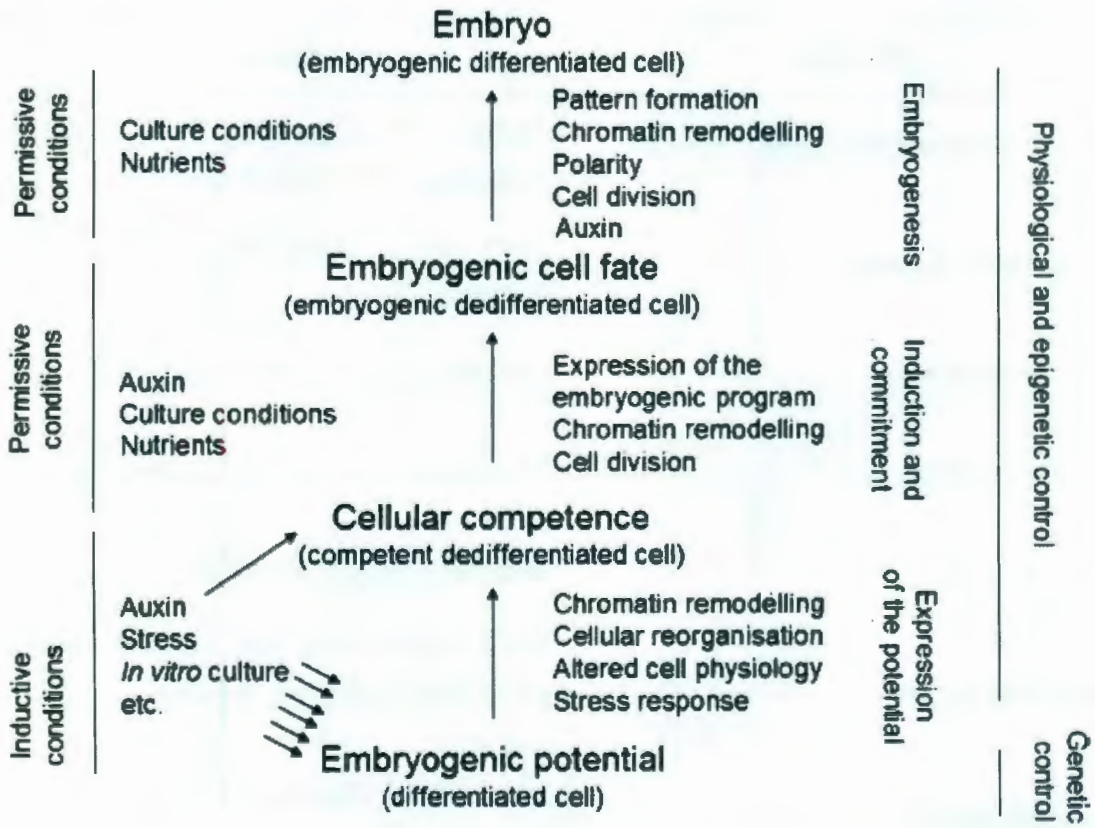

Fig. 1.4: A hypothetical model of events underlying somatic embryogenesis[24].

1.2.1. Gene expression during somatic embryogenesis:

Gene expression during somatic embryogenesis can be evaluated either by isolating genes expressed in somatic embryos and subsequently identifying the function of those genes or by studying the expression of a variety of other genes isolated from non-embryo tissues in the hope that they may also play some role in embryogenesis. A number of genes have been identified that are enhanced in expression in developing embryos, and several of these are being used to analyze mechanisms of gene regulation during embryogenesis[23] (Table 1.1 and 1.2).

Table 1.1: List of various genes identified influencing somatic embryogenesis in higher plants (after Chugh and Khurana[25]).

Process	Genes
Hormone-responsive genes	*DcArg-1, homologs of pJCW1, pJCW2, DcECP63, Em, DcECP-40, DcEP31.*
Housekeeping genes	*Top1, EF-1a, CEM6, H3-1, CGS102, CGS103, CG201.*
Signal transduction	*SERKs, swCDKs, CRKs, MsCPK3.*
Zygotic mutants	*Clv, lec, pkl, pt, stm, wus, zll/pnh*
hsps, germins	*Mshp18, Dchsp1, PcGER1.*
Maturation genes	*Mat1, Dc2.15, Dc3, Dc8, DcEMB1, Em, DcECP31, DcECP40, MsLEC1, MsLEC2.*
Homeotic genes	*Sbh1, EP3-2, PgChi-1, PgGlu-1, EP2, DcAGP1.*
Genes coding for extracellular proteins	*EP3-1, EP3-2, PgChi-1, PgGlu-1, EP2, DcAGP1.*

The advent of molecular techniques has been crucial in identification of genes that exhibit differential activity, e.g. construction of cDNA libraries, differential display analysis, subtracted probe analysis, PCR and its modifications, etc. Quite expectedly, various structural and functional genes like the hormone responsive genes, homeobox genes, *LEA* genes, genes coding for chitinases, kinases regulating somatic embryogenesis and many others have been identified and characterized[25].

Table 1.2: An example of some major genes expressed during carrot somatic embryogenesis (after Chugh and Khurana[25]).

Gene	Function
CEM1, CEM6	Housekeeping (elongation factor1-α)
topI	Housekeeping (topoisomerase I)
CGS102, CGS103, CGS201	Glutamine synthetase
Dcarg-1, Dchsp-1, DNA clones 43,87,93	Hormone responsive (auxin-inducible)
DcEP31, DcEP40, DcECP63, C-AB13	Hormone responsive (ABA-inducible)
DcSERK	Somatic embryogenesis receptor kinases
CHB1-CHB6	Homoebox containing genes
EP3-1, EP3-2	Extracellular proteins (chitinases)
DcAGP1	Extracellular proteins (arabinogalactans)
EP2	Lipid transfer protein
Dc2.15	Maturation gene
Dc3, Dc8, DcECP31, DcECP40, DcEMB1	*LEA* proteins
EMB-1	Homology with *EM* gene

1.2.2. Somatic embryogenesis in banana:

In bananas and plantains, somatic embryogenesis techniques serve for providing a highly performance micropropagation technique which can be used for germplasm maintenance and a cell regeneration system valuable for genetic transformation and somatic hybridization. These techniques rely on the use of growth regulators (mainly auxins) to induce the dedifferentiation of tissues and the formation of embryogenic callus. The callus provides the starting material for the development of embryogenic cell suspensions, from which embryos are produced and plants are regenerated[26].

Several types of explants have been used to induce embryogenic callus and regenerate plants in *Musa* (Table 1.3). Of these explants, immature male flowers appear to be the most responsive starting material for initiating embryogenic cultures of Cavendish banana[30, 36, 40-42] and for mass propagation[43-44]. However, Strosse et al.[34] reported that, the efficiency outcome of embryogenesis using the scalp method and technologies that use immature male flowers are comparable.

Table 1.3: Explant types used for somatic embryogenesis in banana and plantain.

Explant type	Cultivars	Referances
Zygotic embryos	Diploids (AA)	27-30
Rhizome and leaf sheaths	Diploid (AA) Cavendish (AAA) Coking banana (ABB)	31
Proliferating meristems and scalps	Diploid (AA) Cavendish (AAA) Plantain (AAB) Coking banana (ABB)	32-34
Immature male flower	Cavendish (AAA)	30, 35-38
Immature female flowers	Plantain (AAB)	39

Several reports have been published regarding immature male flower method varying in their embryogenic callus response. Escalant et al.[40], found a maximum embryogenic response of 7% within five different banana and plantain genotypes, including *Musa* (AAA) 'Grande Naine'; Navarro et al.[30], obtained a variable 2-6% embryogenic callus response for "Grand Naine" and Strosse et al.[26] reported a mean percentage of ideal embryogenic callus of 8% for "Grand Naine". On the other hand, the scalp-method has been described for 'Williams' with 10% embryogenic response and 0.1 to 6.2% for other Cavendish-type bananas[45]. Strosse et al.[34] reported that both immature male flower and scalp methods yield quite similar results after the induction of embryogenesis.

The effect of immature male flower position on the formation of embryogenic callus in 'Grand Naine' has been established by Escalant et al.[40] who reported that approximately 74% of the embryogenic clusters formed were of the 7th to the 13th position. Simalry, Chong et al.[46] found that the predominant response occurred within flowers from the 8th to the 15th position.

1.3. Somaclonal variation:

Somaclonal variation wàs given, as a general term, by Larkin and Scowcroft[47] and refers to the variation arising in *in vitro* cultures, regenerated plants and their progenies. However, other types of variation arise by specific culture of cells or tissue, which include culture of protoplasts (protoclonal); anthers and microspores (gametoclonal); callus (calliclonal); apical meristem (mericlonal); leaf, stem, root or other somatic tissues (somaclonal)[48]. Somaclonal variation can be created for different approaches, which include: (1) callus or cell suspension cultures for several cycles; (2) regeneration of a large number of plants from such long-term cultures; (3) screening for desirable traits in the regenerated plants and their progenies, eg. *In vitro* selection to select agronomically desirables somaclones for tolerance to various biotic and abiotic stresses using toxic levels of pathotoxins, herbicides, salts, etc.; (4) testing of selected variants in subsequent generations for desired traits; and (5) multiplication of stable variants to develop new breeding lines.

Somaclonal variation has been classified to either that arises from pre-existing variation in the explant or the variation which may be induced by *in vitro* conditions[49]. Additionally, there are several factors affecting somaclonal variation, including; genotype, explant source, *in vitro* period and cultivation conditions in which the culture is established[50]. Variation include mutations, chromosomal rearrangements, changes in chromosome structure (deletions, exchange of chromosome sections, isochromosome formations, inversions, etc.), gene amplification, gene methylations, activation of transposons, exchange of sections of chromosomes, and others[51].

During *in vitro* culture, various environmental factors may lead not only to genetic variation but can also influence gene expression or cause variation in certain interactions between genetic materials. For instance, changes in gene functions (switch on or off), synthesis of certain proteins, cell differentiation, or protein distribution can cause a phenotypic variation which is called epigenetic variation. Although this type of variation cannot be transmitted to offspring *via* sexual reproduction, it can be maintained by asexual propagation[48].

These variations are undesirable in commercial multiplication programs of selected genotypes, which require uniformity or true-to-type plants especially in micropropagation and transgenic plants. On the other hand, somaclonal variation can be exploited to select germ lines with favorable properties, such as early or late fruiting, high yield and quality, resistance or tolerance to biotic or abiotic stress, etc. However, the problem of stability of such traits takes place[48].

1.3.1. Somaclonal variation in banana:

Occurrence of somaclonal variation in banana and plantain micropropagation derived plants with different forms of plant morphology and genetic dissimilarity have been widely reported[52-56]. In this regard, Bairu et al.[54] used RAPD molecular technique to check the incidence of somaclonal variation in Cavendish banana plants derived from the tenth subculture of micropropagation and they found 55% of polymorphism amongst mother plants and their regenerated plants. While, Mohamed[55] found that the polymorphism percentage in plants of sixth subculture of Williams (AAA) ranged from 9.1 to 100%. Additionally, Sheidai et al.[56] analyzed the somaclonal variation in the first, third, fifth,

seventh and ninth subcultures of meristem tip cultures of *M. acuminata* and they found in total 51.40% of polymorphism detected by RAPD marker. However, Abu Harrirah and Khalid[57] reported that there was no somaclonal variation at the molecular level, using RAPD molecular markers, among regenerated plants, derived from male inflorescences direct regeneration (*via* organogenesis) of *M. acuminata* cv. Berangan (AAA), in comparison to their mother plants.

In contrary, few studies have been published on the occurrence of off-types banana plants produced through somatic embryogenesis[58]. In this regard, Côte et al.[59] reported that, a number of plants derived from somatic embryogenesis, in 'Grand Naine', were true to type and have agronomic characteristics comparable to *in vitro* plantlets derived *via* micropropagation. Similar findings were obtained with 'IRFA903' plants derived from seven months old embryogenic cell suspensions[60]. In addition, Cabrera-García et al.[61] evaluated "Dwarf Cavendish" cv. (AAA) plants regenerated from proliferating inflorescence-derived embryogenic suspension cultures, no off-types were observed among the embryogenesis-derived plants during either the in vitro phase or the acclimatization period in the nursery. Contrary to these results, Shchukin et al.[62] found that 3.6% of somatic embryogenesis-derived regenerants of 'Grand Naine' were off-types.

Several types of morphological variations in micropropagated banana have been reported (Table 1.4). On the other hand, molecular markers have been used for detecting somaclonal variation at the molecular level in banana and plantains these include; randomly amplified polymorphic DNA[54-56,63], inter simple sequence repeats[64], sequence characterized amplified region[65], representational difference analysis[66], selective amplification of microsatellite polymorphic loci[53], amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism[67-69].

Table 1.4: Types of morphological variations in micropropagated banana (After Sahijram et al. [52])

Subgroup	Cultivar/s	Type of morphological variation	Reference
Cavendish (AAA)	Williams, Grand Naine	Dwarf, extra dwarf, giant, mosaic, extra mosaic, variegated, deformed lamina, reddish pseudostem, black pseudostem	70
	Shai, Eilon, Arnon	Dwarf, giant	
	Nathan	Extra dwarf, giant	
	Red	Giant, green-red pseudostem	
Musa acuminata (AAA)	Red	Green variants lacking anthocyanidin pigmentation in leaf sheath, petiole, midrib, and fruit rind	71
Not specified	Not specified	Dwarfs, thin and sickly-looking tall plants; abnormal pseudostem pigmentation with or without blotching; variation in leaves viz., twisted and crinkly leaves, narrow and drooping leaves, leaves with unusually wavy margins; abnormal bunch orientation; small, narrow elongated or bloated male bud, absence of male bud or its reversion; small bunch with short/twisted fingers, variation in hand and finger orientation on the bunch; persistent or deciduous floral bracts, hairiness of bunch peduncle and fruit, warty fruits with ugly eruptions	72

Somaclonal variation is undesirable in the context of micropropagation, germplasm conservation and genetic transformation. However, it can be used to get an advantage for genetic improvement of banana[52]. In this regard, several useful somaclonal variants for various attributes have been identified, e.g. TC1-229, semi-dwarf and resistant to *Fusarium* wilt, derived from Cavendish banana[73], Tai-Chiao No.1 and Formosana, which are reported to be tolerant to *Fusarium* wilt Tropical Race 1V, and were derived from Giant Cavendish[74]. However, the majority of somaclonal variants are undesirable such as the mosaic type heterogeneity Cavendish banana[75].

1.4. Molecular markers:

In recent years, molecular markers, and especially DNA-based markers have been extensively used in analysis of genetic diversity. Several polymerase chain reaction (PCR) marker systems are available varying in complexity, reliability and information generating capacity. These include random amplified polymorphic DNA (RAPD), simple sequence repeat polymorphism (SSR), amplified fragment length polymorphism (AFLP) and sequence related amplified polymorphism (SRAP). Each system has its own advantages and disadvantages. For example, RAPD is a simple method to fingerprint genomic DNA, but poor consistency and low multiplexing output limit its use. SSR has the advantage of producing mostly co-dominant markers; however, the development of these is considerably expensive and time- consuming. AFLP has become the method of choice for many studies on plants; spanning numerous disciplines in genetics and ecology[67]. SRAP markers combine simplicity, reliability, and facile sequencing of selected bands. Further, it targets coding sequences in the genome and results in a moderate number of co-dominant markers[77].

1.4.1 Amplified Fragment Length Polymorphism:

Amplified Fragment Length Polymorphism is a DNA fingerprinting technique developed by Zabeau and Vos[78] and Vos et al.,[79]. This method is based on PCR amplification of selected restriction fragments of total digested genomic DNA. Amplified products are separated by electrophoresis to be visualised. The originality of the AFLP method was to design and synthesise arbitrary adaptors first, and then to ligate them to target DNA fragments. The AFLP arbitrary adaptors consist of a known sequence of 20 nucleotides. The target DNA sequences are DNA fragments generated by restriction enzymes. Fragments are produced from genomic DNA by the combined action of two restriction enzymes. Then, adaptors are ligated at each end of a restriction fragment with DNA-ligase. Finally, AFLP primers, complimentary to the adaptors, are used in a PCR to amplify the restriction fragments. AFLP markers reveal a "restriction site" polymorphism and must be treated as dominant markers, since homozygotes and heterozygotes cannot be established unless breeding/pedigree studies are carried out to determine inheritance patterns of each fragment. However, the large number of fragments gives an estimate of

variation across the entire genome, which thus gives a good general picture of the level of genetic variation of the studied organism.

1.4.1.1. Basic steps of AFLP:

a. DNA digestion with restriction enzymes:

Restriction fragments of the genomic DNA are produced by using two different restriction enzymes: a frequent cutter (the four-base restriction enzyme *Mse*I) and a rare cutter (the six-base restriction enzyme *Eco*RI). The frequent cutter serves to generate small fragments, which amplify well and which have the optimal size range for separation on a sequence gel, whereas the rare cutter limits the number of fragments to be amplified (Fig. 1.5).

b. Ligation of oligonucleotide adaptors:

Double-stranded adaptors consist of a core sequence and an enzyme-specific sequence. Therefore, adaptors are specific for either the *Eco*RI site or the *Mse*I site. Restriction and ligation can take place in a single reaction or separately. The core sequence of the adaptors consists of a known DNA sequence of 20 nucleotides, which will be used later as primer in the PCR (Fig. 1.5).

c. Pre-amplification:

This first PCR, called pre-amplification, allows a first selection of fragments by only amplifying the DNA restriction fragments that have ligated an adaptor to both extremities. Additionally to the adaptor sequences, the primers used for the pre-selective amplification have a supplementary base. This extra base enables another first selection by amplifying ¼ of the fragments that have ligated an adaptor to both extremities (Fig. 1.5).

d. Selective amplification:

The aim of this step is to restrict the level of polymorphism and to label the DNA (in the case of using fluorescent labels). For this second amplification, three more nucleotides were added at the 3' end of the primer sequence. These additional nucleotides make the amplification more selective and will decrease the number of restriction fragments

amplified (polymorphism). Moreover, sometimes one of the primers (usually the *Eco*RI primer) can be labelled with a fluorescent dye, and will allow the visualisation of DNA during the migration by the use of UV.

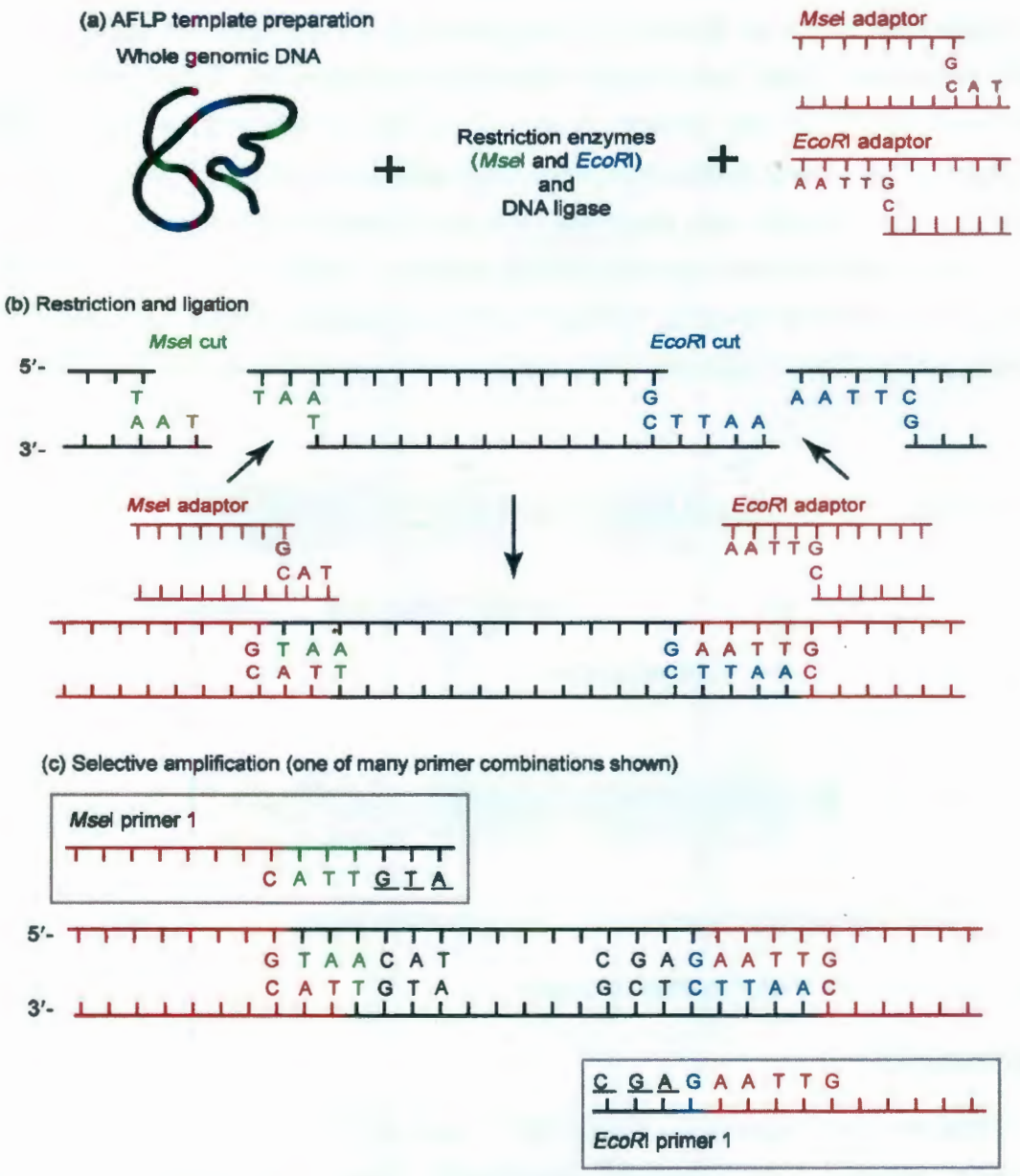

Fig. 1.5: The basic steps of AFLP technique; restriction, ligation, amplification (After Vos et al.,[79]).

1.4.2. Sequence Related Amplified Polymorphism:

Sequence Related Amplified Polymorphism aims for the amplification of open reading frames (ORFs). It is based on two-primer amplification. The primers are of 17 or 18 nucleotides long and consist of the following elements; core sequences, which are 14 to 15 bases long, where the first 10 or 11 bases starting at the 5` end, are sequences of no specific constitution ("filler" sequences), followed by the sequence "CCGG" in the forward primer and "AATT" in the reverse primer. The core is followed by three selective nucleotides at the 3` end. Variation in these three selective nucleotides generates a set of primers sharing the same core sequence. The only rules for construction of the forward and reverse primers are that they do not form hairpins or other secondary structures, and to have a GC content of 40–50%. Further, the filler sequences of the forward and reverse primers must be different from each other and can be 10 or 11 bases long (Fig. 1.6).

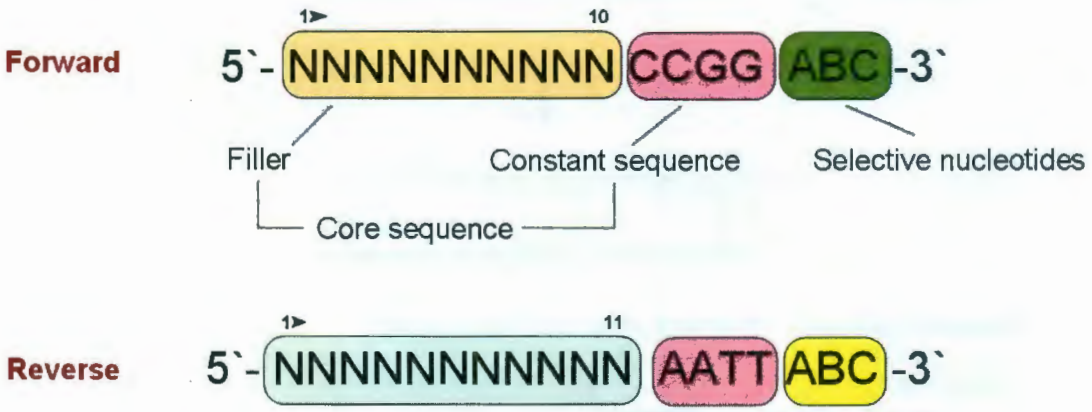

Fig. 1.6: SRAP forward and reverse primers construction (After, Li and Quiros[77]).

1.4.2.1. Protocol for SRAP marker system:

a. DNA amplification:

The first five cycles are run at 94°C, 1 min, 35°C, 1 min, and 72°C, 1 min, for denaturing, annealing and extension, respectively. Then the annealing temperature is raised to 50°C for another 35 cycles. The low initial annealing temperature ensures the binding of both primers to sites with a partial match in the target DNA. The annealing temperature is then increased for the subsequent cycles. This temperature change

ensures that the DNA products amplified at the first five cycles will be efficiently and consistently amplified in exponential fashion during the rest of the reaction. For amplification the cocktail used in other routine PCR-marker applications can be used. The products are separated by denaturing acrylamide gels and detected by autoradiography or silver nitrate.

b. SRAP primer sequence:

The purpose for using the "CCGG" sequence in the core of the first set of SRAP primers was to target exons to open reading frame (ORF) regions. This rationale is based on the fact that exons are normally in GC-rich regions. The second primer set was designed with a core containing the "AATT" sequence near the 3` region in order to aim at AT-rich regions. Normally these are found more frequently on promoters and introns. Since introns, promoters and spacers are usually variable among different individuals; this intrinsic dissimilarity makes it feasible to generate polymorphic bands based on introns and exons[80].

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Justification

Bananas and plantains are very important fruits worldwide. Their improvement is an essential aim. In this regard, understanding of the genetic diversity in the genus *Musa*; sections, species and cultivars could provide an excellent tool for banana breeding and improvement. Molecular markers offer useful and rapid system for genetic diversity analysis and screening of specific bands for certain genotypes and species, which could be helpful in further research for *Musa* improvement.

On the other hand, classical breeding is difficult in the most important cultivars of banana and plantain, due to differing levels of ploidy and sterility, and can only be propagated vegetatively. Consequently, somatic embryogenesis is a valuable tool for use with *Musa* cultivars, for improvement by mass propagation of selected genotypes, mutagenesis, genetic transformation, protoplast fusion or other biotechnological applications. Many factors affect the response of somatic embryogenesis. Among these, the genotype and the developmental age of the plant organ from which the primary explants are obtained are of critical importance in eliciting the desired response to somatic embryogenesis, their effect could be studied.

In addition, plant regenerated from somatic embryogenesis may exhibit variation which may have a genetic basis, known as somaclonal variation. These variations are undesirable in mass propagation programs and genetic transformation techniques. However, they offer prospects for the recovery of useful mutants in tissue culture and for genetic improvement of banana. Molecular markers will be used for the analysis of the somaclonal variation that might be produced via somatic embryogenesis by comparing the regenerated plants with their parental plants.

Hypothesis:

Molecular markers techniques such as SRAP and AFLP can resolve the genetic diversity amongst *Musa* genotypes and detect the somaclonal variation in somatic embryogenesis regenerated plants.

General objective:

Evaluation of the genetic variability amongst *Musa* genotypes and detection of genetic variation resulting from somatic embryogenesis using molecular marker techniques

Particular objectives:

1- Assessment of the genetic variability amongst banana genotypes using molecular markers.
2- Induction of somatic embryogenesis from immature male floral tissues using two developmental stages of explant.
3- Detection of somaclonal variation (if any) in regenerated plants derived from somatic embyogenesis in two cultivars of banana using molecular markers.

Experimental strategy:

To achive the objectives in this study, the experimental strategy displayed in figure 1.7 will be followed.

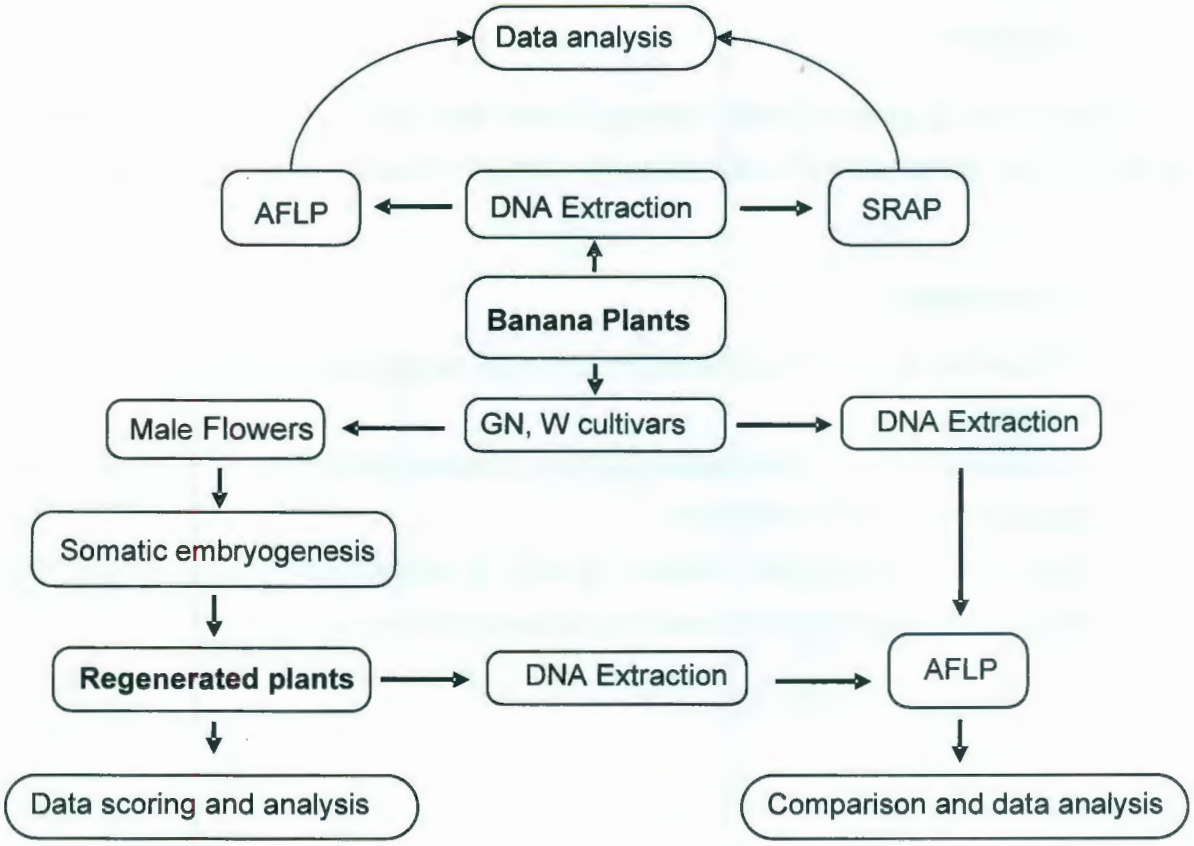

Fig. 1.7: Experimental strategy showing the different parts of the work to be followed to reach the work objectives.

Chapter II

Title: *Musa* genetic diversity revealed by SRAP and AFLP

Main objective:

Assessment of the genetic variability amongst banana genotypes using molecular markers

Particular objectives:

1- Establishment of SRAP technique.
2- Establishment of AFLP technique.
3- Analysis of genetic diversity in different banana genotypes using SRAP and AFLP.
4- Data analysis.

Summary:

In This chapter, both SRAP and AFLP molecular marker techniques were established, primers were selected and different accessiones of *Musa* were analyzed for genetic diversity assessment. Both UPGMA and principal coordinate analyses were used and data from both molecular systems were compeared.

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Chapter II

Musa genetic diversity revealed by SRAP and AFLP*

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Abstract

The sequence-related amplified polymorphism (SRAP) technique, aimed for the amplification of open reading frames (ORFs), vis-â-vis that of the amplified fragment length polymorphisms (AFLP) were used to analyze the genetic variation and relationships amongst forty *Musa* accessions; which include commercial cultivars and wild species of interest for the genetic enhancement of *Musa*. A total of 403 SRAP and 837 AFLP amplicons were generated by 10 SRAP and 15 AFLP primer combinations, of which 353 and 787 bands were polymorphic, respectively. Both cluster analysis of unweighted pair-grouping method with arithmetic averages (UPGMA) and principal coordinate (PCO) analysis separated the forty accessions into their recognized sections (Eumusa, Australimusa, Callimusa and Rhodochlamys) and species. The percentage of polymorphism amongst sections and species and the relationships within Eumusa species and subspecies varied between the two marker systems. In addition to its practical simplicity, SRAP exhibited approximately threefold more specific and unique bands than AFLP, 37% and 13%, respectively. SRAP markers are demonstrated here to be proficient tools for discriminating amongst *M. acuminata*, *M. balbisiana* and *M. schizocarpa* in the Eumusa section, as well as between plantains and cooking bananas within triploid cultivars.

Keywords: AFLP, banana, Genetic variation, *Musa,* germplasm collection, SRAP

Abbreviations: AFLP, amplified fragment length polymorphism; ORF, open reading frame; SRAP, sequence related amplified polymorphism.

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2.1. Introduction

Bananas and plantains are grown in more than 120 countries in tropical and subtropical areas on five continents. Their fruits are an essential food resource and have an important socioeconomic and ecological role[1]. The genus *Musa* L. (Musaceae, Zingiberales) is often divided into five sections: Eumusa, Rhodochlamys, Callimusa, Australimusa and Ingentimusa; these sections are distinguished by their specific chromosome numbers and morphological characters. The Eumusa section is geographically the most widely distributed and contains two major species, *Musa acuminata* Colla. and *M. balbisiana* Colla., which are the origin of the great majority of the edible bananas and plantains[2].

DNA markers are more abundant than morphological markers and are largely unaffected by the environment. The genetic diversity in the genus *Musa* has been assessed using different types of molecular markers, e.g., AFLP[3-6], RAPD[7,8], VNTR[9], RFLP[10,11] and diversity array technology (DArT)[12].

The sequence related amplified polymorphism (SRAP) technique is a simple and efficient marker system that can be adapted for a variety of purposes, including map construction, gene tagging, genomic and cDNA fingerprinting, and map-based cloning. It has several advantages over other molecular marker systems such as simplicity, reasonable throughput rate, disclosure of numerous co-dominant markers, ease of isolation of bands for sequencing and, most importantly, targeting of open reading frames (ORFs)[13]. SRAP has been used in other plant species, e.g., in *Cucurbita pepo* L.[14], *Bixa orellana* L.[15], sugarcane[16], or wheat[17]. Phothipan et al.[18] used SRAP and RAPD molecular markers to study the genetic relationship amongst *M. acuminata, M. balbisiana* and plantains. Their results showed that SRAP was similar to RAPD in detecting genetic polymorphism. However, some SRAP primers were more efficient for detecting the much closer cultivars in the same group.

The combination of SRAP and AFLP markers will be useful to analyze the genetic variability particularly with different accessions of banana and plantain cultivars, as well as different wild *Musa* species. Such an analysis could also be helpful for further improvement of the crop. This study aims to use SRAP and AFLP molecular marker

techniques to detect the genetic variation among *Musa* sections and within sections from Eumusa species and cultivars.

2.2. Materials and methods

2.2.1. Plant materials

Forty accessions consisting of different *Musa* genotypes were used in this study. Young cigar leaves were collected from plants of the *Musa* germplasm collection of the Centro de Investigación Científica de Yucatán (CICY) held at the research station of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP) at Uxmal (Lat. 20º 24` 40.10`` N, Long. 89º 45` 24.90`` E, 8m altitude above sea level, Yucatán, México). All *Musa* accessions with an International Transit Code (ITC) number were provided by the Biodiversity International Centre for *Musa*, located at the Laboratory for Tropical Plant Improvement in the Catholic University of Leuven (Belgium), whereas those without code number, which include accessions 15, 19, and 39, were collected at Teapa (Tabasco), or Cancun (Quintana Roo) in México (Table 2.1), respectively.

2.2.2. DNA extraction and quantification

Total genomic DNA from the 40 *Musa* accessions was extracted from 100 mg of frozen young cigar leaves following the protocol from Dellaporta et al.[19] with some modifications. DNA quality and concentration were determined using a spectrophotometer according to Stulnig and Amberger[20] (Annex-I).

2.2.3. SRAP and AFLP-PCR amplification

The method of Li and Quiros[13] was followed with some modifications for the SRAP marker system. Eighty primer combinations using 8 forward (Me 1-8) and 10 reverse (Em 1-10) primers were tested for amplification efficiency and primer selection with four samples: two samples of accession 9 with genome AA and two of accession 15 with genome BB (Table 2.1), respectively. Each reaction was repeated twice to test for reproducibility of the selected markers. Ten SRAP primer sets were selected based on polymorphism scores greater than 75% and used for the analysis of the different *Musa* accessions (Table 2.2). Each 20µl SRAP amplification reaction consisted of 2µl of 10X PCR buffer, 1.6µl of 50mM $MgCl_2$, 1.6µl of 10µM of each forward and reverse primer, 2.5µl

of 2mM dNTPs, 25 ng template DNA, and 0.25µl of 5U Taq-DNA polymerase (Invitrogen). The PCR was carried out with the initial cycle at 94°C for 2 min, 5 cycles of 94°C for 30 s, 35°C for 30 s and 72°C for 1 min, another 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min, and the final extension at 72°C for 5 min.

The amplified fragment length polymorphism analysis was carried out according to Vos et al.[21] with the following modifications: 250 ng total genomic DNA were endonuclease-digested with 0.2 µl of 10U *Eco*-R1 and 0.2 µl of 5U *Mse*-1 (Invitrogen) with 2 µl of 10X restriction buffer for 3 hrs at 37°C in a final volume of 20 µl. Enzymes were inactivated at 70°C for 15 min. The digested products were ligated by adding 1 µl of each 5 µM EcoR1 and 50 µM MseI double-stranded adaptor followed by adaptor ligation using 1µl of 1U DNA-Ligase (Invitrogen) with 6 µl of 5X Ligase buffer to generate template DNA for pre-amplification. PCR pre-amplification was carried out using AFLP primers, each having one selective nucleotide. Each 25 µl AFLP pre-amplification reaction consisted of 2.5 µl of 10X PCR buffer, 0.75 µl of 50 mM $MgCl_2$, 0.75µl of 30 µM of each primer, 2.5 µl of 2 mM dNTPs, 5µl of (1:10) diluted ligated DNA, and 0.125 µl of 5 U Taq-DNA polymerase (Invitrogen). The PCR was carried out with 20 cycles of 92°C for 1 min, 60°C for 30 s and 72°C for 1 min. The PCR pre-amplified products were diluted 1:25 in H_2O and used as template for selective amplification using AFLP primers, each containing three selective nucleotides. Each 20 µl AFLP selective amplification reaction consisted of 2 µl of 10X PCR buffer, 0.6 µl of 50 mM $MgCl_2$, 0.2 µl of 20 µM of EcoR1 primer, 0.3 µl of 20 µM of Mse-1 primer, 2 µl of 2 mM dNTPs, 5µl of (1:25) diluted pre-amplified DNA, and 0.25 µl of 5U Taq-DNA polymerase (Invitrogen). The PCR was carried out with 11 cycles of 94°C for 30 s, 65°C (-0.7°C/cycle) for 30 s and 72°C for 1min, and 24 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. Out of 26 primer combinations tested, 15 were selected and used. They include the set of 10 primers used in previous studies in *Musa*[22-25], along with five new combinations (Table 2.2). SRAP and AFLP amplification products were separated on a 6% sequencing gel (urea-PAGE) and visualized by staining with silver nitrate according to Bassam et al.[26] with some modifications (Annex-II). As control for eproducibility of the banding patterns, two replicate tissue samples from a single plant of accessions 9 (AA genome) and 15 (BB genome) were included in all experimental steps for both marker system.

Table 2.1: Plant materials of genus *Musa*

No.	Section	Species/hybrid	Subspecies/subgroup	Geno	Name	Abbreviati	ITC[b]
1	Eumusa	*M. acuminata*	*microcarpa*	AA w[a]	Microcarpa	MIC	0253
2	Eumusa	*M. acuminata*	*siamea*	AA w	Siamea	SIA	0660
3	Eumusa	*M. acuminata*	*burmannicoides*	AA w	Calcuta	CAL	0249
4	Eumusa	*M. acuminata*	*malaccensis*	AA w	Pisang Kra	PKR	1345
5	Eumusa	*M. acuminata*	*burmannica*	AA w	Long Tavoy	LON	0283
6	Eumusa	*M. acuminata*	*truncate*	AA w	Truncata	TRU	0393
7	Eumusa	*M. acuminata*	*banksii*	AA w	Banksii	BAN	0623
8	Eumusa	*M. acuminata*	*zebrina*	AA w	Zebrina	ZEB	0966
9	Eumusa	*M. acuminata*	*malaccensis*	AA w	Selangor	SEL	1060
10	Eumusa	*M. acuminata*	-	AA cv[a]	Pisang Berlin	PBE	0611
11	Eumusa	*M. acuminata*	*banksii*	AA cv	Niyarma Yik	NYK	0269
12	Eumusa	*M. acuminata*	-	AA cv	IDN 110	IDN	0413
13	Eumusa	*M. acuminata*	*banksii*	AA cv	Tomolo	TOM	1187
14	Eumusa	*M. balbisiana*	-	BB	Honduras	HON	0247
15	Eumusa	*M. balbisiana*	-	BB	BB-CICY	BB-CICY	-
16	Eumusa	*M. a x M. b.*	Ney Poovan	AB	Kunnan	KUN	1034
17	Eumusa	*M. schizocarpa*	-	SS	Schizocarpa	SCH	0599
18	Eumusa	*M. a x M. s.*	-	AS	Wompa	WOM	1152
19	Eumusa	Dessert banana	Cavendish	AAA	Gran Enain	GEN	-
20	Eumusa	Dessert banana	Cavendish	AAA	Williams	WIL	0570
21	Eumusa	Dessert banana	Gros Michel	AAA	High Gate	HGT	0263
22	Eumusa	Dessert banana	Gros Michel	AAA	Gros Michel	GMI	1481
23	Eumusa	Dessert banana	Ibota	AAA	Yangambi KM5	YAN	1123
24	Eumusa	Dessert banana	Ibota	AAA	JD Yangambi	JDY	1336
25	Eumusa	Plantains	-	AAB	Silk	SIL	0769
26	Eumusa	Plantains	-	AAB	Prata	PRA	0962
27	Eumusa	Plantains	-	AAB	Ihitisim	IHT	0121
28	Eumusa	Cooking banana	-	ABB	Tiparot	TIP	0652
29	Eumusa	Cooking banana	-	ABB	Pisang Awak	PAW	0213
30	Eumusa	Cooking banana	-	ABB	Fougamou	FOU	0101
31	Eumusa	Cooking banana	-	ABB	Bluggoe	BLU	0767
32	Eumusa	Tetraploid *acum.*	Gros Michel	AAAA	SH 3436-6	SH	1284
33	Eumusa	Tetraploid *acum.*	Gros Michel	AAAA	FHIA-23	F-23	1265
34	Eumusa	Tetraploid *M. a.xM.b.*	-	AABB	TMX 5295-1	TMX	1297
35	Eumusa	Tetraploid *M. a.xM.b.*	-	AAAB	FHIA-01	F-01	0504
36	Eumusa	Tetraploid *M. a.xM.b.*	-	AAAB	FHIA-21	F-21	1332
37	Australimusa	*M. beccarii*	-	-	Beccari	BEC	1070
38	Australimusa	*M. textiles*	-	-	Textiles	TEX	1072
39	Rhodochlamys	*M. velutina*	-	-	Velutina	VEL	-
40	Callimusa	*M. coccinea*	-	-	Coccinea	COC	0287

[a] *M. acuminata* AAw = wild type, AAcv = cultivar.

[b] All plant materials that have an ITC were provided by the Biodiversity International Centre for *Musa* located at the Laboratory for Tropical Plant Improvement in the Catholic University of Leuven, Belgium

Table 2.2: Sequences of ten SRAP and fifteen AFLP selected primer combinations.

No.	Code	Forward primer (5`-3`)	Reverse primers (5`-3`)
SRAP marker			
1	ME-1/EM-1	TGAGTCCAAACCGG**ATA**	GACTGCGTACGAATT**AAT**
2	ME-2/EM-1	TGAG**T**CCAAACCGG**AGC**	GACTGCGTACGAATT**AAT**
3	ME-4/EM-1	TGAGTCCAAACCGG**ACC**	GACTGCG**T**ACGAATT**AAT**
4	ME-8/EM-3	TGAGTCCAAACCGG**TGT**	GACTGCGTACGAATT**GAC**
5	ME-3/EM-6	TGAGTCCAAACCGG**AAT**	GACTGCGTACGAATT**GCA**
6	ME-1/EM-7	TGAGTCCAAACCGG**ATA**	GACTGCGTACGAATT**ATG**
7	ME-1/EM-8	TGAGTCCAAACCGG**ATA**	GACTGCGTACGAATT**AGC**
8	ME-2/EM-8	TGAGTCCAAACCGG**AGC**	GACTGCGTACGAATT**AGC**
9	ME-3/EM-8	TGAGTCCAAACCGG**AAT**	GACTGCGTACGAATT**AGC**
10	ME-4/EM-8	TGAGTCCAAACCGG**ACC**	GACTGCGTACGAATT**AGC**
No.	Enzyme	Eco-R1 primer (5`-3`)	Mse-1 primer (5`-3`)
AFLP marker			
1	ECO-1/MSE-1	GACTGCGTACCAATTC**AAC**	GATGAGTCCTGAGTAA**CAA**
2	ECO-3/MSE-1	GACTGCGTACCAATTC**ACA**	GATGAGTCCTGAGTAA**CAA**
3	ECO-4/MSE-1	GACTGCGTACCAATTC**ACG**	GATGAGTCCTGAGTAA**CAA**
4	ECO-1/MSE-3	GACTGCGTACCAATTC**AAC**	GATGAGTCCTGAGTAA**CAG**
5	ECO-3/MSE-3	GACTGCGTACCAATTC**ACA**	GATGAGTCCTGAGTAA**CAG**
6	ECO-4C/MSE-10	GACTGCGTACCAATTC**CAC**	GATGAGTCCTGAGTAA**CCG**
7	ECO-7C/MSE-10	GACTGCGTACCAATTC**CGC**	GATGAGTCCTGAGTAA**CCG**
8	ECO-14C/MSE-10	GACTGCGTACCAATTC**CTG**	GATGAGTCCTGAGTAA**CCG**
9	ECO-15C/MSE-10	GACTGCGTACCAATTC**CTC**	GATGAGTCCTGAGTAA**CCG**
10	ECO-4/MSE-13	GACTGCGTACCAATTC**ACG**	GATGAGTCCTGAGTAA**CTA**
11	ECO-6/MSE-13	GACTGCGTACCAATTC**AGC**	GATGAGTCCTGAGTAA**CTA**
12	ECO-5/MSE-14	GACTGCGTACCAATTC**ACT**	GATGAGTCCTGAGTAA**CTG**
13	ECO-1/MSE-15	GACTGCGTACCAATTC**AAC**	GATGAGTCCTGAGTAA**CTC**
14	ECO-3/MSE-15	GACTGCGTACCAATTC**ACA**	GATGAGTCCTGAGTAA**CTC**
15	ECO-6/MSE-16	GACTGCGTACCAATTC**AGC**	GATGAGTCCTGAGTAA**CTT**

The cells *6*, *7*, *8*, *9* and *11* of AFLP primer sets are new combinations and the remaining are the classical primer set reported in previous studies in *Musa*[22-25].

2.2.4. Data analysis

A binary data matrix indicating the presence (1) or the absence (0) of bands was made from both SRAP and AFLP profiles. Only strong, reproducible and clearly distinguished bands were used for the analysis. The percentage of polymorphism was calculated by dividing the number of polymorphic bands with the total number of regenerated bands. The software NTSYSpc ver. 2.20s (Applied Biostatics Inc.) was used and genetic similarities were computed using Jaccard's coefficient[27] of similarity. Cluster

analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic averages (UPGMA). One thousand repetition counts were used to generate the bootstrapping using the FreeTree program. Principal coordinate analysis (PCO) was also carried out using NTSYSpc program to show the distribution of accessions in a scatter plot.

2.3. Results

The ten SRAP primer sets (Table 2.2) utilized to examine *Musa* genetic variation generated approximately 20 to 59 bands with an average of 40 bands per primer combination. A total of 403 bands were scored, of which 353 (87.59%) were polymorphic and 50 (12.41%) were monomorphic. Approximately 27 to 82 bands, with an average of 56 bands per primer set, were produced with the 15 AFLP primer combinations (Table 2.2). A total of 837 bands were scored, of which 787 (94%) were polymorphic and 50 (6%) were monomorphic across all genotypes examined (Table 2.3).

Table 2.3: Levels of polymorphism in *Musa* spp. as revealed by SRAP and AFLP analysis

Category	Total number of bands		% of polymorphism	
	SRAP	AFLP	SRAP	AFLP
M. acuminata (AA)	204	559	61.76	73.52
M. balbisiana (BB)	167	393	32.34	36.90
M. schizocarpa (SS, AS)	110	358	20.91	40.78
Dessert banana (AAA)	151	431	27.15	46.40
Plantains (AAB)	170	421	33.53	32.54
Cooking banana (ABB)	181	403	28.18	30.77
Plantains and cooking banana	207	491	57.49	53.56
Tetraploid banana (AAAA)	149	323	2.01	8.36
Tetraploid hybrids (AAAB)	172	388	8.72	14.69
M. balbisiana and plantains	296	473	71.62	53.49
M. balbisiana and cooking	199	453	48.74	52.32
Eumusa	282	656	77.30	79.12
Australimusa	149	318	30.20	33.96
Eumusa vs. *Rhodochlamys*	309	550	80.58	81.48
Eumusa vs. *Callimusa*	317	716	83.91	84.92
Eumusa vs. *Australimusa*	361	631	87.53	86.44
Rhodochlamys vs. *Callimusa*	154	314	61.04	72.35
Rhodochlamys vs. *Australimusa*	197	350	68.02	74.15
Callimusa vs. *Australimusa*	197	304	68.02	74.93
Total	403	837	87.59	94.03

The repeatability of both, SRAP and AFLP, systems, was shown by identical band profiles observed within the two replicate tissue samples of each of the control accessions used for each of the primer pairs tested.

2.3.1. *Musa* sectional relationships

The UPGMA cluster analysis of 40 accessions that was constructed with the SRAP markers separated them into two significantly different clusters based on a similarity coefficient of 0.32 (Fig. 2.1). The major cluster split into two branches near a genetic similarity of 0.48. The foremost branch, I, comprises members of the different wild subspecies and cultivars of *Musa acuminata*, *M. balbisiana*, and their hybrids and cultivars belonging to section Eumusa. *M. velutina* (section Rhodochlamys) was separated as a single cluster in branch II. The accessions of sections Australimusa (*M. beccarii* and *M. textilis*) and Callimusa (*M. coccinea*) were grouped into a separated cluster III. Figure 2.2 shows the dendrogram generated by the AFLP data, which has two main clusters. The first cluster included the sections Eumusa and Rhodochlamys (*M. velutina*). The second cluster included section Australimusa (*M. textilis* and *M. beccarii*), whilst Callimusa (*M. coccinea*) formed a single branch out of the dendrogram with a branch showing a similarity of 0.29.

A scatter plot, based on PCO analysis of SRAP and AFLP data, showed the relationships among *Musa* sections (Fig. 2.3, Fig. 2.4). The PCO using SRAP separated Eumusa from the other sections. The Eumusa section was closer to Rhodochlamys than to Callimusa and Australimusa, which were assembled in a separate group. However, the PCO with AFLP data grouped Eumusa with Rhodochlamys, whereas Australimusa and Callimusa formed a separate group.

The percentage of polymorphism (P%) was calculated between each two of the four *Musa* sections using data from both molecular markers (Table 2.3). The P% value was also calculated within each section. However, Rhodochlamys and Callimusa were excluded because both had only one species in this study. There was a highly significant positive correlation between the P% value of the SRAP and AFLP data (r = 0.98). However, there was a highly significant difference between the P% values of the two molecular markers ($P < 0.01$, t-test).

The sequence-related amplified polymorphism and AFLP profiles generated 148 and 111 unique bands, respectively (36.7% and 13.2% of total generated bands), which were specific to certain sections and genotypes (Tables 2.4 and 2.5). These unique bands clearly showed the relationship among *Musa* sections. Both Eumusa and Rhodochlamys shared 4 and 26 bands, whereas Callimusa and Australimusa shared 7 and 10 bands using SRAP and AFLP, respectively. Moreover, there were some unique bands specific for each section, i.e., five bands for Eumusa, 22 for Rhodochlamys, 21 for Callimusa and 32 for Australimusa regenerated by SRAP markers. AFLP markers gave 18 and 32 bands specific for Australimusa and Callimusa, respectively (Tables 2.4 and 2.5). Several of the unique specific bands generated by SRAP and AFLP markers are shown in Figure 2.5.

Musa beccarii clustered close to *M. textilis* (Australimusa) with 30.20 and 33.96% polymorphism using SRAP and AFLP, respectively; i.e., lower than between *M. beccarii* and *M. coccinea* (Callimusa) that was 59.09, 65.95% (data not shown) using SRAP and AFLP, respectively. Furthermore, *M. beccarii* shared 32 and 18 unique bands with *M. textilis,* whereas it shared only three and six bands with *M. coccinea* using SRAP and AFLP, respectively (Tables 2.3, 2.4 and 2.5).

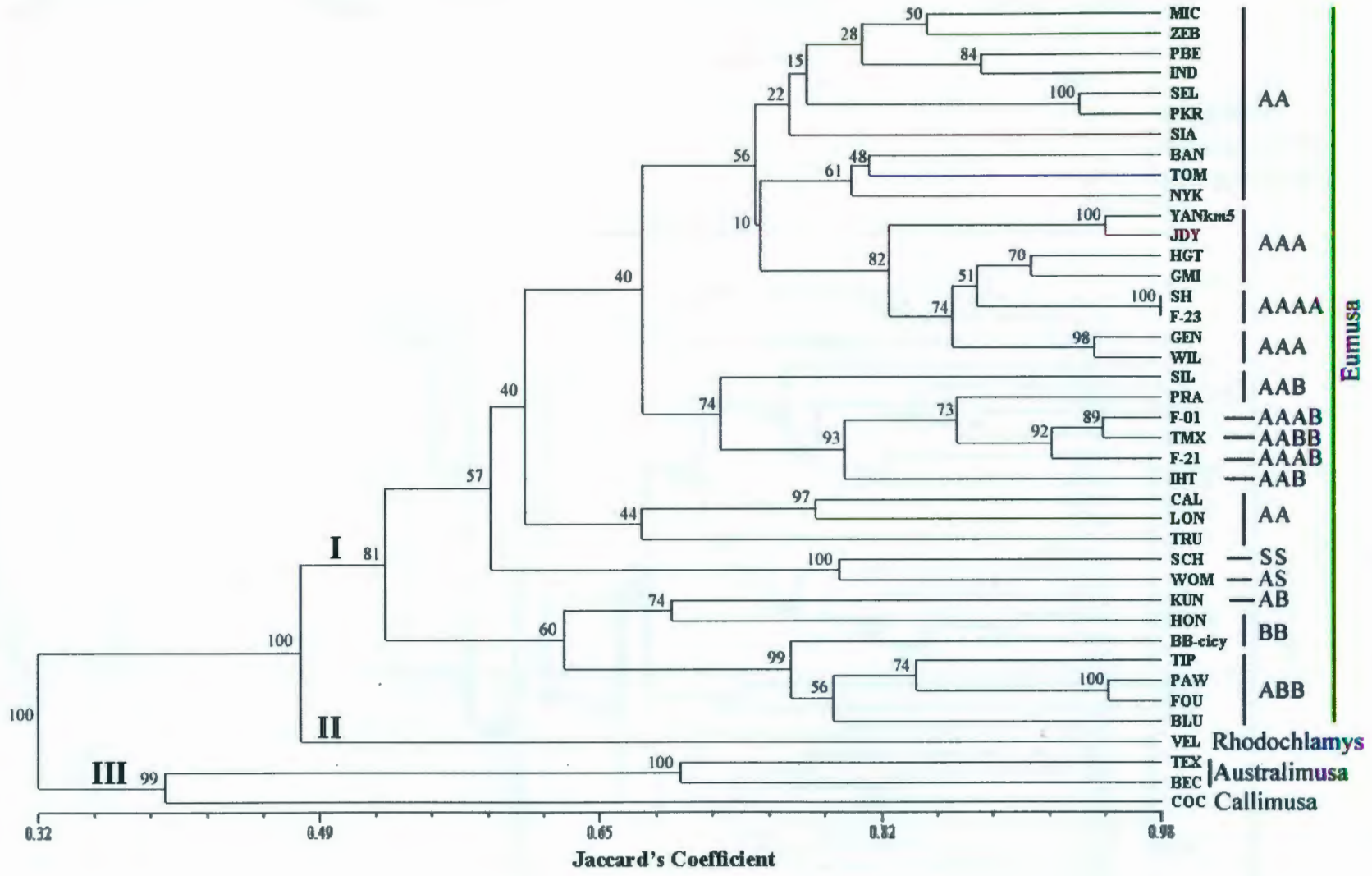

Fig. 2.1: Dendrogram of genetic similarities in *Musa* using SRAP-UPGMA cluster analysis. *Arabic numbers* indicate bootstrapping, whereas *Latin numbers* show *Musa* sectional relationships

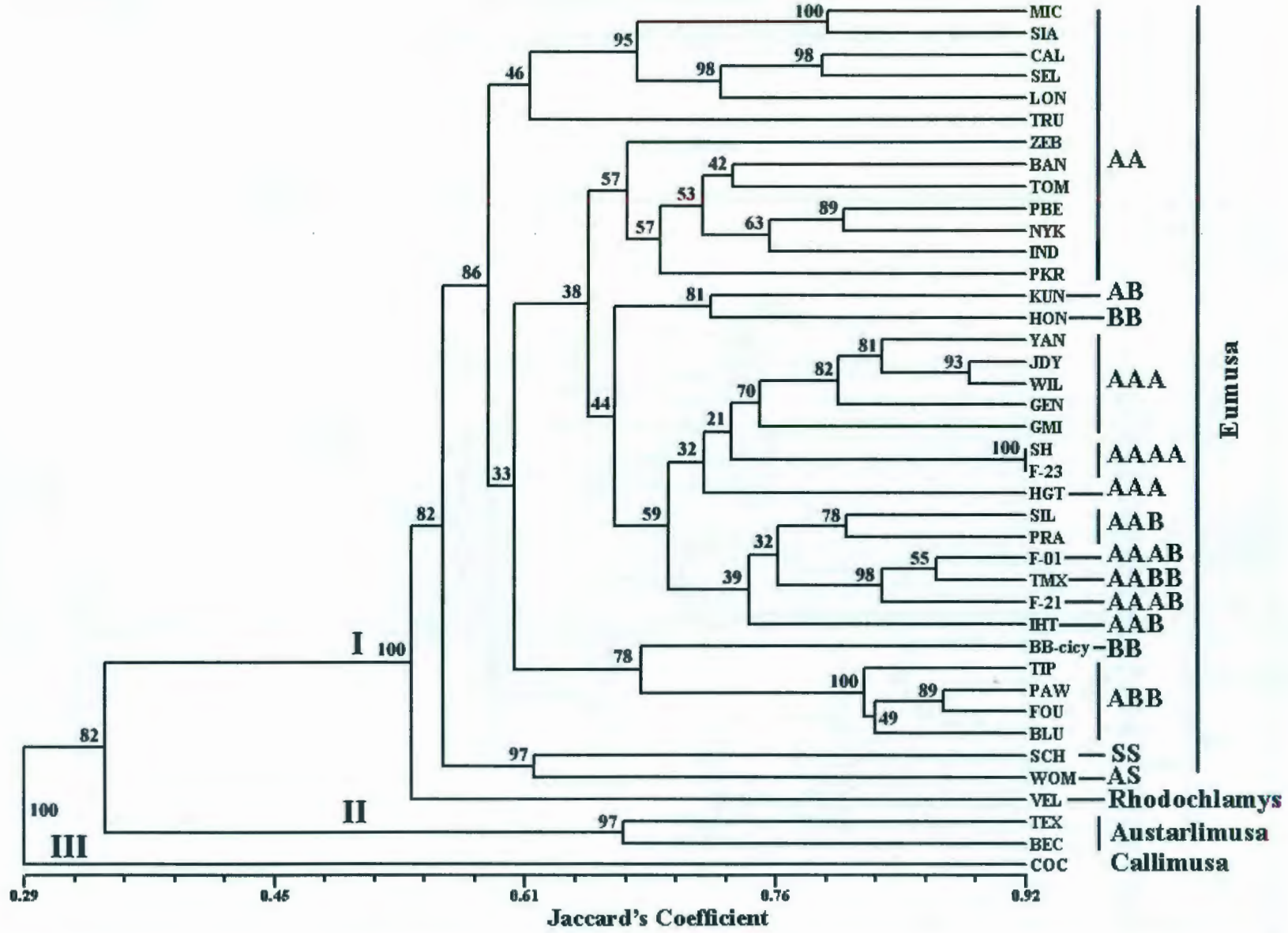

Fig. 2.2: Dendrogram of genetic similarities in *Musa* using AFLP-UPGMA cluster analysis. *Arabic numbers* indicate bootstrapping, whereas *Latin numbers* show *Musa* sectional relationships

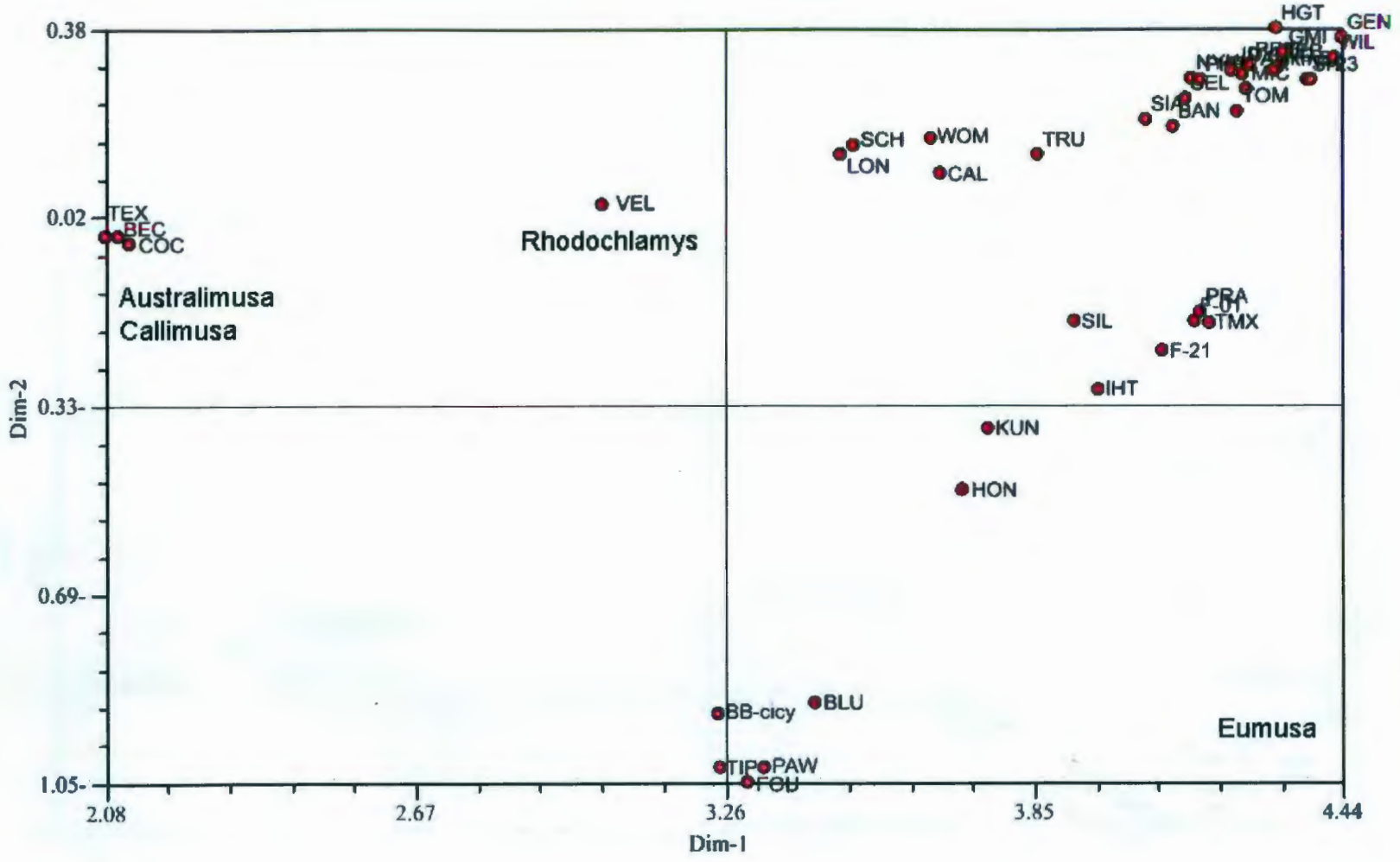

Fig. 2.3: Scatter plot showing the relationships among 40 accessions of *Musa* based on principal coordinate analysis using SRAP. *The vertical line* separates Eumusa from the other sections, and *the horizontal line* separates the Eumusa species

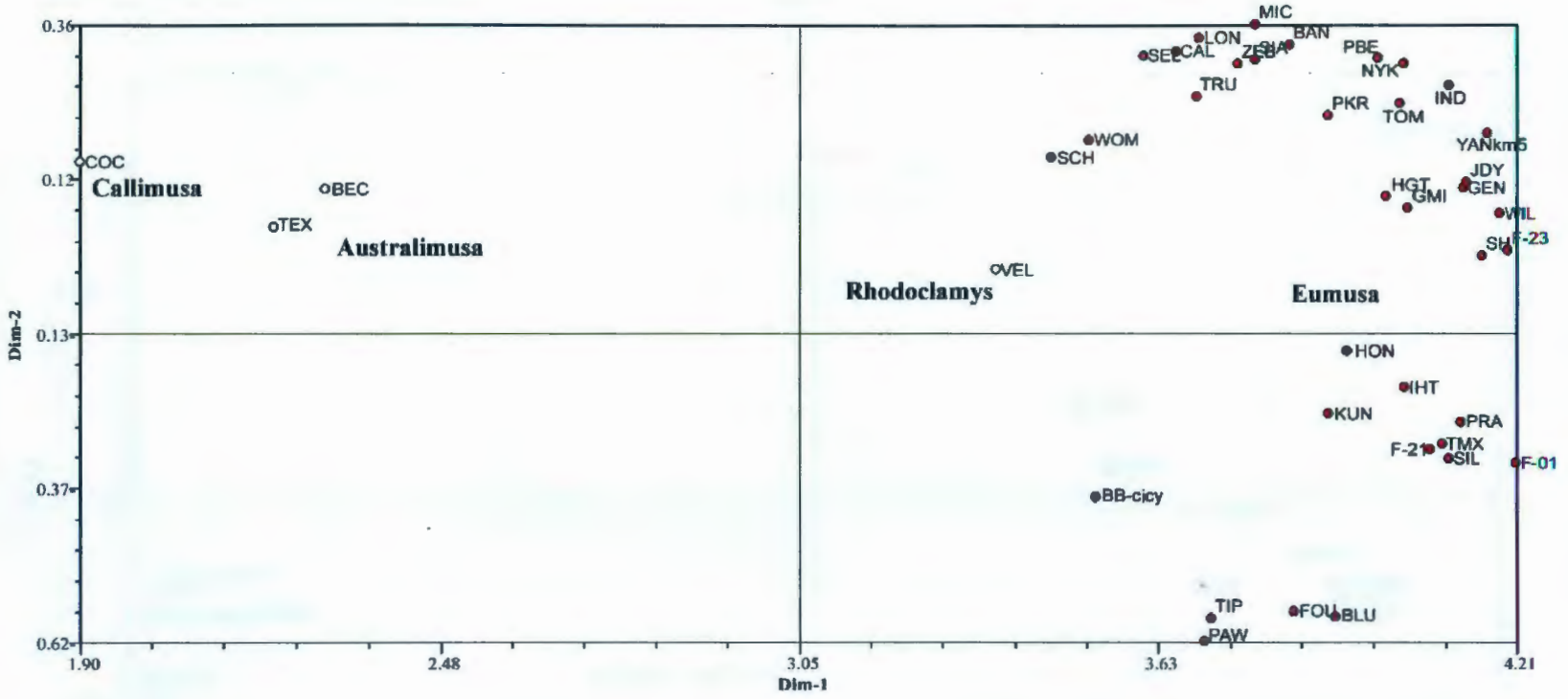

Fig. 2.4: Scatter plot showing the relationships among 40 accessions of *Musa* based on principal coordinate analysis using AFLP. *The vertical line* separates the *Musa* sections, and *the horizontal line* separates the Eumusa species

Table 2.4: Unique SRAP markers and total number of markers characterizing *Musa* genotypes, represented by the number of specific bands per category

Category		Primer combinations[a]										Total
		1	2	3	4	5	6	7	8	9	10	
Eumusa		1	-	-	-	-	-	2	1	-	1	5
Rhodochlamys (*M. velutina*)		3	-	4	3	1	1	5	3	-	2	22
Callimusa (*M. coccinea*)		4	-	1	-	1	6	4	3	2	-	21
Australimusa		8	1	2	4	2	1	1	3	8	2	32
Eumusa – Rhodochlamys		1	-	-	-	-	-	1	-	-	2	4
Callimusa – Australimusa		1	1	-	-	-	1	1	-	-	3	7
M. beccarii		2	1	1	1	-	-	2	2	1	2	12
M. textiles		1	1	3	1	-	1	1	3	5	3	19
M. coccinea – M. beccarii		-	-	-	-	-	2	-	-	-	1	3
B Genome		2	1	1	1	-	-	-	-	-	2	7
S Genome		-	-	-	-	-	1	-	-	-	-	1
M. acuminata ssp.	*microcarpa*	-	-	-	-	-	-	-	1	-	-	1
	malaccensis	-	-	-	-	1	1	-	-	-	-	2
	zebrina	-	-	-	-	-	-	-	1	-	-	1
	burmanica	-	-	-	-	-	-	1	1	1	-	3
	banksii	-	-	-	-	-	-	-	1	-	-	1
	truncata	-	-	-	-	-	-	-	-	1	-	1
	burmanica-burmanicoides	-	-	1	2	-	1	-	-	1	1	6
Total		23	5	13	12	5	15	18	19	19	19	148

[a] Numbers of primer combinations related to Table 2.2.

Table 2.5: Unique AFLP markers and total number of markers characterizing *Musa* genotypes, represented by the number of specific bands per category

Category	Primer combinations[a]															Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Eumusa - Rhodochlamys	3	3	6	-	-	-	-	3	1	2	1	3	3	1	-	26
Callimusa - Australimusa	-	2	1	-	-	-	1	1	-	1	1	1	1	1	-	10
Australimusa	1	-	3	-	-	-	-	-	1	2	1	5	4	-	1	18
M. coccinea – M. beccarii	-	-	-	-	-	-	1	-	1	1	2	-	-	1	-	6
M. coccinea	1	3	-	-	4	1	1	-	3	4	1	2	3	2	7	32
M. beccarii	-	-	-	-	1	1	-	1	5	2	-	-	-	-	-	10
M. textilis	-	-	-	-	1	-	1	-	2	1	-	-	1	-	-	6
B Genome	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3	3
Total	5	8	10	0	6	2	4	5	13	13	6	11	12	5	11	111

[a] Numbers of primer combinations related to table 2.2.

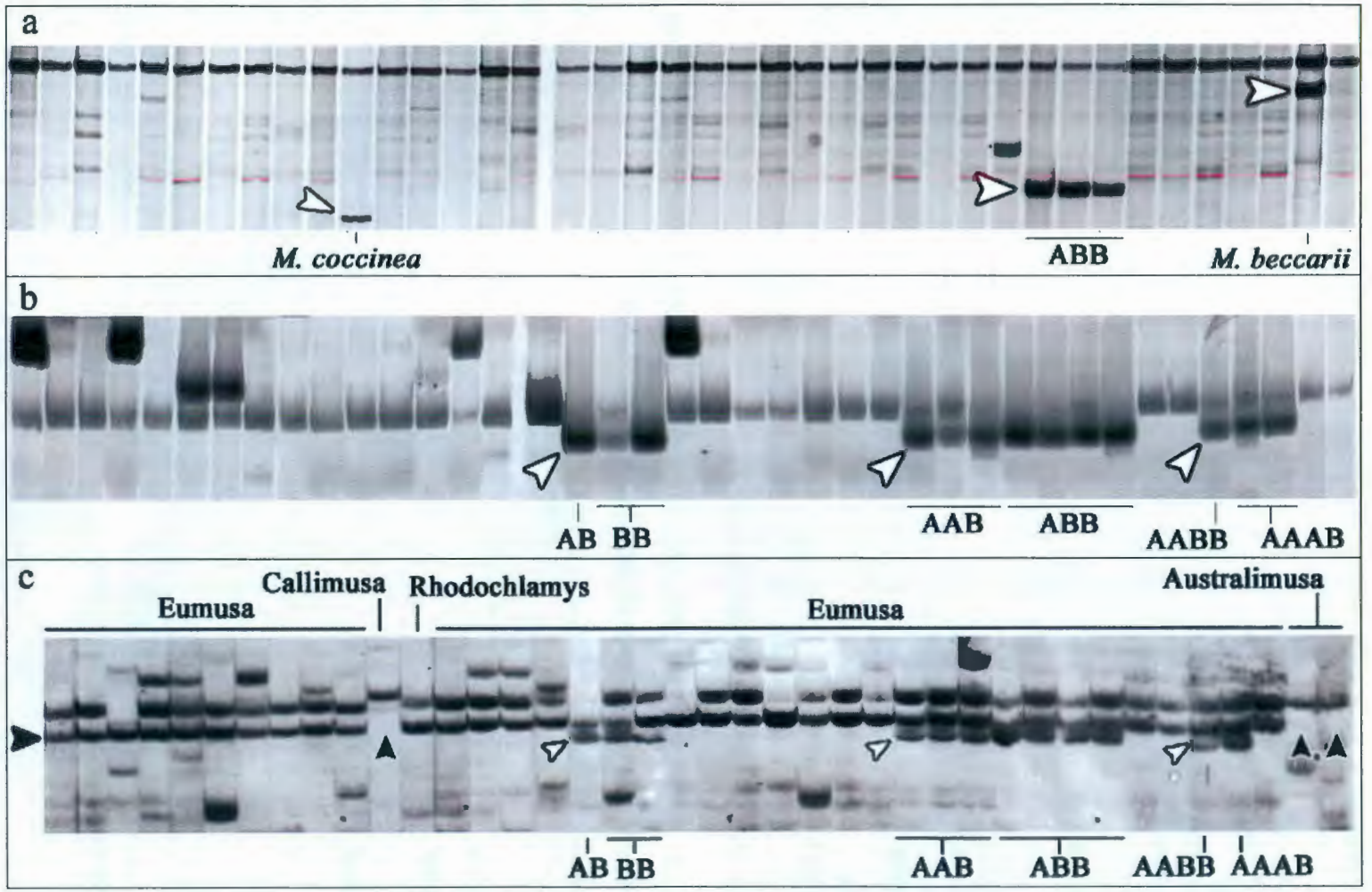

Fig. 2.5: Unique bands, specific for certain genotypes and sections generated by SRAP and AFLP markers. **a**: SRAP-specific bands for *M. coccinea*; ABB (cooking bananas); *M. beccarii*, **b**: a SRAP-unique band specific for B genome **c**: AFLP-specific bands for Eumusa and Rhodochlamys (*horizontal black arrow head*) and absent from Callimusa and Australimusa (*vertical black arrow heads*); and B genome unique band (*white arrow heads*)

2.3.2. Relationships within Eumusa

The UPGMA dendrogram based on the SRAP data, showed a precise separation of Eumusa accessions according to their genome composition (Fig. 2.1). The *M. acuminata* subspecies and cultivars clustered with dessert banana (AAA), tetraploid banana cultivars (AAAA), plantains (AAB), and tetraploid hybrids (AABB, AAAB), whereas the *M. balbisiana* genotypes (BB) were well separated and clustered with cooking banana (ABB) and 'Kunnan' (AB) hybrid. The two accessions of '*M. schizocarpa*' (SS) and 'Wompa' (AS) clustered together within Eumusa. One unique band generated by the Me-1/Em-7 primer combination was specific to these accessions and does not occur in other genotypes, which suggests that it could be specific for the S genome.

The amplified fragment polymorphism cluster analysis within section Eumusa was slightly different from SRAP cluster analysis (Fig. 2.2). The dendrogram splits into four clusters; the first cluster included six subspecies of *M. acuminata*. The second cluster consisted of two subspecies of *M. acuminata* and diploid cultivars. In addition, this subgroup included a diploid hybrid of *M. acuminata* x *M. balbisiana* (Kunnan) which clustered with *M. balbisiana* (Honduras). Dessert banana cultivars (AAA), plantains (AAB), tetraploid banana cultivars (AAAA) and tetraploid hybrids (AAAB and AABB) were included in the second cluster. The third cluster comprised *M. balbisiana* (BB-CICY), clustered with the cooking bananas (ABB). The two accessions of '*M. schizocarpa*' (SS) and 'Wompa' (AS) formed the fourth cluster.

The PCO analysis based on SRAP divided Eumusa accessions into three groups (Fig. 2.3). The first group included *M. acuminata* subspecies and cultivars with '*M. schizocarpa*' accessions (SS, AS). The second group consisted of plantains, tetraploid hybrids (AABB, AAAB), the hybrid 'Kunnan' (AB) and *M. balbisiana* type 'Honduras'. The third group was formed by *M. balbisiana* type 'BB-CICY' and cooking bananas. The PCO analysis of Eumusa accessions based on AFLP (Fig. 2.4) was similar to SRAP-PCO. However, AFLP-PCO placed plantains and tetraploid hybrids (AABB, AAAB) near to *M. balbisiana* more than to *M. acuminata*.

The sequence-related amplified polymorphism and AFLP results showed, respectively, 61.76 and 73.52% polymorphism within *M. acuminata*; they also showed

32.34, 36.90% and 20.91, 40.78% polymorphism within *M. balbisiana* and *M. schizocarpa* accessions, respectively (Table 2.3). The lowest genetic variability in Eumusa using both SRAP and AFLP (2.01 and 8.36%, respectively) was between tetraploid banana cultivars (AAAA) 'SH 3436-6' and 'FHIA-23', which clustered with triploid cultivars, whereas the tetraploid hybrids (AAAB) 'FHIA-01' and 'FHIA-21' showed 8.72 and 14.69% polymorphism using SRAP and AFLP, respectively, and clustered with the hybrid cultivar 'TMX 5295-1' (AABB) of the plantain group (Figs. 2.1 and 2.2, Table 2.3).

2.3.3. Relationships amongst *M. acuminata* accessions

Musa acuminata has been divided into eight subspecies: *banksii, burmannica, burmannicoides, malaccensis, microcarpa, truncata, siamea, zebrina*[2]. Our results showed that the clustering of the eight subspecies depended on the molecular marker system. SRAP grouped *M. acuminata* subspecies into the following clusters: (1) *microcarpa-zebrina*, (2) *malaccensis*, (3) *siamea*, (4) *banksii*, (5) *burmannica-burmannicoides* and (6) *truncata* (Fig. 2.6). AFLP clustering defined the following groups: (1) *microcarpa-siamea*, (2) *burmannica-burmannicoides-malaccensis*, (3) *truncata*, (4) *zebrina* and (5) *banksii*. Additionally, the two accessions of ssp. *malaccensis* (type 'Selangor' and type 'Pisang Kra') were clustered together in the SRAP dendrogram, whereas 'Selangor' clustered with ssp. *burmannica* and *burmannicoides*; 'Pisang Kra' clustered with ssp. *banksii* and the diploid cultivars (Fig. 2.6) in the AFLP dendrogram.

The sequence-related amplified polymorphism profiles were more informative than AFLP profiles and generated certain unique bands specific for *M. acuminata* subspecies (Table 2.5). Six unique bands were shared by 'Long Tavoy' (ssp. *Burmannica*) and 'Calcutta-4' ssp. (*burmannicoides*). The SRAP cluster analysis grouped 'Pisang Berlin' and 'IDN-110' with subspecies *microcarpa* and *zebrina*, which may indicate their relationships with these subspecies. 'Tomolo' and 'Niyarma Yik' clustered also with their related subspecies *banksii* (Fig. 2.6). All the diploid cultivars clustered with subspecies *banksii* and *malaccensis* type 'Pisang Kra' (Fig. 2.6) when using AFLP data.

2.3.4. Relationships within triploid dessert banana cultivars

Six cultivars of three different dessert banana subgroups were used in this study. The percentage of polymorphism within these cultivars was 27.15 and 46.40%, generated with SRAP and AFLP, respectively (Table 2.4). All dessert banana cultivars clustered near *M. acuminata* based on SRAP and AFLP data. However, SRAP results showed the dessert banana cultivars clustered near ssp. *banksii* and together according to their subgroups (Fig. 2.1). These were 'Yangambi-km5' and 'JD-Yangambi' (subgroup "Ibota"), 'High Gate' and 'Gros Michel' (subgroup "Gros Michel"), and 'Williams' and 'Gran Naine' (subgroup "Cavendish"). The grouping with AFLP data was not according to the known subgroups, i.e., JD-Yangambi' and 'Williams' from different subgroups clustered together followed by 'Yangambi-km5', whereas the other cultivars formed single branches (Fig. 2.2).

All the dessert banana cultivars were well separated from the cluster of plantains (AAB) after PCO analyses of SRAP data (Fig. 2.1, Fig. 2.3). The AFLP dendrogram showed that the dessert banana cultivars clustered together with the sub-cluster of plantains (AAB) (Fig. 2.2).

2.3.5. Relationships within *M. balbisiana*

The cluster analysis based on SRAP data (Fig. 2.1) showed that both *M. balbisiana* accessions (Honduras and BB-CICY) grouped together with cooking banana (ABB) with a P% of 48.74, whereas they were separated from plantains (AAB) with 71.62% polymorphism (Table 2.3). However, scatter plot of PCO separated 'Honduras' from 'BB-CICY' and grouped it near to plantains (Fig. 2.3). AFLP data showed that both BB genotypes clustered together when the data of new primer combinations (Table 2.2) were used. Data from the 15 primers (classic and new sets, Table 2.2) showed that 'Honduras' clustered with 'Kunnan' (AB hybrids) and plantains and tetraploid hybrids. In contrast BB-CICY clustered with the cooking bananas (ABB) (Fig. 2.2). AFLP markers showed 53.49 and 52.32% polymorphism between *M. balbisiana* genotypes and both of plantains and cooking bananas, respectively (Table 2.3).

The sequence-related amplified polymorphism and AFLP profiles generated seven and three unique bands, respectively, which are specific to the B genome that existed in all

genotypes with the genome B (Tables 2.4 and 2.5). The polymorphism between the two BB genotypes was low (32.34, 36.90% using SRAP and AFLP, respectively) (Table 2.3).

2.3.6. Relationships between plantains and cooking bananas

Plantains (AAB) and cooking bananas (ABB) cultivars were completely separated in the cluster and PCO analyses using both SRAP and AFLP data, which corresponded with the composition of their genomes (Figs. 2.1, 2.2, 2.3 and 2.4). The AAB cultivars 'Silk', 'Prata' and 'Ihitisim' clustered with the tetraploid hybrids (AABB and AAAB) and were near to *M. acuminata*. The cooking bananas 'Tiparot', 'Pisang Awak', 'Fougamou' and 'Bluggoe' clustered together with *M. balbisiana*. The P% generated from our SRAP and AFLP data was 33.53; 32.54 and 28.18; 30.77 within plantains and cooking banana respectively', whereas it was 57.49; 53.60 between them (Table 2.3).

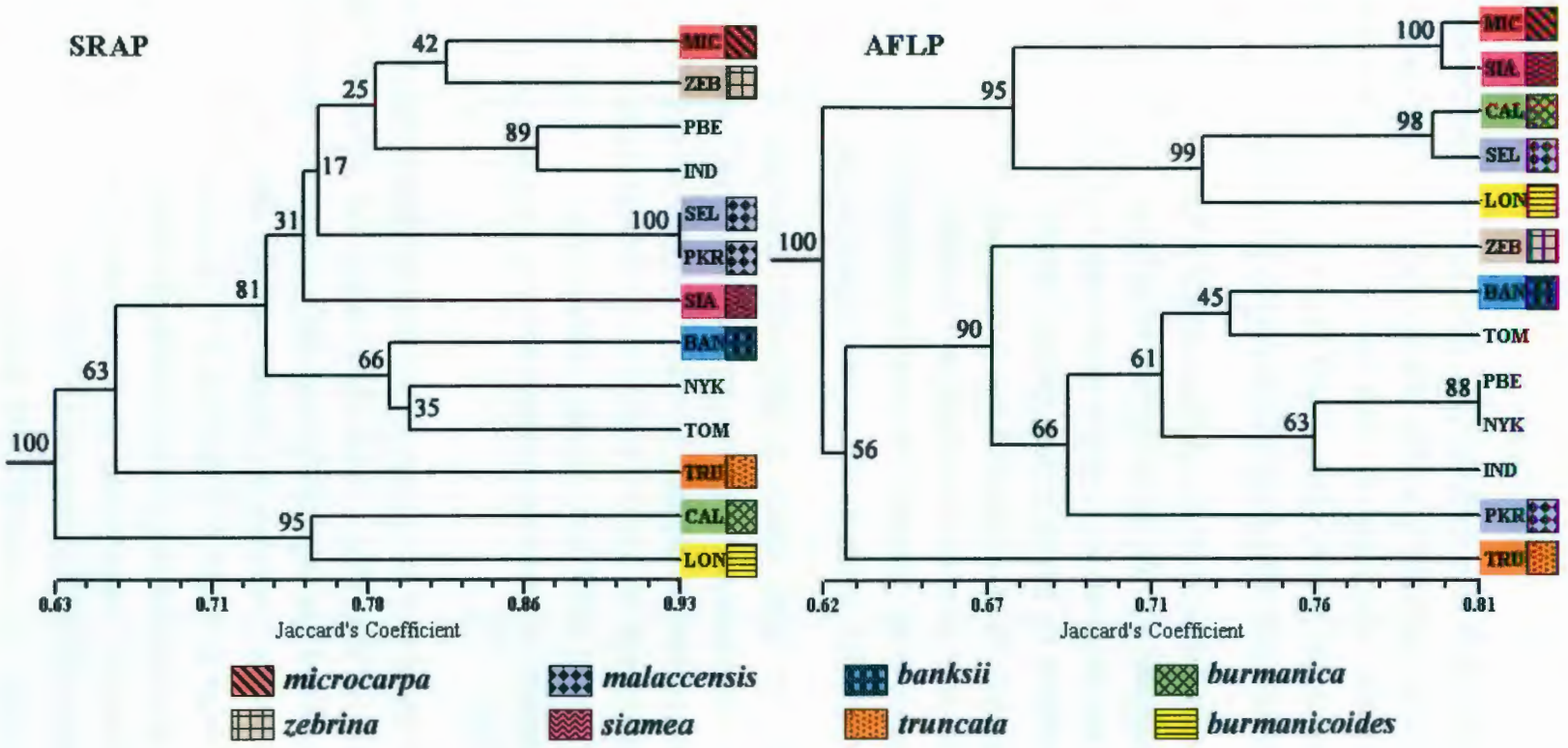

Fig. 2.6: Dendrogram of genetic similarities and relationships among *M. acuminata* subspecies using SRAP and AFLP UPGMA cluster analysis. *Numbers* indicate bootstrapping.

2.4. Discussion

SRAP and AFLP molecular markers were used to detect the genetic diversity in *Musa*. Both UPGMA cluster analysis and PCO analysis based on SRAP and AFLP clearly separated the accessions according to their sections and species. However, the percentage of polymorphism among sections and species, as well as the relationships within the Eumusa species and subspecies, varied between the two marker systems.

The relationships between *Musa* sections ensuing from both SRAP and AFLP analysis in our study agreed with the AFLP analysis of Wong et al.[22]. They suggested that Australimusa should be combined into a single section with Callimusa, and both Eumusa and Rhodochlamys should be joined in one section. Bartoš et al.[28] also showed that species from Eumusa and Rhodochlamys formed a subgroup in a phylogenetic analysis based on genome size, number of chromosomes, and number of 45S rDNA loci. However, both Loh et al.[3] and Wong et al.[4] reported that *M. ornata* (Rhodochlamys) formed a distinct cluster separated from the Eumusa cultivated bananas based on their AFLP analysis.

Musa beccarii has been placed in the section Callimusa following numerical taxonomic analysis of wild bananas by Simmonds and Weatherup[29], despite the fact that its basic chromosome number is different ($x = 9$)[28,30]. Based on our SRAP and AFLP analyses, we recommend that *M. beccarii* should be considered as Australimusa. In this regard, Wong et al.[22] using AFLP analysis and Risterucci et al.[12] using DArT analysis found *M. beccarii* closed to Australimusa species and they considered it as a species of the Australimusa section.

The relationships among *M. acuminata* subspecies varied between the two DNA marker systems used in this study, which may reflect the difference between the techniques. *Musa acuminata* has been divided into eight subspecies: *banksii, burmannica, burmannicoides, malaccensis, microcarpa, truncata, siamea, and zebrina*[2]. However, our SRAP results were close to those of Careel et al.[31] who reported that *M. acuminata* accessions could be grouped into four groups or poles of diversity: (1) *banksii-errans,* (2) *malaccensis,* (3) *microcarpa- zebrina* and (4) *burmannica-burmannicoides-siamea*. Our SRAP and AFLP results agreed with Ude et al.[24] who found that 'Long Tavoy' clustered with 'Calcutta-4'. Moreover, Carreel et al.[31] and Shepherd[30] reported a close relationship

between 'Long Tavoy' and 'Calcutta-4', recognizing that both accessions belong to the subspecies *burmannica*.

Both SRAP and AFLP results in this study showed that *M. acuminata* ssp. *malaccensis* and ssp. *microcarpa* clustered together but were well separated from ssp. *truncata*, which is in accordance with AFLP analysis by Wong et al.[4]. However, our results did not match those of Simmonds[32], who placed ssp. *truncata* as a synonym of ssp. *microcarpa* or Hotta[33], who placed ssp. *truncata* as a synonym of ssp. *malaccensis*. Furthermore, Gawel and Jarret[34] obtained their results using cpDNA RFLP on *M. acuminata* ssp. *truncata, microcarpa* and *malaccensis.* Their results showed that ssp. *truncata* collected from two different sources clustered separately; the clone form FHIA clustered with ssp. *malaccensis*, whereas the clone from the Catholic University of Leuven clustered with spp. *microcarpa*.

The sequence-related amplified polymorphism and AFLP successfully separated dessert bananas (AAA) into a single cluster. However, SRAP was more effective at grouping the cultivars according to their subgroup. AFLP analysis by Ude et al.[23,24] showed that 'Yangambi-km5' was close to 'Gros Michel' even though they are from different subgroups. El-khishin et al.[6] used AFLP and found that dessert banana cultivar 'Williams' clustered with 'Muhammad Ali', a plantain (AAB) cultivar, which correspond with our AFLP results but not with our SRAP results.

Our SRAP and AFLP results showed a close relationship between the two accessions of *M. balbisiana*. However, PCO analysis of both DNA marker systems placed 'Honduras' (BB) close to plantains (AAB), whereas BB-CICY was placed with cooking bananas (ABB), which may indicate the origin of these groups. More BB accessions should be included in further research to clarify this issue.

Similar to our results, Phothipan et al.[18] found that plantains clustered with *M. acuminata* and separated from *M. balbisiana* using RAPD and SRAP markers. However, AFLP results of Ude et al.[24] showed that cooking bananas (ABB) and plantains (AAB) formed two subgroups that shared a main cluster with *M. balbisiana* and was fully separated from *M. acuminata*.

In conclusion, this study showed that SRAP and AFLP markers are a powerful tool for evaluating genetic polymorphisms and relationships in *Musa*. They also serve for discriminating amongst *Musa* sections and within Eumusa species, as well as between plantains and cooking bananas. The unique bands regenerated by SRAP (since it based on ORF amplification), could be helpful for further use in *Musa* genetic enhancement.

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Chapter III

Title: Influence of the genotype and age of the explant source on the capacity for somatic embryogenesis of two Cavendish banana cultivars (*Musa acuminata* Colla, AAA)

Main Objective:

Induction of somatic embryogenesis (SE) from immature male floral tissues using two developmental stages of explant

Particular objectives:

1- Collection of two developmental ages of male bud from two commercial cultivars.
2- Induction of embryogenic callus.
3- Studying the effect of explant developmental age and genotype on SE induction.
4- Establishment of embryogenic cell suspension.
5- Studying the effect of two growth regulators on the percentage of germination of somatic embryos.

Summary:

In this chapter, two developmaenal ages of banana male bud, as a source of the explant, were used of two commercial banana cultivars to study their effect on the induction of somatic embryogenesis. Embryogenic cell suspension, maturation and germination of somatic embryos as well as plant conversion were accomplished. Experimental data were statisticaly analyzed and results were discussed.

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Chapter III

Influence of the genotype and age of the explant source on the capacity for somatic embryogenesis of two Cavendish banana cultivars (*Musa acuminata* Colla, AAA)*

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Abstract

The embryogenic capacities of flower explants from one- and two-week-old male inflorescence buds from *Musa acuminata* Cavendish, AAA, genotypes 'Williams' and 'Grand Naine' were investigated. Explants of hands with immature flowers were excised and induced for embryogenesis. Highly significant differences were found in the embryogenic response, depending on the genotype and the developmental stage of the buds from which the explants were excised. After four months of induction, the total percentage of callus formed ranged from 97.81% in explants of two–week-old 'Williams' buds to 52.11% in explants of two–week-old 'Grand Naine' buds. Embryogenic callus formation was, on average, higher in the two-week-old 'Williams' explants (10.01%) than in the one–week-old explants (0.78%). The opposite was true for 'Grand Naine', with 7.51% embryogenic callus produced in explants of one-week-old buds compared to 2.49% in two-week-old buds. Selected embryogenic calluses that were successfully established on proliferation medium led to embryogenic cell suspensions with good regeneration capacities. Fifteen to thirty-five percent of the embryos germinated, demonstrated high plant-conversion capacity (99%). The effect of the interaction between the developmental age of explant and the genotype on the embryogenesis response is discussed.

Keywords: *Musa*, embryogenic callus, genotype, male bud age, Cavendish banana.

Abbreviations: MS, Murashige and Skoog; IAA, indole-3-acetic acid; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; NAA, 1-naphthaleneacetic acid; SE, somatic embryogenesis; IMFs, immature male flowers; SCV, settled cell volume; ECS, embryogenic cell suspension; MM, maturation medium; GM, germination medium, ABA, abscisic acid.

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3.1. Introduction

Bananas and plantains (*Musa* spp.) are two of the world's major fruit crops. They are the staple food for millions of people in the tropics[1]. Cavendish bananas dominate the world market for dessert bananas. Classical breeding is difficult in edible *Musa* spp. due to differing levels of ploidy and sterility. Consequently, somatic embryogenesis is a valuable tool for use with *Musa* cultivars, for improvement by mass propagation of selected genotypes, mutagenesis, genetic transformation, protoplast fusion or other biotechnological applications[2]. In bananas and plantains, different types of explants have been used to induce embryogenic callus and regenerate plants. These include zygotic embryos[3-6], rhizome and leaf sheaths[7], proliferating meristems and scalps[8,9] and immature male[6,10-13] and female[14] flowers. Of these explants, immature male flowers appear to be the most responsive starting material for initiating embryogenic cultures of Cavendish banana[6,11,15-17] and for mass propagation[18,19]; however, recent findings show that the efficiency outcome of embryogenesis using the scalp method[20] and technologies that use immature male flowers are comparable.

Several endogenous and exogenous factors exert influences on the growth, development and performance of plant cells in culture[21]. Among these, the genotype, the developmental status of the "mother plant" and the type and developmental status (or age) of the plant organ from which the primary explants are obtained are of critical importance in eliciting the desired response to somatic embryogenesis[21-23]. The effect of the genotype and explant age on somatic embryogenesis has been investigated in several plant species, e.g., in coffee[24,25], triticale[26], *Brassica napus* L.[27], *Solanum trilobatum* L.[28] and *Anacardium occidentale* L.[29]. These studies demonstrate that these factors are of enormous significance for the outcome of the inducement of somatic embryogenesis. In banana, some data suggest that the parameters mentioned above are equally important for the development of somatic embryogenesis[15,30].

To gain insights into the mechanisms of somatic embryogenesis in *Musa*, the present study assesses the effect of the genotype and the developmental age of the male inflorescence bud, the source of the explant, and the interaction between these two factors on the somatic embryogenesis capacities of two *Musa acuminata*, AAA, Cavendish banana cultivars, namely, 'Williams' and "Grand Naine".

3.2. Materials and methods

3.2.1. Plant materials and explant preparation

To evaluate the influence of the developmental stage of the male inflorescence bud on somatic embryogenesis (SE), the buds were classified as age one or two week(s), according to their growth stage, defined as follows: After the bract subtending the most basal node or the first cluster of female flowers (fingers) opens, the flowers are at anthesis on day 1 to day 7; when the 7th cluster is completely exposed, the bud age is classified as one week. After the 8th bract has opened and has passed anthesis over the next 14-15 days and after the successive flowers have emerged, the bud age is classified as two weeks.

'Grand Naine' and 'Williams' (AAA) male inflorescence buds of one and two weeks of age were collected from field-grown plants in a commercial plantation at Teapa, Tabasco, Mexico and used for *in vitro* culture within two days of collection. The collected male buds were washed in tap water and shortened to 6-8 cm in length by successively removing the bracts and the hands of the male flowers subtended by them. The buds were disinfected with 70% (v/v) ethanol for five minutes, rinsed three times with sterile double-distilled water and reduced in size further to tips that were 3.0 cm long x 1.0 cm wide, under a laminar flow hood. To prevent dehydration of the tissues, the reduced tips were kept in a jar with 500 µL of sterile double-distilled water during further processing.

3.2.2. Induction of somatic embryogenesis

The explants, that is, the hands of immature male flowers (IMFs), were isolated aseptically from the reduced buds by removing the bracts under a binocular stereomicroscope. The immature flower hands that were excised from positions 16 to 8 (1 being the hand closest to the meristematic dome of the male bud[2]) of each bud were inoculated and cultured in jars containing semi-solid callus induction medium (M1). The medium[15] is composed of MS[31] salts and vitamins supplemented with 5.71 µM IAA, 18 µM 2,4-D, 5.4 µM NAA, 4.1 µM biotin, 87 mM sucrose, and 2 g/L Gelrite. The medium was adjusted to pH 5.7 before autoclaving at 120°C for 20 min. The cut surface of the explant hand base, with its two rows of IMFs, was placed in contact with 25 mL of M1 medium in 100 mL containers (baby food jars), and the jars were sealed with plastic film (Kleen-

Pack). The cultures were kept in a growth room at 27 ± 2°C under total darkness and maintained without subculture for four months. The cultures were examined twice a week to record any visible changes in the appearance of the explants in culture. Following four months on M1, the percentages of embryogenic callus, yellow nodular callus, white compact callus and translucent callus formed were recorded after Strosse et al.[2].

3.2.3. Embryogenic callus proliferation

After four months of culture, selected embryogenic calluses with expression of somatic embryos were placed on a modified proliferation medium based on Navarro et al.[6], consisting of 1/2 strength MS salts supplemented with vitamins of Morel[32], 4.5 µM 2,4-D, 1.47 mM KH_2PO_4, 174 mM sucrose, and 2 g/L Gelrite. The medium was adjusted to pH 5.8. The culture containers were sealed and maintained under induction conditions, and the medium was refreshed each month.

3.2.4. Initiation and maintenance of the embryogenic cell suspension

For initiation of embryogenic suspension cultures, approximately 0.75g fresh weight of embryogenic calluses with expression of early stage transparent embryos were transferred to 25 mL of proliferation medium (without the gelling agent and with full-strength MS salts and 87 mM sucrose) into 125 mL Erlenmeyer flasks. To allow for air exchange, the flasks were sealed with rubber stoppers with pre-drilled holes that were plugged with cotton. The cultures were agitated on an orbital shaker at 85 rpm in a growth room that was maintained at 27±2°C in total darkness. The suspensions were filtered through a 380 µm pore size metallic mesh after the second week of culture, separating the primary calluses from the fine filtrate. Once the filtrate was recovered into a sterile 50 ml Falcon tube, after allowing the cells to sediment for 15 minutes, the settled cell volume (SCV) was measured. Two thirds of the liquid medium was refreshed, thereby maintaining ~ 3% SCV, as suggested by Strosse et al.[20]. The medium was changed every week during the first two months of establishment of the embryogenic suspension. Thereafter, the medium was changed every two weeks.

3.2.5. Embryo germination and plant regeneration

The somatic embryos were germinated after Navarro et al.[6]. In addition, the regeneration capacities of two embryogenic suspension lines were tested by plating 0.2 ml of each embryogenic cell suspension (ECS) onto semi-solid maturation medium (MM) (25 ml in baby food jars), consisting of MS salts and vitamins supplemented with 0.43 µM kinetin, 0.23 µM zeatin, 87 mM sucrose, and 2 g/L Gelrite. Five replicates were plated for each line, and these were maintained for three to four weeks in darkness. The number of somatic embryos recovered per plated suspension was recorded by counting the embryos from each container before they were transferred to the germination medium. Next, the embryos were transferred to medium containing MS salts and vitamins supplemented with 87 mM sucrose, 2 g/L Gelrite and different combinations of 6-benzylaminopurine (BAP) and IAA, for one month. The combinations that we tested were GM1: 11.4 µM IAA and 2.22 µM BAP, GM2: 1.14 µM IAA and 0.22 µM BAP, GM3: 0.22 µM BAP and GM4: free growth regulator. The plantlets were rooted on medium that was composed of 1/2 strength MS supplemented with 87 mM sucrose, and 2 g/L Gelrite (pH 5.8) for one month. The germination and rooting cultures were incubated at 27 ± 2°C under total darkness for three days, afterwards using a 16-hours (60 $\mu E.m^{-2}.s^{-1}$) photoperiod.

3.2.6. Experimental design and statistical analysis

To induce calluses, two independent experiments were used, each with three replicates. Each replicate consisted of 20 jars, and each jar contained 9 explants (IMF hands from positions 16-8). We carried out the germination experiment using somatic embryos from the 'Grand Naine' cultivar derived from two independent embryogenic cell suspension lines, using four replicates per treatment. Each replicate used ten somatic embryos per container. The experimental design for both experiments used a randomised complete block (RCBD). Analysis of variance (ANOVA) was carried out using the MSTAT-C statistical program[33]. The treatment means were compared using Duncan's multiple-range test.

3.2.7. Scanning electron and light microscopy

For scanning electron microscopy, the tissues, IMFs hands and embryogenic callus with well-developed embryoids were fixed overnight in FAA fixative solution (formalin:

acetic acid: alcohol) at 4 ± 1°C. After this, the FAA fixative was exchanged twice with fresh FAA, using additional 4-h incubations at 4 ± 1°C. Then, the embryoids were dehydrated by washing in an ethanol/water series that progressed to absolute ethanol. The samples were dried using the critical-point method and coated with a thin layer of gold. The samples were observed and photographed using a Jeol JSM-6460 LC scanning electron microscope operating at 18 kV. Light microscopy micrographs were taken using a Carl Zeiss ICM-405 inverted microscope.

3.3. Results

3.3.1. Effect of the explant source age on the response to somatic embryogenesis

Explants from IMF-hands (Fig. 3.1a), of the *Musa acuminata* AAA cultivars 'Williams' and 'Grand Naine', formed calluses on M1 medium after four months. This medium supported the growth and development of callus for the two cultivars and the two ages of buds. Four types of calluses were observed, that is, embryogenic callus, yellow nodular callus, white compact callus and translucent callus (Fig. 3.1b-e, respectively). The percentage of explants that initiated the formation of callus depended more on the developmental stage of the male bud from which the explants were excised than on the cultivar. This is shown by the percentage of the response estimated for each type of callus (Table 3.1). The total percentage of callus formed ranged from 97.81% in two-week-old explants of 'Williams' to 52.11% in two-week-old explants of 'Grand Naine'. The results showed that the age of explant source had the greatest effect on the percentage of embryogenic callus that was induced. That is, explants derived from two-week-old male buds of 'Williams' showed the highest percentage of embryogenic callus development (10.01 ± 0.19%), whereas explants derived from one-week-old buds of the same genotype yielded a significantly lower percentage of embryogenic callus development (0.78 ± 0.78%). The inverse relation was observed for 'Grand Naine'; explants from the one-week buds were associated with highest percentage of embryogenic callus growth (7.51 ± 0.19%), three times that of the two–week buds (2.49 ± 0.40%). Among the other callus types, the highest percentage was found in the yellow nodular callus that ranged from 51.39 to 42.08%, followed by translucent callus that ranges from 40.03 to 1.94% and white compact callus that ranged from 3.71 to 0.00% (Table 3.1). The analysis of variance showed highly significant differences ($P < 0.01$) between the genotypes for total callus and

translucent callus formation, and between the two ages of the explant source in the development of embryogenic callus ($P < 0.01$) and white compact callus ($P < 0.05$). Moreover, the interaction between the genotype and age of the explant source was significantly different ($P < 0.01$) for the embryogenic callus, translucent callus and total callus, and significant ($P < 0.05$) for the yellow nodular callus produced.

3.3.2. Embryogenic callus proliferation

Cultures of 'Williams' and 'Grand Naine' were examined twice a week for further development; after four months on induction medium, the explants (Fig. 3.1a) formed embryogenic and yellow nodular calluses that predominated in the cultures (Fig. 3.1b-c). On transfer of the selected embryogenic callus onto proliferation medium, somatic embryos were produced. Some individual embryos separated from the embryogenic callus, cultured on proliferation medium, showed enhanced formation of secondary embryogenic callus after one month. White, compact and translucent calluses (Fig. 3.1d, left panel and Fig. 3.1e, respectively) were non-embryogenic and were eliminated from the cultures before the transfer to the proliferation medium. These calluses were not used during initiation of embryogenic suspension cultures.

3.3.3. Embryogenic cell suspension and plant regeneration

Cell suspensions, initiated with calluses like those depicted in Figure 3.1b and Figure 3.1g, were generated after about 6-8 weeks of culture. Fine ECS were recognised with high proportion (≥75-80%) of cell aggregates and pro-embryos (Fig. 3.2a, b). The first sign of the formation of somatic embryos occurred approximately 3-4 weeks after the plating of ECS onto MM. This was revealed by an increase in size and development of the somatic embryos, and by the presence of the cotyledonary slit (Fig. 3.1f, marked "s" in the figure). On the average, the suspensions yielded 1.2×10^5 embryos per ml of SCV. The emergence of the plumule in the embryos on MM (Fig. 3.1f, marked "p" in the figure) was a clear early sign of the appropriate time for the transfer onto GM (Fig. 3.2c). On transfer of the embryos onto different GM media, the embryos germinated after about one month. The percentage of germination ranged from 15 to 35%, depending on the combination of the growth regulators used (Fig. 3.3). Thus, the average regeneration frequency for the 'Grand Naine' somatic embryos ranged from 1.67×10^4 to 3.9×10^4 plants per ml of the

settled cells. These values were calculated for the germinated embryos on medium that was free of growth regulators (GM4) and GM2 (0.22 µM BAP and 1.14 µM IAA), respectively. There were highly significant differences ($P < 0.01$) in the percentage of germination between the growth regulator combinations.

When the plant-growth regulators were omitted from the medium (GM4), the lowest percentage of embryo germination was observed (15%). This was followed by 17.5% for the GM3 medium (0.22 µM BAP). In contrast, GM1 medium (2.22 µM BAP and 11.4 µM IAA) showed an intermediate germination percentage (22.5%) and GM2 (0.22 µM BAP and 1.14 µM IAA) was the best (35%). Figure 3.2c illustrates the germination of somatic embryos on GM medium. In the figure, the more advanced embryo showed first the growth of a green shoot, then root development. After germination, the plantlets were transferred to rooting medium. Later rooted plants were transferred successfully (99%) to potting medium (peat moss). Figure 3.2d shows some of the regenerated *Musa* plants that were derived from somatic embryogenesis and ready for transfer to the field.

Table 3.1: The effect of the genotype and age of the male bud floral explant (in weeks) on callus formation after four months in culture on induction medium

Genotypes / age of MB	Percent of response as different callus types ± SE				Total callus
	Embryogenic	Yellow nodular	White compact	Translucent	
W* 1 week	0.78^{D}±0.78	51.39^{A}±1.54	3.71^{A}±0.71	21.83^{C}±3.28	77.71^{B}±0.73
W 2 weeks	10.01^{A}±0.19	46.67^{AB}±1.67	1.11^{AB}±1.11	40.03^{A}±0.78	97.81^{A}±1.10
GN* 1 week	7.51^{B}±0.19	42.08^{B}±2.65	1.56^{AB}±0.31	30.10^{B}±2.11	81.25^{B}±2.13
GN 2 weeks	2.49^{C}±0.40	47.68^{AB}±3.65	0.00^{B}±0.00	1.94^{D}±1.30	52.11^{C}±5.96

W* = 'Williams' cv., GN* = 'Grand Naine' cv.; the means within the columns that have the same letters are statistically similar (Duncan's multiple range test, $P < 0.05$). The values reported are the means ± standard error, SE ($n = 3$).

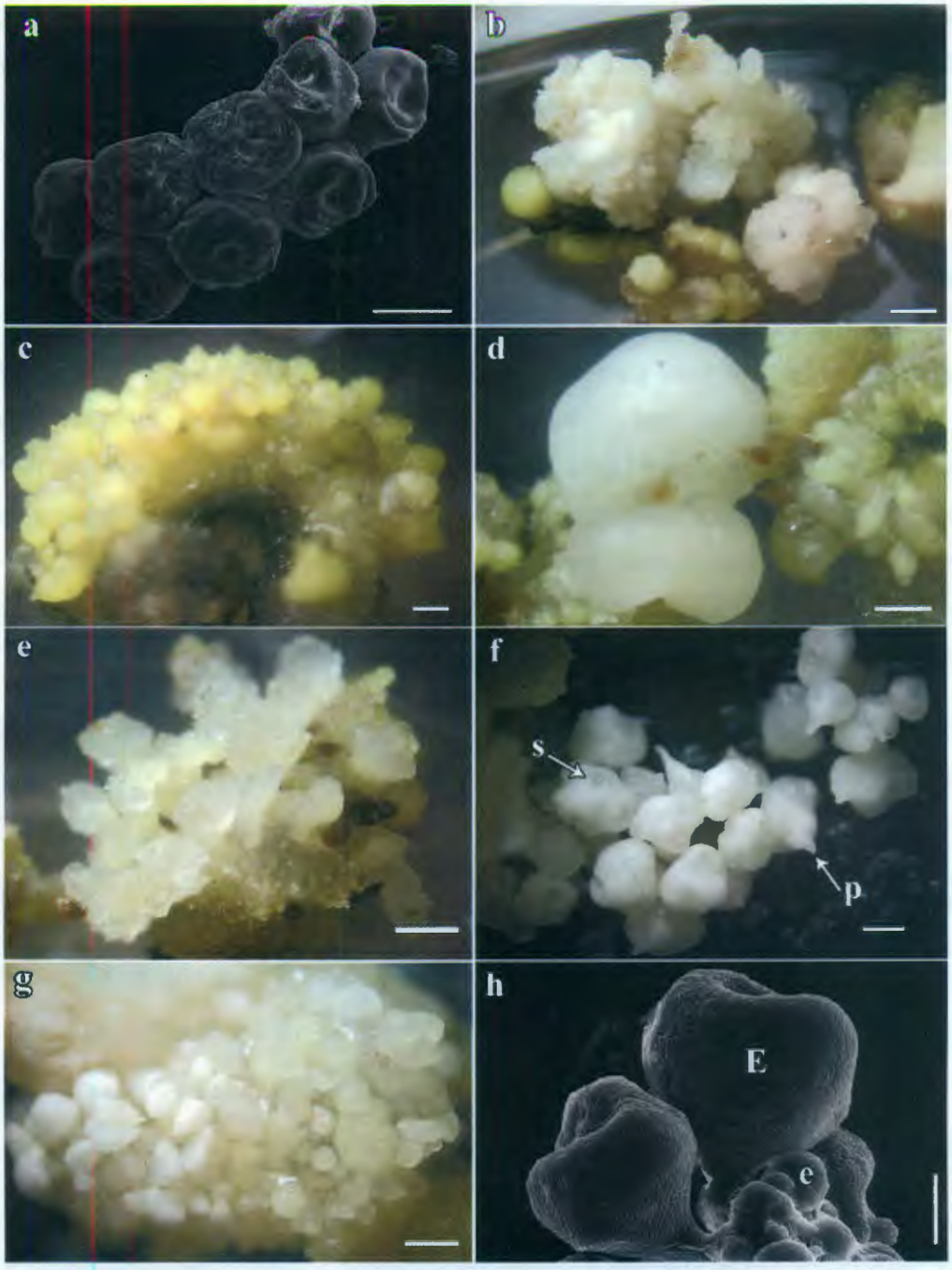

Fig. 3.1: The somatic embryogenesis response of the initial explant (male flower primordia cultured on M1 medium) and differentiation of the somatic embryos on MM medium. (a) A scanning electron micrograph of the flower primordia. (b) An embryogenic callus with expression of embryos. (c) Yellow nodular callus. (d) White compact callus. (e) Translucent callus. (f) Individual developed somatic embryos on MM; at this stage, the cotyledonary slit (*s*) and the plumule (*p*) which are emerging from some embryos, are visible. (g) A cluster of developing somatic embryos at different stages. (h) A scanning electron micrograph of well-developed somatic embryos (*E*) and numerous embryoids (*e*). Scale *bars*: (a, f, g) 500 µm, (b, c, d, e) 2.5 mm and (h) 200 µm.

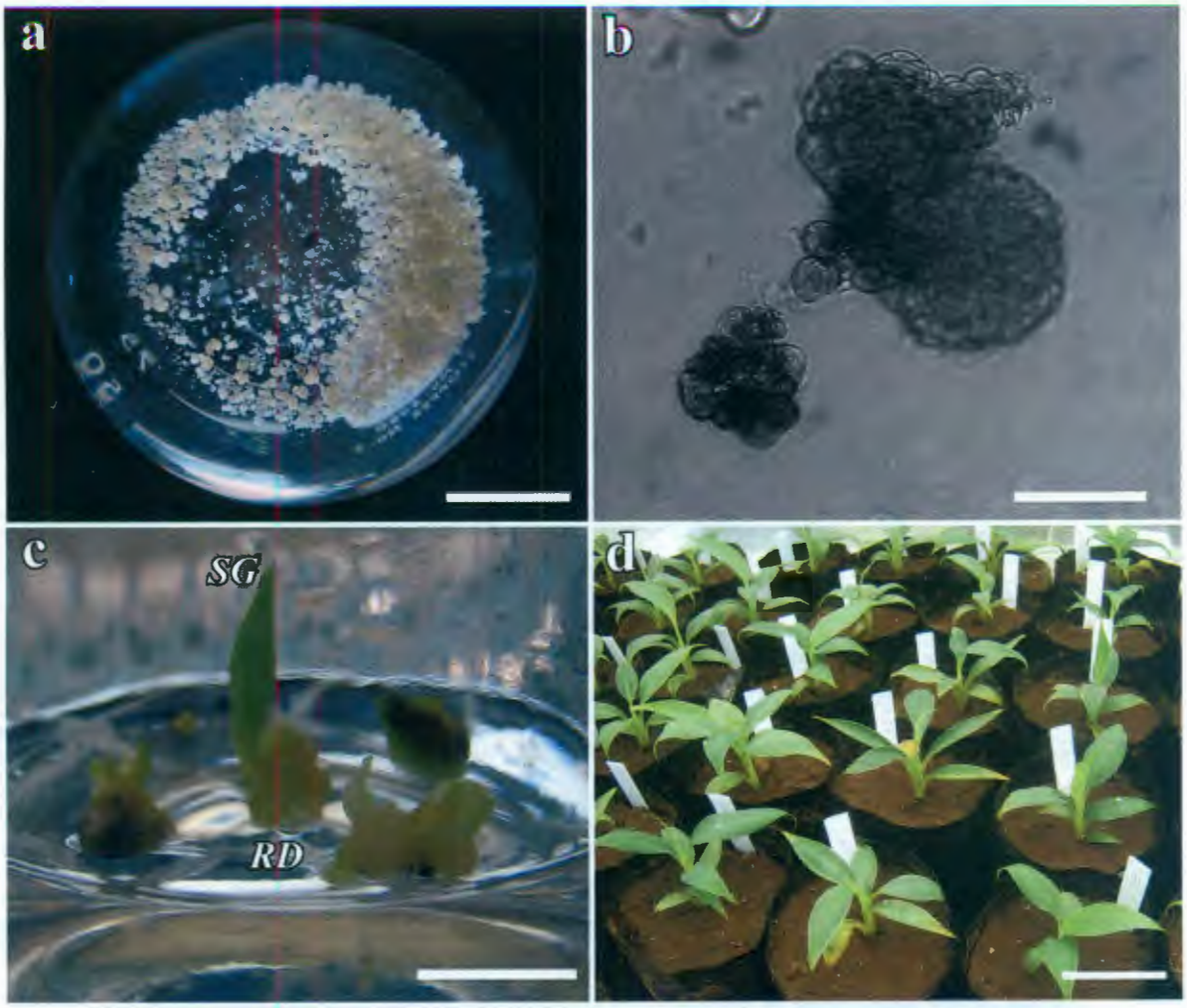

Fig. 3.2: (a) The embryogenic cell suspension derived from 'Grand Naine' embryogenic calluses cultured in liquid medium, one month after the start of the culture. (b) ECS with cell aggregates. (c) The germination of somatic embryos on GM medium, showing shoot growth (*SG*) and root development (*RD*). (d) SE-regenerated banana plants. Scale *bars*: (a) 2 cm, (b) 100 µm, (c) 1 cm and (d) 6 cm.

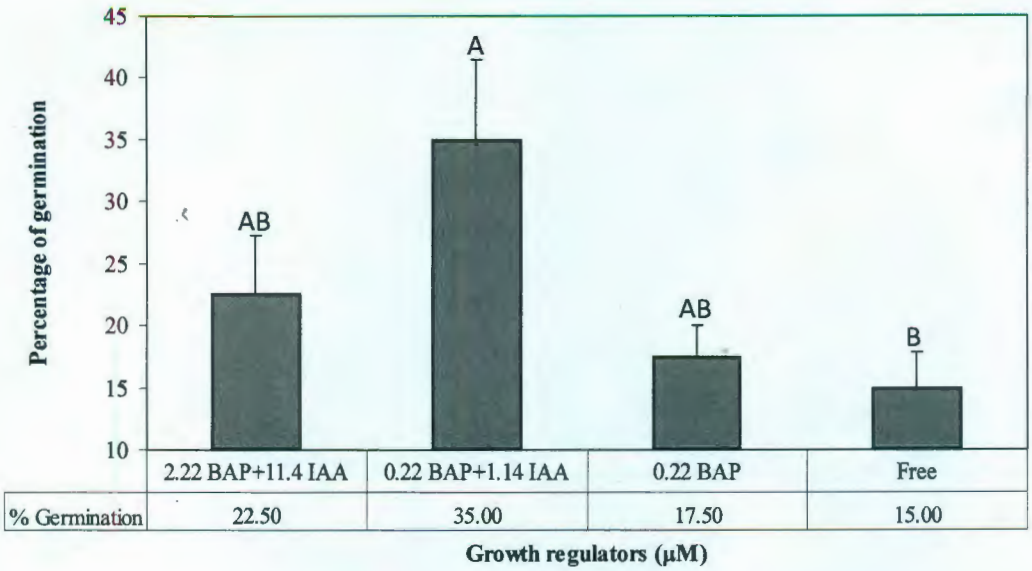

Fig. 3.3: The average germination percentage of 'Grand Naine' somatic embryos on germination medium with different growth-regulator combinations. The bars with the same letters are statistically similar (using Duncan's multiple range test, $P < 0.01$). The columns and bars represent the mean values and the standard errors ($n = 4$), respectively.

3.4. Discussion:

To our knowledge, this is the first report that addresses the effect of the age of the inflorescence male bud and its interaction with the genotype on the formation of embryogenic callus during the somatic embryogenesis induction phase in bananas. The evidence presented here supports the widely-held idea that these parameters play a critical role in the outcome and the progress of embryogenic competence that is triggered in the cells of the explant by the auxins that are present in the culture medium.

Using two *Musa* Cavendish AAA genotypes, we were able to demonstrate in this study that the maturation stage or age of the male bud, from which the explant was sourced, plays a key role during the earliest steps of the standardization of the somatic embryogenesis using the IMF-method in bananas. Of the two bud-ages investigated for the 'Williams' genotype, the explants derived from the two-week-old IMF-buds showed superior embryogenic callus formation to the one-week-old buds, indicating that matured explant sources formed more embryogenic callus than the younger ones in this genotype.

The inverse relation was observed for 'Grand Naine'. These inverse responses to the age of the explant sources show that the effect is dependent on the genotype during initiation of the somatic embryogenesis process. The callus-induction responses found here for both Cavendish cultivars are comparable to the levels reported using the IMF-method by others. For example, Escalant et al.[15], found a variable 0-7% embryogenic response within five different banana and plantain genotypes, including *Musa* (AAA) 'Grande Naine'; Navarro et al.[6], obtained a variable 2-6% embryogenic callus response for "Grand Naine" and Strosse et al.[2] reported a mean percentage of ideal embryogenic callus of 8% for "Grand Naine". Moreover, our data is also within the range that has been described for the scalp-method for 'Williams' (10%, Xu et al.[34]) and other Cavendish-type bananas (0.1 to 6.2%). These observations support the opinion of Strosse et al.[20] that both methods yield rather similar results once embryogenesis has been induced.

It is generally accepted that the successful culture of plant material *in vitro* is influenced greatly by the developmental age of the tissues or organs that are used as the source of the explants[35]. Here, we show the effect of the ontogenetic age of the male bud of the inflorescence on the induction of somatic embryogenesis. It is reasonable to suggest that these results are attributable to the maturation age of the inflorescence bud, and not to effects arising from the position of the IMF-hands that were used for induction, as the experimental design minimised this possibility by using the same position (That is, flowers from the 8^{th} to 16^{th} position in each container). In addition, the environmental effects were minimised by using explants that had been collected simultaneously from both genotypes growing under similar cultural and field conditions. The IMF position effect on the formation of embryogenic callus in 'Grand Naine' has been established by Escalant et al.[15] who reported that *ca.* 74% of the embryogenic clusters formed were on IMF positions 7 to 13, and that the embryogenic potential was affected not only by differences in the genotype, but also by seasonal effects. In agreement with this finding, Chong et al.[30] reported that the predominant response occurred within flowers from the 8^{th} to the 15^{th} position. This positional effect is perhaps more related to the age of the explant tissue (in terms of its degree of differentiation, along a gradient in the organ) than to the maturation stage of the organ as judged by our results. Considering the outcome that has been discussed above, together with the significant ($P < 0.05$) difference that we found between the genotypes in terms of the total callus induction responses, this work suggests that the protocols that are

used should be broad enough to capture the full range of possible genetic and developmental differences for each genotype. This is especially important because the availability of embryogenesis-competent explants is a common goal when establishing good quality *Musa* embryogenic suspension cultures. Therefore, to increase the initial number of good responsive explants, the first step in the protocol should be to identify the optimum window of competence for each explant-genotype and take advantage of factors that give the highest response. The regeneration competence of the embryogenic cell lines has been estimated from the average number of somatic embryos that are differentiated in the maturation media from the plated fixed volume and the average percentage of geminated embryos. The present data is comparable to that found previously in the literature. For example, Cote et al.[11] reported an average of 3.7 x 10^5 embryos per mL of settled cell volume with a 3-20% germination percentage for *Musa* AAA, 'Grande Naine', Chong et al.[30] reported an average of 3.0 x 10^5 embryos per mL with a 80.5% germination percentage for *Musa* AAA 'Grande Naine' and Xu et al.[34] obtained an ECS yield of 0.92-2.17x10^5 embryos per mL with a 4.12- 10.5% conversion frequency for *Musa* AAA 'Williams'.

The germination of somatic embryos and their conversion into vigorous plantlets depends on the history of the tissue in culture, the presence and type of growth regulators and the type of cell aggregates in the suspension. In this work, we found that there was a twofold increase in germination when a proper combination of IAA and BAP was used. Comparable values of 13 to 25% embryo/plant conversion rates using GM1 have been reported by Navarro et al.[6] for 'Grand Naine' (AAA), whereas, using the same genotype and a medium of the same composition as the GM2 combination, Cóte et al.[11] reported a range of 3 to 20% germination percentage. In the latter work, the authors found that the rate of germination depended on the size of the embryos at the stage when they were transferred to the germination medium. Domergue et al.[16] reported a range of 0 to 91.4% germination percentage, and this was dependent upon the type of cell aggregates from which the embryos were differentiated. Daniels et al.[36], using a GM1 medium in combination with 0.01 mg/L Biobras-6 and the tetraploid cultivar FHIA-21 (AAAB), found a range from 36.25 to 81.25% germination percentage that depended on the cell density. More recently, the presence of abscisic acid (ABA) and BAP during the differentiation of the embryos in the maturation phase has been found to enhance germination up to 80% in

a plantain (AAB) cultivar[37]. In summary, the precise role of different growth regulators such as IAA, BAP, brassinosteroids and ABA (together with other osmotic stressors) remains to be determined during the maturation of somatic embryos in different *Musa* genotypes.

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Chapter IV

Title: Analysis of regenerated plants derived from somatic embryogenesis in banana using AFLP

Main objective:

Detection of somaclonal variation (if any) in regenerated plants derived from somatic embyogenesis in two cultivars of banana using AFLP molecular marker

Particular objectives:

1- Establishment of AFLP technique for DNA extracted from regenerated plants and their mother plants.

2- Analysis of regenerated plants for genetic variation by comparing them to their mother plants.

Summary:

In this chapter, one hundred plants regenerated from somatic embryogenesis of two banana commercial cultivars were used for the detection of somacloanl variation. Thirty AFLP primer compinations were used. Data were analyzed by both UPGMA and principal coordinate analyses and results were discussed.

Chapter IV

Analysis of regenerated plants derived from somatic embryogenesis in banana using AFLP

Abstract

Regenerated plants derived from somatic embryogenesis, using immature male flower technique of two banana Cavendish cultivars namely 'Grand Naine' and 'Williams', were used for somaclonal variation analysis. Thirty primer combinations of the amplified fragment length polymorphism marker system (AFLP) were used. A total of 1293 and 1302 bands were generated, of which 1275 (98.6%) and 1281 (98.4%) were monomorphic, and 18 (1.4%) and 21 (1.6%) bands were polymorphic in 'Grand Naine' and 'Williams', respectively. Both cluster analysis of unweighted pair-grouping method with arithmetic averages (UPGMA) and principal coordinate (PCO) analysis separated the two cultivars, and grouped each cultivar with its regenerated plants. AFLP analysis showed 8 and 16 bands specific to the regenerated plants of 'Grand Naine' and 'Williams', respectively, these were absent in their parents, in addition, we found evidence of the absence of 10 and 5 bands in the regenerated plants, respectively, which were presented exclusively in their parental plants. Such specific bands presented in the regenerated plants could be useful for further investigation on the genetic identification of somaclonal variation in banana. On the other hand, regenerated plants of both cultivars were transferred to the field; no gross phenotypic alteration has been detected until the beginning of the flowering period. However, further field evaluation of individual plants is required for the observation of possible phenotypic somaclonal variants that may show useful characters, e.g. resistance to biotic or abiotic stress as well as high yield and fruit quality.

Keywords: *Genetic variation, somatic embryogenesis, molecular marker, banana.*

Abbreviations: AFLP, amplified fragment length polymorphism; PCO, principal coordinate analysis; UPMGA, unweighted pair-grouping method with arithmetic averages.

4.1. Introduction:

Plants regenerated from somatic cell cultures may exhibit variation which may have a genetic or non-genetic basis. Genetic variation that occurs through plant tissue culture has been termed as 'somaclonal variation'[1]. For obtaining true to type plants from a selected genotype, somaclonal variation is undesirable. On the other hand, somaclonal variation offers prospects for the recovery of useful mutants in tissue culture and for genetic improvement of banana[2].

Somaclonal variants derived from banana and plantain micropropagation with different types of plant morphology and genetic variation have been widely reported[2-6]. Unlikely, few studies have been published on the occurrence of off-types banana plants produced through somatic embryogenesis[7]. In this regard, Côte et al.[8] found that, a number of plants derived from somatic embryogenesis, in 'Grand Naine', were true to type and have agronomic characteristics comparable to *in vitro* plantlets derived via micropropagation. Similar findings were obtained with 'IRFA903' plants derived from seven months old cell suspensions[9]. Contrary to these results, Shchukin et al.[10] found that 3.6% of somatic embryogenesis-derived regenerants of 'Grand Naine' were off-types.

Several molecular markers have been used for detecting somaclonal variation at the molecular level in banana and plantains including; randomly amplified polymorphic DNA[4-6,11], inter simple sequence repeats[12], sequence characterized amplified region[13], representational difference analysis[14], selective amplification of microsatellite polymorphic loci[3], amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism[15-18].

The objective of this part of the present study was to detect the extent, if any, of somaclonal variation in plants derived from somatic embryogenesis, using immature male flowers method, of two banana cultivars namely 'Grand Naine' and 'Williams' (*Musa acuminata* Colla, AAA), using AFLP marker system.

4.2. Materials and methods:

4.2.1. Plant materials:

Plants were derived via somatic embryogenesis using the immature male flowers from the two Cavendish cultivars 'Grand Naine' and 'Williams'[19] (Chapter III). Young cigar leaves from; 50 randomly selected regenerated plants and 20 mother plants from each cultivar were collected from the Instituto Nacional de Investigacion Forestales, Agricolas y Pecuaries (INIFAP) experimental research farm at Uxmal, Yucatán, México (Lat. 20º 24` 40.10`` N, Long. 89º 45` 24.90`` E, 8m altitude above sea level). Tissues were disinfected for one minute each step, with sodium hypochlorite 6% (v/v) and ethanol 70% (v/v), rinsed with distilled water, and excess of water removed with paper towel. Subsequently, leaf samples (100 mg) were weighted, wrapped in aluminium foil and frozen using liquid nitrogen and stored at -80°C until their use.

4.2.2. DNA extraction:

Total genomic DNA from all samples under study was extracted according to Dellaporta et al.[20] with some modifications. DNA concentration was determined using a spectrophotometer according to Stulnig and Amberger[21] (Annex I).

4.2.3. AFLP analysis:

AFLP analysis was performed according to Vos et al.[22] with some modifications (see Chapter II, section 2.2.3). Two DNA-bulk samples from 20 mother plants of each Williams and Grand Naine genotypes, and two DNA-bulk samples from 50 regenerated plants from each cultivar, were used. Each bulk was made by mixing constant concentration of DNA from a mother or regenerated plants. Two hundred and fifty nano-grams of DNA from each bulk was digested with the restriction enzymes; *Eco*-R1 and *Mse*-1 (Invitrogen), followed by adaptor ligation using DNA-Ligase (Invitrogen) to generate template DNA for pre-amplification. PCR pre-amplification was carried out using AFLP primers each having one nucleotide. The PCR pre-amplified products were diluted to 1:25 in H_2O and used as templates for AFLP selective amplification using two AFLP primers each containing three selective nucleotides. Thirty primer combinations were used to detect the somaclonal variation in this study (Table 4.1). The final AFLP-PCR products

were separated on a 6% sequencing gel (urea-PAGE) and visualised by staining with silver nitrate according to Bassam et al.[23] with some modifications (Annex II).

4.2.4. Data analysis:

The polymorphic bands were scored independently as being either present (1) or absent (0) in each cultivar parental plants-bulk and regenerated plants-bulk. Only strong, reproducible and clearly distinguished bands were used for the analysis. A binary data matrix indicating the presence (1) or the absence (0) of bands was made from AFLP profiles. The percentage of polymorphism was calculated by dividing the number of polymorphic bands with the total number of regenerated bands.

The software NTSYSpc ver. 2.20s (Applied Biostatistics Inc.) was used to calculate the genetic similarities using Jaccard's coefficient[24] of similarity. Cluster analysis was carried out on similarity estimates using the unweighted pair-group method with arithmetic averages (UPGMA). Genetic distances were calculated as [(1- Jaccard's similarity) x 100]. One thousand repetition counts were used to generate the bootstrapping using FreeTree program. A 3D Scatter plot of the principal coordinate analysis (PCO) was also carried out, using NTSYSpc program.

Table 4.1: Sequences of thirty AFLP primer combinations

No.	Code	Sequence (5`-3`)	No.	Code	Sequence (5`-3`)
1	Eco-1 Mse-1	GACTGCGTACCAATTC**AAC** GATGAGTCCTGAGTAA**CAA**	16	Eco-4 Mse-1	GACTGCGTACCAATTC**ACG** GATGAGTCCTGAGTAA**CAA**
2	Eco-1 Mse-3	GACTGCGTACCAATTC**AAC** GATGAGTCCTGAGTAA**CAG**	17	Eco-4 Mse-3	GACTGCGTACCAATTC**ACG** GATGAGTCCTGAGTAA**CAG**
3	Eco-1 Mse-15	GACTGCGTACCAATTC**AAC** GATGAGTCCTGAGTAA**CTC**	18	Eco-4 Mse-15	GACTGCGTACCAATTC**ACG** GATGAGTCCTGAGTAA**CTC**
4	Eco-1 Mse-16	GACTGCGTACCAATTC**AAC** GATGAGTCCTGAGTAA**CTT**	19	Eco-4 Mse-16	GACTGCGTACCAATTC**ACG** GATGAGTCCTGAGTAA**CTT**
5	Eco-1 Mse-14	GACTGCGTACCAATTC**AAC** GATGAGTCCTGAGTAA**CTG**	20	Eco-4 Mse-14	GACTGCGTACCAATTC**ACG** GATGAGTCCTGAGTAA**CTG**
6	Eco-2 Mse-1	GACTGCGTACCAATTC**AAG** GATGAGTCCTGAGTAA**CAA**	21	Eco-5 Mse-1	GACTGCGTACCAATTC**ACT** GATGAGTCCTGAGTAA**CAA**
7	Eco-2 Mse-3	GACTGCGTACCAATTC**AAG** GATGAGTCCTGAGTAA**CAG**	22	Eco-5 Mse-3	GACTGCGTACCAATTC**ACT** GATGAGTCCTGAGTAA**CAG**
8	Eco-2 Mse-15	GACTGCGTACCAATTC**AAG** GATGAGTCCTGAGTAA**CTC**	23	Eco-5 Mse-15	GACTGCGTACCAATTC**ACT** GATGAGTCCTGAGTAA**CTC**
9	Eco-2 Mse-16	GACTGCGTACCAATTC**AAG** GATGAGTCCTGAGTAA**CTT**	24	Eco-5 Mse-16	GACTGCGTACCAATTC**ACT** GATGAGTCCTGAGTAA**CTT**
10	Eco-2 Mse-14	GACTGCGTACCAATTC**AAG** GATGAGTCCTGAGTAA**CTG**	25	Eco-5 Mse-14	GACTGCGTACCAATTC**ACT** GATGAGTCCTGAGTAA**CTG**
11	Eco-3 Mse-1	GACTGCGTACCAATTC**ACA** GATGAGTCCTGAGTAA**CAA**	26	Eco-8 Mse-1	GACTGCGTACCAATTC**ACC** GATGAGTCCTGAGTAA**CAA**
12	Eco-3 Mse-3	GACTGCGTACCAATTC**ACA** GATGAGTCCTGAGTAA**CAG**	27	Eco-8 Mse-3	GACTGCGTACCAATTC**ACC** GATGAGTCCTGAGTAA**CAG**
13	Eco-3 Mse-15	GACTGCGTACCAATTC**ACA** GATGAGTCCTGAGTAA**CTC**	28	Eco-8 Mse-15	GACTGCGTACCAATTC**ACC** GATGAGTCCTGAGTAA**CTC**
14	Eco-3 Mse-16	GACTGCGTACCAATTC**ACA** GATGAGTCCTGAGTAA**CTT**	29	Eco-8 Mse-16	GACTGCGTACCAATTC**ACC** GATGAGTCCTGAGTAA**CTT**
15	Eco-3 Mse-14	GACTGCGTACCAATTC**ACA** GATGAGTCCTGAGTAA**CTG**	30	Eco-8 Mse-14	GACTGCGTACCAATTC**ACC** GATGAGTCCTGAGTAA**CTG**

4.3. Results:

Regenerated plants from somatic embryogenesis, using immature male flowers method, of two banana cultivars namely 'Grand Naine' and 'Williams' were used in this study to detect the somaclonal variation using AFLP molecular marker technique. AFLP primer sets (Table 4.1) generated a range of 31 to 62 bands with an average of 46 bands per primer combination. A total of 1293 and 1302 bands were scored for Grand Naine and Williams, respectively (Table 4.2).

Out of the 30 AFLP primer sets used in this study, 14 primers (46.7%) showed polymorphism, of which 8 primers (i.e. 2, 5, 8, 12, 14, 18, 20, 27) were polymorphic for Grand Naine and generated 18 (1.39%) polymorphic bands, while 12 primers (i.e. 2, 3, 4, 6, 8, 12, 13, 14, 16, 18, 20, 22) were polymorphic for Williams, and generated 21 (1.61%) polymorphic bands (Table 4.1 and 4.2). The number of polymorphic bands ranged from 1 to 5 and 1 to 3 bands in Grand Naine and Williams, and the percentage of polymorphism ranged from 1.89 to 10.20% and 1.72 to 8.57%, respectively (Table 4.2).

In addition, regenerated plants of Grand Naine and Williams represented 8 and 16 additive bands, respectively, which were specific and not found in their parents' bulk profile, meanwhile 10 and 5 bands, existed only in the parents' bulk profile (Table 4.2, Fig. 4.1). The maximum number of specific bands for regenerated plants of Grand Naine and Williams was five and three, respectively. These were generated by Eco-ACA/Mse-CTT, and by both of Eco-ACC/Mse-CTT and Eco-AAG/Mse-CAA primer combinations, respectively (Table 4.2).

On the other hand, the UPGMA dendrogram showed the relationship between each donor parent and its regenerated plants, in which the similarity between the parents and their regenerated plants were 98.6 and 98.4% , with genetic distance of 1.4 and 1.6% in Grand Naine and Williams, respectively (Fig. 4.2 and Table 4.3). Moreover, scatter plot of PCO analysis demonstrated the association of these parents with their regenerated plants, in which the two cultivars were separated from each other and each cultivar was placed near to its regenerated plants (Fig. 4.3).

Regenerated plants of the two cultivars were transferred to the field for phenotypic evaluation and comparison to their parental plants. No gross phenotypic alteration, e.g.

mosaics, variegation, dropping leaves and dwarfs, etc., has been detected, in both cultivars, until the beginning of the flowering period.

Table 4.2: Levels of polymorphism between parents and their regenerated plants, and a survey of unique bands in two banana cultivars as revealed by AFLP analysis

	Primers[a]	Grand Naine				Williams			
		TNB	NPB	+/- REG	%P	TNB	NPB	+/- REG	%P
1	Eco-1/Mse-1	41	0		0.00	42	0		0.00
2	Eco-1/Mse-3	52	1	-1	1.92	52	1	-1	1.92
3	Eco-1/Mse-15	57	0		0.00	58	1	+1	1.72
4	Eco-1/Mse-16	31	0		0.00	35	3	+3	8.57
5	Eco-1/Mse-14	56	3	+1, -2	5.36	58	0		0.00
6	Eco-2/Mse-1	44	0		0.00	44	3	+3	6.82
7	Eco-2/Mse-3	45	0		0.00	45	0		0.00
8	Eco-2/Mse-15	42	2	-2	4.76	42	1	+1	2.38
9	Eco-2/Mse-16	40	0		0.00	39	0		0.00
10	Eco-2/Mse-14	38	0		0.00	38	0		0.00
11	Eco-3/Mse-1	43	0		0.00	43	0		0.00
12	Eco-3/Mse-3	46	3	+2, -1	6.52	46	1	+1	2.17
13	Eco-3/Mse-15	44	0		0.00	45	1	+1	2.22
14	Eco-3/Mse-16	49	5	+5	10.20	49	2	+2	4.08
15	Eco-3/Mse-14	31	0		0.00	31	0		0.00
16	Eco-4/Mse-1	38	0		0.00	37	3	+1, -2	8.11
17	Eco-4/Mse-3	45	0		0.00	47	0		0.00
18	Eco-4/Mse-15	32	2	-2	6.25	31	1	-1	3.23
19	Eco-4/Mse-16	62	0		0.00	62	0		0.00
20	Eco-4/Mse-14	53	1	-1	1.89	52	3	+2, -1	5.77
21	Eco-5/Mse-1	50	0		0.00	50	0		0.00
22	Eco-5/Mse-3	35	0		0.00	36	1	+1	2.78
23	Eco-5/Mse-15	45	0		0.00	45	0		0.00
24	Eco-5/Mse-16	38	0		0.00	38	0		0.00
25	Eco-5/Mse-14	50	0		0.00	51	0		0.00
26	Eco-8/Mse-1	40	0		0.00	40	0		0.00
27	Eco-8/Mse-3	39	1	-1	2.56	39	0		0.00
28	Eco-8/Mse-15	37	0		0.00	37	0		0.00
29	Eco-8/Mse-16	34	0		0.00	34	0		0.00
30	Eco-8/Mse-14	36	0		0.00	36	0		0.00
	Total	129	18	+8, -10	1.39	1302	21	+16, -5	1.61

[a] Primer numbers relate to table 4.1, **TNB**: total number of bands, **NPB**: number of polymorphic bands, **+/-REG**: addition or missing of specific bands in regenerated plants, **%P**: percentage of polymorphism.

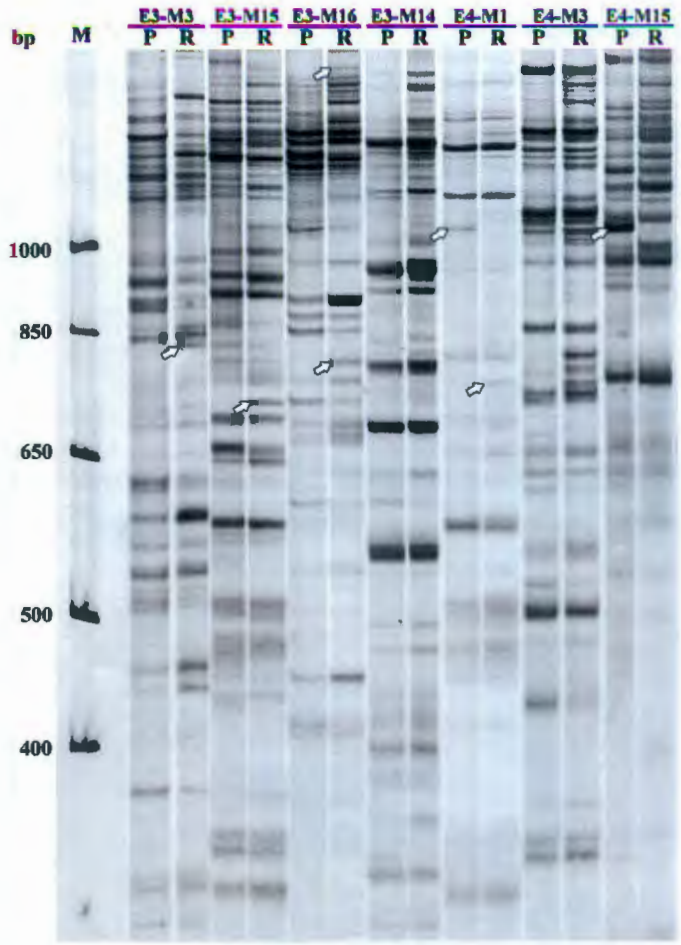

Fig. 4.1: Unique bands (*arrows*), specific for Williams parental plants (P) and their regenerated plants (R), generated by some AFLP primer combinations.

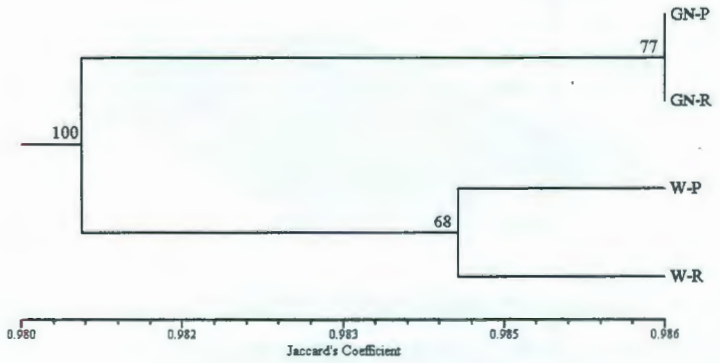

Fig. 4.2: AFLP-UPGMA dendrogram of the donor parents of Grand Naine (GN-P) and Williams (W-P) and their regenerated plants (GN-R and W-R) based on the data of all tested primers, *numbers* indicate the bootstrapping.

Table 4.3: Jaccard's similarity coefficient matrix (*above diagonal*) and genetic distance (*below diagonal*) between the donor parents and their regenerated plants calculated form data of all tested primers

	GE-P	GE-R	W-P	W-R
GE-P	-	98.6	98.4	98.0
GE-R	1.4	-	97.8	98.1
W-P	1.6	2.2	-	98.4
W-R	2.0	1.9	1.6	-

GE-P: Grand Enain Parental plants, GE-R: Grand Enain Regenerated plants, W-P: Williams Parental plants, W-R: Williams Regenerated plants.

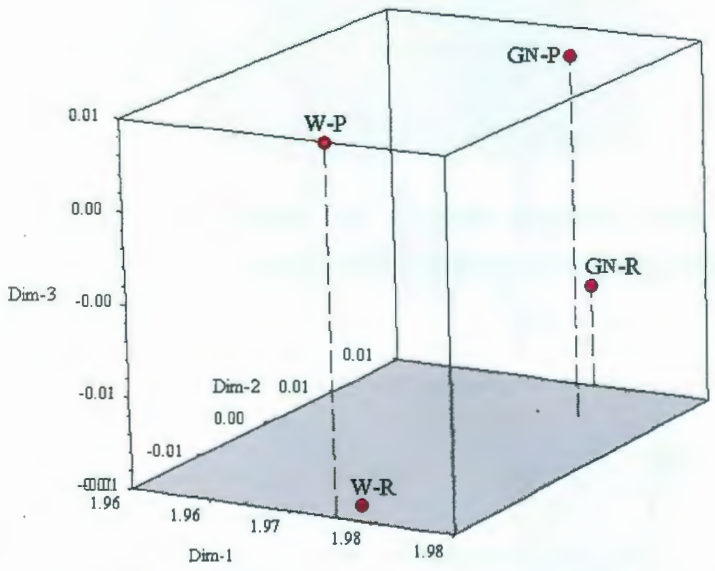

Fig. 4.3: Scatter plot showing the relationships amongst banana donor parents of Grand Naine (GN-P) and Williams (W-P) and their regenerated plants (GN-R and W-R) based on principal coordinate analysis using AFLP.

4.4. Discussion:

Somaclonal variation has been classified to either that which arises from pre-existing variation in the explant or the variation which may be induced by tissue culture conditions[25]. Additionally, there are several factors affecting somaclonal variation, including; genotype, explant source, *in vitro* period, number of subcultures and cultivation conditions in which the culture is established[26]. Variation may occur in chromosomes (structure or number), DNA rearrangement, or point mutations.

In this study, plants derived from somatic embryogenesis were used for detection of somaclonal variation. Only a small number of studies have been published on the occurrence of off-types banana plants produced through somatic embryogenesis in comparison to other tissue culture techniques[7]. In this regard, Shchukin et al.[10] found that, the rate of somaclonal variation in plants produced via somatic embryogenesis was less than that in shoot-tip-propagated plants, using 'Grand Naine'. Furthermore, Cabrera-García et al.[27] evaluated Cavendish plants regenerated from proliferating inflorescence-derived embryogenic suspension cultures, and reported that no off-types were observed among the embryogenesis-derived plants during either the *in vitro* phase or the acclimatization period in the nursery. Furthermore,

The percentage of polymorphism detected between regenerated plants and their parents, in this study, was significantly less than previous reports. For example, Bairu et al.[4] reported 55% of RAPD-polymorphism in Cavendish banana plants derived from the tenth subculture of micropropagation. While, Mohamed[5] found that the polymorphism percentage in plants of sixth subculture of Williams, using RAPD markers, ranged from 9.1 to 100%. In addition, Sheidai et al.[6] analyzed the somaclonal variation in the first, third, fifth, seventh and ninth subcultures of meristem tip cultures of *M. acuminata* and they found in total 51.40% of polymorphism detected by RAPD. On the other hand, our results here are different from those of Abu Harrirah and Khalid[28] who used male inflorescences of *M. acuminata* cv. Berangan (AAA) for direct regeneration (*via* organogenesis) and found no genetic variations among regenerated plants in comparison to their mother plants, using RAPD molecular markers. This difference could be attributed to the use of different growth regulators, since they have used Benzylaminopurine for direct regeneration and we used 2, 4-dichlorophenoxyacetic acid for embryogenic callus induction.

Although somaclonal variation is undesirable in the context of micropropagation, but it can be used to get an advantage for genetic improvement of banana[2]. In this regard, several useful somaclonal variants for various attributes have been identified, e.g. TC1-229, semi-dwarf and resistant to *Fusarium* wilt, derived from Cavendish banana[29], Tai-Chiao No.1 and Formosana, which are reported to be tolerant to *Fusarium* wilt Tropical Race 1V, and were derived from Giant Cavendish[30]. However, the majority of somaclonal variants are undesirable such as the mosaic type heterogeneity in Cavendish banana[31].

Our AFLP results showed the presence and absence of unique bands specific for parental plants as well as regenerated plants in each cultivar. The presence of these specific bands in the parental plants and loss of them in the regenerated plants indicates the loss or alteration of certain loci during tissue culture due to somaclonal variation, while the occurrence of specific bands in the regenerated plants and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants[6].

When there is no observation of phenotypic alteration in plants derived through tissue culture, genetic variation could be detected by molecular markers[32,33]. Our results can be compared with those of Côte et al.[8,9] who found that, in 'Grand Naine' and 'IRFA903', a number of plants derived from four or seven months old embryogenic cell suspensions, respectively, were observed phenotypically to be true to type and have agronomic characteristics comparable to *in vitro* plantlets. However, our AFLP analysis was successful in detecting the genetic variation between regenerated plants and their mother plants. This phenomenon could be because these genetic variations may occur in non-coding regions in the genome. Additionally, since the banana cultivars used in this study are triploid 'AAA' and have three copies of each chromosome, the genetic alteration might be redundant.

In conclusion, the AFLP marker technique was shown to be a good tool for detection of genetic variation in somatic embryogenesis-derived plants. However, further field evaluation of individual plants is required for the observation of possible phenotypic somaclonal variants that may show useful characters, e.g. resistance to biotic or abiotic stress as well as high yield production and fruit quality. Furthermore, such specific bands

presented in the regenerated plants may be of importance in understanding the genetic basis of somaclonal variation in *Musa*.

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Chapter V

General discussion and conclusion

Plant improvement programs, either by conventional breeding or recombinant DNA technology, often rely on the understanding of genetic diversity and relationship amongst different species and cultivars of the plant. This information could help the breeder to select reliable genotypes to be used in the breeding or biotechnological programs as well as it could help in germplasm establishment and reservation.

In this study (Chapter II) two molecular marker systems, SRAP and AFLP, were used, both proved to be useful tool for analyzing and assessment of genetic diversity amongst *Musa* species, wild subspecies as well as commercial cultivars. The use of two molecular marker techniques differing in the area of the genome they target; enables a broad analysis of *Musa* accessions. Although both markers used in this study were able to discriminate amongst the different analyzed accessions, SRAP marker was more informative than AFLP since it gave more unique bands and showed relationships amongst the different genotypes related more to the phenotypic characteristics relationships. The relationships amongst *Musa* sections resulting from both SRAP and AFLP analyses in the present study agreed with the AFLP analysis of Wong et al.[1], in which they found that Australimusa section was closed to Callimusa, and both Eumusa and Rhodochlamys grouped together. However, SRAP results, in the present, showed a separation of Rhodochlamys from Eumusa more than those of AFLP.

Regarding the relationships amongst different *Musa* species, the molecular markers used in this study conflicted with the classical classification of some species. For example, Simmonds and Weatherup[2] have placed *M. beccarii* in section Callimusa following their numerical taxonomic analysis of wild bananas, despite the fact that its basic chromosome number is different ($x = 9$)[3,4]. In this study, according to SRAP and AFLP results *M. beccarii* should be considered as Australimusa, since it shares species of Australimusa the number of unique bands five times more of that it shares Callimusa species, of both SRAP and AFLP. In addition, these findings supported the previous reports of Wong et al.[1] using AFLP analysis and Risterucci et al.[5] using DArT analysis who found *M. beccarii* closed to Australimusa species.

Furthermore, SRAP and AFLP analyses gave several unique bands which were specific for certain genotypes and species. However, SRAP bands are of high importance since it targets the open reading frames (ORFs). In this regard, *Musa acuminata* has been divided into eight subspecies[6], both molecular markers used in this study showed the separation of these subspecies in agreement with the previous classification. Moreover, SRAP marker, unlike AFLP, gave various unique bands specific for each of these different subspecies of *M. acuminata*, these SRAP bands could help in differentiation amongst the wild types of *M. acuminata* and their derived cultivars and hybrids as well.

On the other hand, somatic embryogenesis, as a tool of vegetative propagation, provides a good method to preserve superior genotypes, in the existence of high inherent heterozygosity, self-incompatibility or sterility mechanisms. This technique is very important as a good way for mass propagation of selected genotypes and providing preliminary materials for further biotechnological techniques such as genetic transformation, protoplast fusion or mutagenesis. Consequently, somatic embryogenesis could serve for banana improvement in view of the fact that it is impossible using classical breeding in banana's sterile cultivars. In the present study (Chapter III), the influence of the genotype and age of the explant source on the capacity of somatic embryogenesis was studied. The effect of immature male flower (IMF) position on induction of somatic embryogenesis has been established previously[7,8]. However, this is the first study of the effect of the age of the inflorescence male bud, from which the IMFs are isolated, and its interaction with the genotype on the formation of embryogenic callus in banana. It was clear observed that each cultivar has its optimum response in terms of physiological age of the explant source, which can be exploited to obtain the highest possible response for embryogeneic callus formation.

Plants derived from tissue culture techniques such organogenesis or somatic embryogenesis may present somaclonal variation. This variation is undesirable in micropropagation programs and genetic transformation techniques. However it might provide useful mutations related to resistance or tolerance to biotic or abiotic stresses. In banana, somaclonal variants derived from organogenesis have been widely reported[9-13] at both phenotypic and molecular level, unlike those produced from somatic embryogenesis[14]. In this regard, Cabrera-García et al.[15] evaluated Cavendish plants

regenerated from proliferating inflorescence-derived embryogenic suspension cultures, and reported that no off-types were observed among the embryogenesis-derived plants during either the *in vitro* phase or the acclimatization period in the nursery. However, Shchukin et al.[16] found that 3.6% of somatic embryogenesis-derived regenerants of 'Grand Naine' were off-types. In this study (Chapter IV), the AFLP marker technique used was useful to detect the genetic variation between parental plants and their regenerants, which could not be observed in phenotype (until the beginning of flowering). The percentage of polymorphism was significantly less than what has been reported for banana micropropagatoin through organogenesis[4-6]. However, AFLP marker in the present study generated several unique bands specific to the regenerated plants and could be used in the genetic identification of the somaclones and might be helpful in banana improvement. Moreover, further evaluation at phenotypic level is required for observation of any useful somaclonal variants.

In a general conclusion, the main objective in this study, that was; SRAP and AFLP molecular markers could resolve the genetic diversity amongst *Musa* genotypes and detect the somaclonal variation in banana, was accomplished successfully, and the results provided large amount of information which could be used for further research work in *Musa* improvement.

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Perspectives

This study showed that SRAP is a powerful molecular marker tool for evaluating *Musa* genetic polymorphism and relationships. It also serves for discriminating amongst *Musa* sections and within Eumusa species, as well as between plantains and cooking bananas. It regenerated various unique bands specific for *Musa* sections, A-genome (subspecies of *M. acuminata*), B-genome (*M. balbisiana*, plantains and cooking banana) and S-genome (*M. schizocarpa* and its hybrids). The sequencing and analyzing of the mentioned unique bands could be helpful for further use in genetic identification and subsequently in *Musa* improvement.

The results presented in this study of somatic embryogenesis are relevant for future tissue culture purposes of banana, since they will enhance the capacity of embryogenic callus induction. This will be by selecting the suitable developmental explant age for each genotype to obtain the highest possible response in somatic embryogenesis formation. In addition, since the genetic variation produced by somatic embryogenesis in this study was notably less than what has been reported for organogenesis, this reflects the fact that somatic embryogenesis is a reliable technique for speeding up of breeding program for genetic transformation or for massive multiplication of superior genotypes. Moreover, the unique bands generated by AFLP specific for regenerated plants are highly important. Molecular identification and understanding of the somaclonal variation in banana could be achieved by sequencing and analyzing of these unique bands.

Annex-I

DNA Isolation from Plant Tissues

(Dellaporta *et al.*, 1983 with modifications)

1- Incubate the extraction buffer in water bath at **65°C** for **15** min.
2- Grind **100 mg** plant tissue using liquid nitrogen; put the powder in 2 ml tube stand on ice.
3- Add **1 ml** extraction buffer and **100 µl SDS** (20%) to each sample.
4- Incubate in water bath **65°C** for **30 min** (**with inversion each 5 min**).
5- Leave samples to take the room temperature (RT).
6- Add half volume of **5M Potassium Acetate** (pH=8) with **mixing**; incubate on **ice** for **30 min**, leave samples to take **RT**.
7- Centrifuge at **13000** rpm for **30** min at **10°C.**
8- Take the supernatant to a new tube and **re-centrifuge** 13000 rpm for 20 min at 10°C.
9- Add 5 µl RNAase (**10mg/ml**), incubate at **37°C** for **1h**.
10- Add equal volume cold Isopropanol, incubate **-20°C** for **1h**.
11- Centrifuge at 13000 rpm for **20 min** at **4°C**.
12- Remove the supernatant and wash the pellet 2 times with ethanol 70% (500µl, 10000 rpm, 5min)
13- Let the pellet completely dry on the bench.
14- Dissolve the pellet in **600 µl TE** buffer.
15- Centrifuge 13000 rpm for **10 min** at **10°C**.
16- Transfer the supernatant to a new tube.
17- Add equal volume of cold isopropanol with 1/10 volume **sodium acetate (3M, pH=5.2)** invert the tube gently.
18- Incubate at **-20°C** for **1 hr**.
19- Centrifuge at **13000 rpm** for **15 min** at **4°C**.
20- Remove the supernatant and wash the pellet two times with ethanol 70% (**500µl, 12000 rpm, 5 min**).
21- Let the pellet completely dry on the bench.
22- Dry the pellet; dissolve in 50 µl TE or ddH_2O.

DNA Extraction buffer:	TE Buffer:
100 mM Tris HCl (pH=8)	10 mM Tris HCl (pH=8)
50 mM EDTA (pH=8)	1 mM EDTA (pH=8)
1.5 M NaCl	
1.0% (w/v) Poly Ethylene Glycol^{8000}	
0.5% (w/v) Sodium metabisulfate	

DNA quantification:

DNA concentration was determined using the spectrophotometer. DNA concentration was estimated using the conversion factor formula of 1.0 OD at 260_{nm} is equivalent to 50 µg/ml of dsDNA. To assess DNA quality the optical densities at four additional wavelengths, 230_{nm}, 270_{nm}, 280_{nm} and 320_{nm} were recorded[2]. Pure preparations of DNA have an OD_{260}/OD_{280} value near to 1.8. Nucleic acid preparations free of phenol should have OD_{260}:OD_{270} ratios of ~1.2, significant absorption at 230 nm indicates contamination by phenolate ion, thiocyanates, and other organic compounds, whereas absorption at higher wavelengths (320_{nm} or higher) is usually caused by light scattering, indicates the presence of particulate matter, and used for background correction. To confirm the concentration and quality of DNA samples, 100ng from each sample was run in 1% agarose gel stained in ethidium bromide solution and compared visually with 1kb ladder standard under UV illumination.

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Annex-II

Urea-Poly Acrylamide Gel Electrophoresis and Silver Nitrate Staining Protocol

Gel preparation:

1- Wash the large glass plate with soap (**its own scotch**) and ethanol 70% and dry completely with towel paper.
2- Add 2ml repel and spread it on the glass, wait 5 min and add more. At the first time of using the glass is better to put repel 5-8 times.
3- Wash the other plate (small) with soap and its own scotch, then with ethanol 70% and dry well.
4- Add 1ml of binding silane and spread it completely on the glass plate, let to dry 10-15 min.
5- Put the spacers on the large plate and carefully place the small plate on it without touching the other plate, put the third spacer in the bottom and connect the clips.
6- Prepare the polyacrylamide and pour it inside the two glass plates. Don't make bubbles.
7- Place the comb on the top of the gel from its flat side (*in the case of shark teeth comb*); let the gel dry for 2h.
8- Remove the clips, combs and the bottom spacer and carefully wash the glass plates well with water and soap and dry it with paper.
9- Place the plates on the electrophoreses equipment and put the two rubber pieces in the two sides.
10- Pour the TBE 1X buffer in the upper and down trays.
11- Connect the power at constant watts at 60wt, 2000 volt and 50 mA to preheating the gel, until the gel temperature arrives 50°C.
12- Wash the comb well with soap and dry it and put some repel on the teeth.
13- Wash the gel surface with buffer five times with injector. Place the comb until the teeth touch the gel (*1mm inside*).
14- Load your samples, with washing the tip each time and wash the next well.
15- Turn the power supply on ($60wt^{constant}$, 2000 volt, and 50 mA).

Silver nitrate staining (after Bassam et al.[1]):

1- Remove the comb and spacers, with clip remove the large glass plate.
2- Put the plate on large tray on shaker.
3- Fix the gel with fix-stop solution (10% acetic acid + 2% ethanol) with shaking until the complete remove of repel. You can check with water test (***very important***).
4- Rinse the gel (*three times for five min each*) with distilled water with shaking.
5- Stain the gel with silver nitrate (1g/l silver nitrate and 1.5 ml of 37% formaldehyde, *which has to be added immediately before staining*) with shaking for 30-45 min. *Complete the preparation of developing solution by adding 1.5ml/l of 37% formaldehyde and 8µl/l of 0.2g/ml sodium thiosulfate to the* ***cold*** *sodium carbonate.*
6- Rinse the gel in distilled water for 5-10 sec. **No more.**
7- Add half of the developing solution on the gel and agitate hardly until the first bands are visible, remove the solution in a glass and add the other half and continue agitation until all bands are visible.
8- Add the old fix-stop solution with shaking for 3 min.
9- Rinse the gel with distilled water for 5 min and let the gel dry on the bench.

Solutions used in polyacrylamide gel electrophoresis and silver nitrate staining:

40% Stock
193.3g Acrylamide 6.7g Bis acrylamide Up to 500 mlH_2O

6% Acrylamide
150 ml Acrylamide 40% 450 g Urea (final conc. 8M) 100 ml TBE 10X Up to 1000 ml H_2O

6% Acrylamide gel preparation
70 ml 6% Acrylamide 500 µl APS 40 µl TEMED

Binding silane
100µl binding silane 24.25 ml ethanol 96% 750µl acetic acid

Fix-stop solution
10% Glacial acetic acid

Staining solution
1g/l of silver nitrate **Before using** add 1.5 ml of 37% formaldehyde

Developing solution
30 g/l sodium carbonate, incubate at 4°C until use **Before using** add 1.5ml of 37% formaldehyde and 8µl of 0.2 g/ml sodium thiosulfate.

TBE buffer
5X (1 liter) 54.0 g Tris base 27.5 g Boric acid 20.0 ml EDTA (0.5M, pH=8) H_2O up to 1L *use 1X for polyacrylamide gel*

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