DOCTORADO EN CIENCIAS Y BIOTECNOLOGÍA DE PLANTAS

Identification of Biochemical and Molecular Markers with Potential Application in the Genetic Improvement of Coconut Palms

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Rolando Cardeña López

Centro de Investigación Científica de Yucatán, A.C.

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Commonly used abbreviations

AFLP	Amplified fragment length polymorphism
CRD	'Cameroon Red Dwarf'
CP	Coomassie blue-stained proteins
DNA	Deoxyribonucleic acid
ENP	Endopeptidases
LY	Lethal yellowing
MAS	Marker-assisted selection
MYD	'Malayan Yellow Dwarf'
PCR	Polymerase chain reaction
PB111	Port Bouet 111 ('Cameroon Red Dwarf' x 'West African Tall')
PB121	Port Bouet 121 ('Malayan Yellow Dwarf' x 'West African Tall')
PER	Peroxidases
QTL	Quantitative trait loci
RAPD	Randomly amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RLT	'Rennell Tall'
WAT	'West African Tall'

Contents

Abstract

The conventional genetic improvement of the coconut palm *(Cocos nucifera* L.) is severely restricted by its characteristic reproductive biology, which includes a long generation time, lack of asexual reproduction, and low seed production. Additionally, the attempts aimed to its in vitro cloning have met so far with inefficient rates of propagation. This situation, and the threat imposed to its cultivation by lethal yellowing (LY), a devastating phytoplasma disease, have resulted in a need for markers that assist in the genetic improvement of this perennial crop. With that objective, analyses of proteins and randomly amplified polymorphic DNA (RAPD) were performed with coconut germplasm considered suitable for breeding LY-resistant plants. The protein polymorphisms detected fitted a pattern of expression of two alleles of a dimeric peroxidase, two alleles of a monomeric endopeptidase, and a pair of active and null alleles of a coomassie blue stained protein. In the materials analysed, four distinct genotypes were identified: one specific to the 'West African Tall' (WAT), another specific to the 'Rennell Tall' (RLT), a third common to the 'Malayan Yellow Dwarf' (MYD) and the 'Cameroon Red Dwarf' (CRD), and the last common to the hybrids PB121 (MYD x WAT) and PB111 (CRD x WAT). No significant differences (P = 0.05) were found in the distributions of the protein alleles studied between the LY survivors from an 'Atlantic Tall' (AT) population, and the unselected progeny of the original AT plants. RAPD analyses revealed a total of 82 scorable markers in the DNA pools from MYD (resistant) and WAT (susceptible). Twelve of those RAPDs appeared at frequencies 0.85 or greater in one of the original populations, and 0.15 or lesser in the other. Three of the markers appearing at minimum frequency of 0.85 in MYD were found at frequencies 0.6, 0.8 and 1.0, respectively, in the LY survivors from an AT population (susceptible) which is genetically related to WAT. These markers are considered to be potentially linked with LY resistance, and could find applicability in marker assisted breeding of LY-resistant coconuts. The protein markers, and the RAPDs selected in the MYD and WAT populations, can be used in such genetic improvement aspects as cultivar differentiation and legitimization of hybrids. Together, all of the protein and RAPD markers identified in this study are valuable tools in research concerning genetic variability and reproductive biology of the coconut palm.

Resumen

El mejoramiento genético de la palma de coco *(Cocos nucifera* L.) está severamente restringido por características de su biología reproductiva que incluyen un tiempo de generación largo, la ausencia de reproducción asexual y un bajo rendimiento de semillas. Adicionalmente, los intentos dirigidos a su clonación in vitro se han tenido que enfrentar hasta el momento con tasas de propagación ineficientes. Esta situación, y la amenaza impuesta a su cultivo por el amarillamiento letal (AL), enfermedad devastadora causada por fitoplasmas, han planteado la necesidad de encontrar marcadores que ayuden en el mejoramiento genético de este cultivo perenne. Con tal objetivo, se efectuaron análisis de proteínas y ADN polimórfico amplificado al azar (RAPD) en germoplasma de cocotero considerado adecuado para la selección y propagación de plantas resistentes al AL. Los polimorfismos detectados en los análisis de proteínas concuerdan con la expresión de dos alelos de una peroxidasa dimérica, dos alelos de una endopeptidasa monomérica, y un par de alelos, activo y nulo, de una proteína teñida con azul de coomassie. Se identificaron cuatro genotipos en los materiales analizados: uno específico del 'Alto del Oeste Africano' (WAT), otro específico del 'Alto Rennell' (RLT), otro común al 'Enano Malayo Amarillo' (MYD) y al 'Enano Rojo de Camerún' (CRD), y el último común a los híbridos PB121 (MYD x WAT) y PB111 (CRD x WAT). No se encontraron diferencias significativas (P = 0.05) en las distribuciones de los alelos correspondientes a las proteínas estudiadas, entre los sobrevivientes al AL de una población 'Alto del Atlántico' (AT) y la progenie no seleccionada de las plantas AT originales. Los análisis de RAPD revelaron un total de 82 marcadores fácilmente reconocibles en las pozas de ADN del MYD (resistente) y el WAT (susceptible). Doce de esos RAPDs aparecieron con frecuencias de 0.85 o mayores en una de las poblaciones originales, y 0.15 o menores en la otra. Tres de los marcadores que aparecieron a una frecuencia mínima de 0.85 en MYD aparecieron en frecuencias 0.6, 0.8 y 1.0, respectivamente, en los sobrevivientes al AL de una población AT (susceptible), la cual está genéticamente relacionada con WAT. Se considera que estos marcadores están potencialmente ligados con resistencia al AL, y podrían ser de utilidad en la selección asistida por marcadores de plantas resistentes a la enfermedad. Los marcadores proteicos analizados, así como los RAPDs seleccionados en las poblaciones de MYD y WAT, pueden ser usados en aspectos del mejoramiento genético del cocotero tales como la diferenciación de cultivares y la legitimación de híbridos. En conjunto, la totalidad de los marcadores identificados en este estudio representa una valiosa herramienta para estudios sobre diversidad genética y biología reproductiva de la palma de coco.

Foreword

The coconut palm is a tropical crop particularly in need of concerted efforts to keep it from the undeserved relegation caused by the current trends of the food oil market. Such a loss of demand for coconut oil, the main product with economic importance of the coconut palm, is by far a consequence of the threat posed by other oil crops, cultivated in developed countries under mechanised agricultural systems, and for which genetically improved varieties are available. Another, biological threat, is the lethal yellowing disease which for over a century has devastated coconut plantations in Africa and the Americas. Research in breeding improved coconut materials, specially varieties that are resistant to lethal yellowing, underwent a marked lag. Thus, prevalence of the coconut palm in the tropical landscape is probably explained by its remarkable capacity to withstand the inclemency of low input agricultural systems, which, along with its multiplicity of uses, fit well with the limitations of the subsistence economies that rule the regions where it is cultivated.

The socio-economic aspects underlying the above situation are far beyond the scope of this presentation, which intends to introduce the framework that justifies this doctoral dissertation. Several features contribute to make the coconut palm recalcitrant to the efforts aimed to its genetic improvement. As a perennial tree, its reproductive age is reached after several years of planting. It does not produce any natural structures of vegetative propagation, nor has this goal been achieved by artificial means in the field. For many years, it has resisted the attempts undertaken worldwide to achieve efficient protocols for *in vitro* cloning. Conventional breeding of coconuts is inefficient too, as this crop rarely produces more than 100 seeds per plant per year. To worsen this scenario, selection of coconuts for such an important trait as resistance to lethal yellowing is only possible through long-lasting, resource-demanding field trials.

These facts are obvious constraints to obtaining the sustained funding required for such a long-term endeavour, as would be the case for any program on coconut genetic improvement. They also explain why so few research groups are working in this area. The present project was undertaken as a response to this problem, in the context of a research program which is particularly focused on the lethal yellowing disease. It approached a strategy that has proved a valuable help in applied aspects concerned with the genetic improvement of many crops, and which represents a promising alternative for the panorama prevailing over the coconut palm. That is the use of genetic markers. In addition to gathering the results for a doctoral project, hopefully this work will encourage the continuity of the efforts needed for the coconut palm to become a more competitive crop, for the benefit of those who, at the very least, regret its loss from the landscape.

Introduction

CURRENT STATUS OF COCONUT GENETIC IMPROVEMENT.

The reproductive biology of the coconut palm (*Cocos nucifera* L.) poses severe constraints to its genetic improvement through conventional breeding. Generation time may be as long as seven years (Sangare, 1992), there is no vegetative propagation, and seed production peaks around the low number of 100 nuts per plant per year (Dhamodaran *et al.,* 1991). Thus, coconut genetic improvement relies on breeding materials for increased heterosis, mainly through production of F_1 hybrids between dwarf and tall cultivars. These hybrids yield better than their parents, possibly because the resulting heterosis causes a complementarity of their yield components (Harries, 1991).

PB121 ('Malayan Yellow Dwarf' x 'West African Tall') and PB111 ('Cameroon Red Dwarf' x 'West African Tall') are high yielding hybrids produced on a commercial scale in The Ivory Coast. They have been introduced to at least 12 countries, and PB121 is claimed to be the most widely cultivated coconut hybrid in the world (Bourdeix *et al.,* 1992; Nuce de Lamothe and Benard, 1985). The commercial production of PB121 and PB111 is based on the assisted pollination technique, which involves emasculation and manual pollination of the mother palms (Nuce de Lamothe and Rognon, 1973). To apply this technique, dwarf cultivars are preferred as female progenitor for their precocity, higher rate of inflorescence emission, higher number of female flowers per inflorescence, and shortness, as compared to tall cultivars (Nuce de Lamothe and Benard, 1985).

The production of hybrids between contrasting genotypes has become the most important approach to improve yield in coconuts. The characteristics of PB121 and PB111, and other high yielding hybrids produced worlwide, have been reviewed by Dhamodaran *et al. (op. cit.).*

BREEDING FOR LETHAL YELLOWING RESISTANCE.

Lethal yellowing (LY) is a phytoplasma disease that has devastated coconut cultivation in the Americas, of which the earliest report dates probably back to 1834. Similar phytoplasma diseases occur in both eastern and western Africa (Eden-Green, 1997). By use of molecular techniques like DNA probe hybridization, restriction endonuclease

digestion of PCR products, and DNA sequencing, it has been shown that the phytoplasmas associated with the complex of coconut lethal decline diseases are closely related (Harrison and Oropeza, 1997; Harrison *et al.,* 1994; Tymon and Jones, 1997). At the moment, the only efficient way to control LY consists of replanting with resistant genotypes. Since no method of experimental transmission exists, screening of coconut germplasm for LY resistance relies on performing field trials where the arrival and spread of the disease may span over several years. Thus, such evaluations represent long-term, resource-demanding tasks, and urge for alternative approaches that assist in the selection of LY-resistant materials (Been, 1995).

As LY is deadly, the resistance of a genotype refers to the survival of its populations after years of field exposure to the disease (Been, 1995). Current knowledge on the resistance of different materials comes from planned evaluations (Been, 1981; Schuiling *et al.,* 1992; Kullaya *et al.,* 1997; Sangare *et al.,* 1992; Dery *et al.,* 1997; Zizumbo *et al.,* in press), and from the natural spread of the disease. Both lines of evidence assign the highest resistance (over 85 per cent) to domesticated genotypes like 'Malayan Dwarf', 'Indian Dwarf', 'Sri Lanka Dwarf' and 'King Coconut', and the lowest resistance (under 15 per cent) to wild-type populations like 'West African Tall', 'Jamaica Tall', 'Indian Tall', 'Vanuatu Tall' and 'Atlantic Tall' populations from Mexico. The most resistant genotype, 'Malayan Dwarf', is preferred as female progenitor in F_1 breeding programs intended to increase both LY resistance and yield. As discussed in the previous section, this preference is due to practical reasons related with the hybrid production process. However, it limits the genetic base available to coconut breeders (Been, 1995).

Thus, breeding for LY resistance has become a main goal for coconut conservation and genetic improvement programs. For this purpose, alternatives are urged which reduce the duration and increase the efficiency of the current process of producing hybrids from few resistant genotypes.

GENETICS OF LETHAL YELLOWING RESISTANCE.

Over the years, different models for inheritance of LY resistance have been proposed. It was first assumed, and later disproved, that the resistance trait is governed by a single, dominant locus (Whitehead, 1968). An opposing view is that it is a polygenic trait (Been, 1981), whereas another interpretation considers it influenced by a single major co-dominant locus and minor modifier loci (Harries, 1995). Evidence that LY resistance is controlled by quantitative trait loci (QTL) was recently provided by statistical analyses of results from the most extensive field trial so far performed, that conducted by the Coconut Industry Board in Jamaica (Ashburner and Been, 1997). Such analyses show

significant effects of environment, and of genotype x environment interactions, on the resistance level of various genotypes. Also, the narrow-sense heritability of LY resistance was estimated to range between such high values as 0.72 and 0.79.

The genetics underlying LY resistance is a crucial aspect in the design and implementation of strategies for the conventional breeding of resistant germplasm. For this purpose, the aforementioned analysis proposes a model intended to suit the specific case of the Caribbean region. That LY resistance is controlled by QTL also affects the choice of novel approaches aimed at assisting such conventional breeding procedures. Some of those approaches, like use of molecular markers for tagging LY resistance genes, and use of such molecular tags as tools in marker assisted selection (MAS) of LY-resistant plants, will be discussed in the next sections.

ALTERNATIVES FOR COCONUT GENETIC IMPROVEMENT.

The need has long been recognised for alternatives that overcome the constraints imposed on coconut genetic improvement by the special features of its reproductive biology. In this context, a promising strategy is the *in vitro* propagation of elite plants. However, independent efforts for over two decades have not yielded an efficient cloning protocol (Chan *et al.,* 1998). Another approach has been to identify genetic markers that assist in such aspects of conventional breeding as cultivar differentiation, identification of hybrids and, eventually, MAS of desirable traits. For this purpose, different methodologies have been used to address a key issue, the characterisation of germplasm. A broad range of morphological, physiological, biochemical and molecular markers have been studied, which will be detailed in the next section.

At the moment, only petiole color, one of the genetic markers that have been characterised in coconut, is used to assist in a specific aspect of its genetic improvement. That is the certification of progeny from certain crosses (Saint and Nuce de Lamothe, 1987). As explained below, such crosses involve a very limited number of genotypes. Furthermore, analysis of petiole color is subjective and prone to error. For this and other applications, new genetic markers remain to be characterised. The recent advent of molecular techniques for coconut research has opened avenues for such an urgent need as is the identification of markers applicable in the selection of desirable traits, particularly LY resistance. Those techniques have broadened the alternatives for genetic improvement of this perennial crop, and offer promising perspectives for that purpose.

GENETIC MARKER STUDIES IN THE COCONUT PALM.

Different types of markers have been used to approach basic and applied aspects of coconut genetic diversity, including evolution, reproductive biology, germplasm characterisation, and certification of hybrids. Petiole color and germination speed are genetic markers which have found some application in the production of hybrids by assisted pollination. They are used in the nursery stage to differentiate the hybrid seeds from those produced by self pollination of the mother palms. Germination speed has proved useful whenever it differs notably in the progenitors (Whitehead, 1965). However, petiole color is preferred as a selective marker. Seedlings with the same petiole color as the mother palm are considered self pollinations. This marker has been used to select yellow x green, and red x green hybrids (Saint and Nuce de Lamothe, 1987), to detect pollen contamination in seed orchards (Nuce de Lamothe and Rognon, 1975), and to estimate outcrossing rates (G.R. Ashburner, unpub.).

Analyses of petiole color inheritance indicated that this trait fits the expression of two diallelic, unlinked loci (Bourdeix, 1988). The proposed determinism implies that selection of hybrids by petiole color is totally reliable only if the progenitors: i) are homozygous for yellow, red or green petiole, ii) have different color, and iii) are crossed in combinations other than green x yellow or red x yellow. Thus, although practical, petiole color poses important disadvantages as a marker for selection of hybrids:

1) It limits the use of the variability found in the coconut palm,

2) it can underestimate the yield of true hybrids, and

3) it is subjective and hence prone to error.

Other markers have been studied in coconut with an aim to characterise the intraspecific genetic variability, including polyphenols, proteins (mainly isozymes), and randomly amplified polymorphic DNAs (RAPDs). After initial, encouraging results, use of polyphenols was abandoned as their expression was found to be influenced by the environment (Jay *et al.*, 1989; Sociento, unpub., quoted by Meunier, 1992). The groups working with proteins (Benoit and Ghesquiere, 1984; Canto-Canché *et al.,* 1993; White *et al.,* 1987) found that leaf extracts were easily oxidised, gave low enzyme activities, and inconsistent results. They succeeded in obtaining good extracts from either pollen, embryos or endosperm. This implies that any genetic markers identified in these tissues would be worthless to differentiate progeny in hybrid production programs. For this application, genetic markers are required which can be detected in vegetative tissues. Molecular markers, as normally unaffected by the tissue source, or by physiological or environmental effects, are well suited for this purpose. In this context, only a RAPD analysis of the genetic diversity in populations from the South Pacific region had been published (Ashburner, 1994).

CURRENT TOOLS FOR PLANT GENOME ANALYSIS.

The advent of the PCR technology brought about a boom in the number of techniques available for molecular genetic analyses. It is usual to see modifications, variants and even novel methods continuously appearing in the literature. Consequently, the acronyms used to refer to all those techniques have become a matter of confusion to the non-familiar reader. An accurate understanding of the state of the art in this field can only be obtained from the most recent publications, amongst which two comprehensive reviews are particularly useful. One presents guidelines for the choice of molecular techniques applicable in germplasm characterisation and conservation (Karp *et al.,* 1997), and the other details the methodological aspects concerned with the different types of molecular markers used in plant genome analysis (Rafalski *et al.,* 1996).

From these works it can be noted that the application of definite criteria for the classification of the numerous existing techniques is not an easy task. As this is a fast-evolving field, new terms are regularly coined to account for modifications to established methods. On the contrary, new developments call for reconsidering the original scope of some classifications and thus to make it broader, or to generate new categories. Such is the case of the multiple arbitrary amplicon profiling (MAAP), term coined to refer to those procedures employing single arbitrary sequence primers in a PCR reaction (Caetano-Anollés, 1994). The ulterior development of the amplified fragment length polymorphism (AFLP) analysis (Vos *et al.,* 1995) posed the question as to whether consider it as a type of MAAP. Indeed, it fits the general meaning of MAAP but it also differs in several aspects from the methods to which this acronym originally referred, *i.e.* randomly amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF).

Additionally, a single procedure may be given several names as occurs with the restriction enzyme digestion of PCR products, referred to as PCR-RFLP (Akopyans *et al.,* 1992), cleaved amplified polymorphic sequence (CAPS) analysis (Konieczny and Ausubel, 1993) and, in a specific application, mutant analysis by PCR and restriction enzyme cleavage (MAPREC) (Lu *et al.,* 1993). To bring this scenario more confusing, it is also the case that two independent techniques are combined into a single procedure which, of course, is assigned its corresponding acronym. Consider, for example, the use of microsatellite primers in conjunction with AFLP analysis in a procedure named as SAMPLE (Morgante and Vogel, 1994). In the middle of the apparently overwhelming complexity of this topic, the classification scheme used by Karp *et al.* (1997) provides a quick reference to the more general features on which the different techniques for molecular genetic analysis are based. Such a scheme recognises four general categories:

1. Non-PCR based methods: RFLP analysis with probes for single- or low-copy number DNA sequences, or for some kind of those repetitive sequences known collectively as variable number of tandem repeats (VNTRs) *(e.g.* minisatellite or microsatellite DNA (simple sequence repeats [SSRs]).

2. Arbitrarily (or semi-arbitrarily) primed PCR: MAAP (RAPD, AP-PCR, DAF), AFLP.

3. Site-targeted PCR: Amplification of a sequence tagged site (STS) from chloroplast or mitochondrial genomes, or from nuclear ribosomal RNA (NOR) loci, followed by electrophoresis of the products in any of various gel systems *(e.g.* TGGE, DGGE, SSCP, heteroduplex [HD] formation); PCR sequencing; STMS.

4. Variations of the basic techniques: CAPS (also known as PCR-RFLP), SCAR, DAMD, SPAR, ISSR, SAMPLE.

The applicability of any of the above molecular markers to coconut research and breeding would need to be considered in the light of their comparative advantages, and through the previous experience on similar applications to other crops.

With regards to the analysis of data generated by molecular techniques, there are over 20 different computer programmes aimed to genetic diversity studies (Karp *et al., op. cit.).* Such programmes are designed for either co-dominant or dominant markers, or nucleic acid sequences. Some of them are intended for use with a specific type of marker (*e.g.* RAPDistance for RAPDs, or MICROSAT for microsatellites) and, as concerns the analysis of genetic relationships, they apply either phenetic or cladistic approaches, or both of them (*e.g.* PHYLIP, MEGA). The software available to determine linkage of markers with traits of interest, and for the genetic mapping of such markers, is restricted to some few versions of the MAPMAKER programme (Lander *et al.,* 1987). They enable the analysis of different types of segregant populations, and some of them were specifically designed for QTL analysis (Paterson *et al.,* 1988).

MOLECULAR MARKERS APPLICABLE TO COCONUT BREEDING.

A review on the potential and past uses of different genetic markers in coconut genetics and breeding has already been published (Ashburner, 1995). In that review, the applicability of markers consisting of morphological traits, the expression of disease resistance, isozymes, and different molecular markers, is discussed in the context of such genetic improvement aspects as characterisation of genotypes and breeding systems, and genetic mapping. The molecular markers discussed comprised RFLP, RAPD, VNTR and STS markers. In the context of genetic improvement applications such as cultivar differentiation, gene tagging and MAS of desirable traits, it was

concluded that PCR-based STS markers have the greatest potential since they are amenable to become a simple and cheap product that can be used by breeders.

The above review points at the long generation time of the coconut palm as a main constraint for the application of molecular markers to gene tagging stategies in this crop. Indeed, it makes the production of such useful materials as near-isogenic lines (NILs) and recombinant inbred lines (RILs) an unthinkable endeavour. Even breeding segregant populations for gene mapping purposes (F_2 or backcrosses) would need to be undertaken as a long-term task. Given this situation, the eventual success of any project aimed to identifying markers for such an important trait as is LY resistance would rely on joining efforts between breeders, farmers, pathologists and molecular geneticists. As regards to the question on what techniques could fit in this purpose, preference for STS markers is firmly increasing, but their development demands a great deal of expertise, time and resources, and it is justified as long as they are based on polymorphisms previously characterised and of proven utility.

At the time this doctoral project started, no STS markers had been developed for the coconut palm. In this context, the use of primers for conserved regions of the mitochondrial or chloroplast genomes (Demesure *et al.,* 1995) was a possibility, in which case their utility for detecting useful polymorphisms would have to be evaluated. RFLPs had been succesfully used for tagging QTL that control disease resistance in different plant-pathogen systems (see, for example, Wang *et al.,* 1994; Young *et al.,* 1994; Sandbrink *et al.,* 1995; Tamulonis *et al.,* 1997). However, no RFLP studies had been done in coconut and, although heterologous probes could be used, their applicability would need to be tested. The lack for published information on coconut genome sequence pointed the arbitrarily primed PCR techniques as promising tools for the identification of markers applicable in coconut genetic improvement, especially for tagging of LY resistance genes.

Amongst these techniques, RAPD analysis had proved successful in identifying markers linked to disease resistance in plants, both in cases involving single genes (Eastwood *et al.,* 1993; Bai *et al.,* 1995; Young and Kelly, 1997) and, more recently, QTL (Hnetkovsky *et al.,* 1996). This technique was also applied in a genetic diversity study of coconut populations from the South Pacific region (Ashburner, 1994), which was the only molecular marker-based study in the coconut palm to have been published. By the time when decisions were taken regarding the techniques to be used for this thesis, the principles of the AFLP analysis had not even appeared in the scientific literature. This technique, as RAPD and other MAAP analyses, does not require any sequence knowledge of the genome to be analysed. Compared with them, it has a greater ability to generate polymorphisms and is generally considered to be more reproducible. However, it is more demanding in terms of technical skills, equipment and consumables (Karp *et al.,* 1997).

For diversity studies, isozyme markers were known to work better than MAAP and AFLP analyses because of their ability of revealing co-dominant polymorphisms, which enables the detection of heterozygotes. Compared with molecular markers in general, their analysis is relatively easy and inexpensive, but shows a number of limitations which have taken them to gradually fall into disuse. These include the narrow proportion of the genome they allow to analyse, the commonly fewer polymorphisms they can reveal, and their eventual susceptibility to environmental influences. As regards to their applicability in tagging disease resistance genes, it would be favoured if protein polymorphisms that are potentially involved with such resistance are analysed, and provided that those markers are genetic in nature and not artifacts resulting from environmental effects.

Modifications done to the extraction procedures previously used to analyse coconut isozymes showed promise to overcome the problems previously met with the use of leaves. By such modifications, protein polymorphisms that were stable before developmental and environmental factors were detected (Cardeña and Zizumbo, 1993). Some of those polymorphisms appeared in the electrophoretic patterns of peroxidases and endopeptidases (Cardeña *et al.,* 1994), and coomassie blue stained proteins from genotypes which express contrasting LY resistance. These polymorphisms might be associated with this important trait. Moreover, studies to establish their genetic basis could reveal markers for genetic improvement applications that require the analysis of vegetative tissues.

The application of RAPD analysis in the above context appeared to be essential, as it would provide the large number of polymorphisms desirable to be screened for increasing the chances of an eventual identification of markers associated with LY resistance. For this purpose, the strategy proposed by Waugh and Powell (1992), based on the analysis of DNA pools from individuals showing contrasting phenotypes for the trait of interest, was considered suitable. Such DNA pools would come from available coconut genotypes with appropriate characteristics for this study.

Hypothesis and objectives

HYPOTHESIS.

The genetic variability existing in coconut palms is exploited to produce high yielding hybrids by crosses which favour heterosis in the progeny. Lethal yellowing resistance is a desirable trait which shows variable expression within coconut populations, to the extent that both resistant and susceptible genotypes have been identified. This trait is controlled by loci occurring with different allele combinations in such contrasting materials. In genotypes expressing intermediate resistance, exposure to LY results in a selection of the alleles linked with the resistance trait, such as they become fixed in the survivors.

Hence, biochemical and molecular analyses of coconut germplasm could reveal genetic markers applicable in such aspects of genetic improvement as cultivar differentiation and certification of hybrids. Especially, if such germplasm includes populations known to express either resistance or susceptibility to LY, and others, genetically related and previously subjected to selection by the disease, genetic subtraction strategies could be applied that reveal markers potentially linked with LY resistance. The markers revealed by these studies could also be applied in research on genetic diversity and reproductive biology, as well as in the implementation of strategies for germplasm conservation.

OBJECTIVES.

General.

To identify proteins and RAPDs that can assist in practical aspects of coconut genetic improvement, with special emphasis in cultivar differentiation, certification of hybrids, and selection of LY-resistant plants.

Specific.

1. To establish experimental conditions that are appropriate for the electrophoretic analysis of protein polymorphisms from coconut leaves.

2. To investigate the genetic basis of the polymorphisms identified.

3. To determine the possibility of using such polymorphisms as genotype fingerprints for the cultivars 'West African Tall', 'Rennell Tall', 'Malayan Yellow Dwarf' and 'Cameroon Red Dwarf', and the hybrids PB121 ('Malayan Yellow Dwarf' x 'West African Tall') and PB111 ('Cameroon Red Dwarf' x 'West African Tall').

4. To investigate whether the protein polymorphisms identified could be linked with LY resistance.

5. To apply RAPD analysis in a genetic subtraction strategy envisaged to identify markers potentially linked with LY resistance.

PROTEIN ANALYSES.

Genotypes.

Analyses were performed with genotypes present in México, which were either traditionally cultivated, or introduced about 20 years ago. The introduced materials comprised the cultivars 'West African Tall' (WAT), 'Rennell Tall' (RLT), 'Malayan Yellow Dwarf' (MYD) and 'Cameroon Red Dwarf' (CRD), and the hybrids PB121 (MYD x WAT) and PB111 (CRD x WAT). These genotypes were introduced as seeds from the Marc Delorme Station of the Institut de Recherches pour les Huiles et Oléagineux (IRHO) in Ivory Coast, Africa, from 1977 to 1979 (Manciot, 1978; Meunier, 1982). All the seedlings were quarantined before release, and no selection for true hybrids was known to occur in the nurseries. The plants are in the experimental station "Benito Juárez" (state of Guerrero), an area still free of LY, and are 19 years old. Local materials consisted of two genetically related 'Atlantic Tall' populations (AT). One of them (Ca1) was from Ciudad del Carmen, Campeche, and had been exposed to selection pressure by LY. At the time of collection, the age of the plants was around 20 years, and their survival was estimated to be 20 per cent. The other AT population (Ca2) was the progeny of Ca1, obtained before the attack of LY and mantained in a field trial in the coast of Yucatán (Zizumbo *et al.,* in press), free of the disease until it was collected. At the time of collection these plants were four years old.

The evaluation of extraction protocols, as well as the analyses of developmental and environmental effects, were done with a three years old AT plant grown in a nursery at CICY (Mérida, Yucatán). For the search of polymorphisms, five plants from each of the MYD, WAT, RLT and PB121 populations were analysed. Unless otherwise indicated, the population analyses involved groups of 21 plants, which were different for each protein visualisation.

Evaluation of isozyme extraction protocols.

A novel extraction buffer was tested for its capability to give stable protein extracts, which result in reproducible electrophoretic patterns. The buffer combined various strategies that have been described as useful in the extraction of proteins from tissues with high contents of phenolics:

1) To keep these compounds out of solution. This was approached by grinding the tissues in the presence of insoluble polyvinylpyrrolidone (PVPP), which has been shown as an effective phenol-complexing polymer (Gray, 1978).

2) To retard the oxidation of phenolics that might have solubilized despite the use of PVPP, or which formed in the extract, by two approaches:

i) To retard chemical oxidations. Previous observations that an acidic pH hinders the ionization of phenolics and thus their chemical oxidation (Loomis, 1974; Van Driessche *et al., op. cit.)* were considered, and consequently an ascorbate-based buffer was used. As there is little change in the pK_a of ascorbic acid over oxidation (Windholz *et al.,* 1983), this event would not alter the buffer's ability to mantain pH.

ii) To avoid the action of polyphenoloxidases. This was intended by using thiourea, a chelator which deprives these enzymes of their metal cofactor copper (Van Driessche *et al.,* 1984). As copper is known to accelerate the oxidation of ascorbic acid by air (Windholz *et al., op. cit.),* thiourea would act too in increasing the stability of the buffer.

3) To revert some of the oxidative reactions which may nevertheless have occurred, including:

i) Disulphide bond formation. Cysteine was used as a sulfhydril protector for this purpose. As redox potential of the pair cysteine/cystine is similar to that of the pair ascorbate/dehydroascorbate (White *et al.,* 1978), it was expected that combining them could offer the means for cysteine to act as an electron carrier reducing disulphide bonds at the expense of ascorbate oxidation.

ii) Other, not thiol-involving, oxidations. General antioxidant properties of ascorbic acid are well known (White *et al., op. cit.;* Windholz *et al., op. cit.).* Thus, in addition to act as a pH regulator, and to indirectly revert thiol group oxidation, this would be an additional role of ascorbic acid in the buffer.

The buffer was composed of 0.1 M ascorbic acid, 50 mM cysteine and 5 mM thiourea, pH 5.50. For the extraction, intermediate pinnae from the youngest open leaves were washed and tissue-blotted. 0.1 g of the foliar lamina was ground in a pre-chilled mortar with 1 ml of cold buffer, in the presence of 70 mg of PVPP. To minimize the exposure of the extract to the cellular debris, the homogenate was pressure-filtered to clarity through filter paper in a device specially designed for this purpose (Figure 1). Finally, the filtrate was immediately stored in either liquid nitrogen or an ultrafreezer (-80 °C). This protocol was compared with that used by Bennaceur *et al.* (1991) for the extraction of isozymes from date palms, modified to use the same ratio of buffer and tissue, and to include the final step of freezing storage. The quality of the extracts was evaluated according to three criteria: i) colour, ii) intensity, sharpness and number of bands in the electrophoretic patterns, and iii) reproducibility of such electrophoretic patterns after a six month storage at -80 °C.

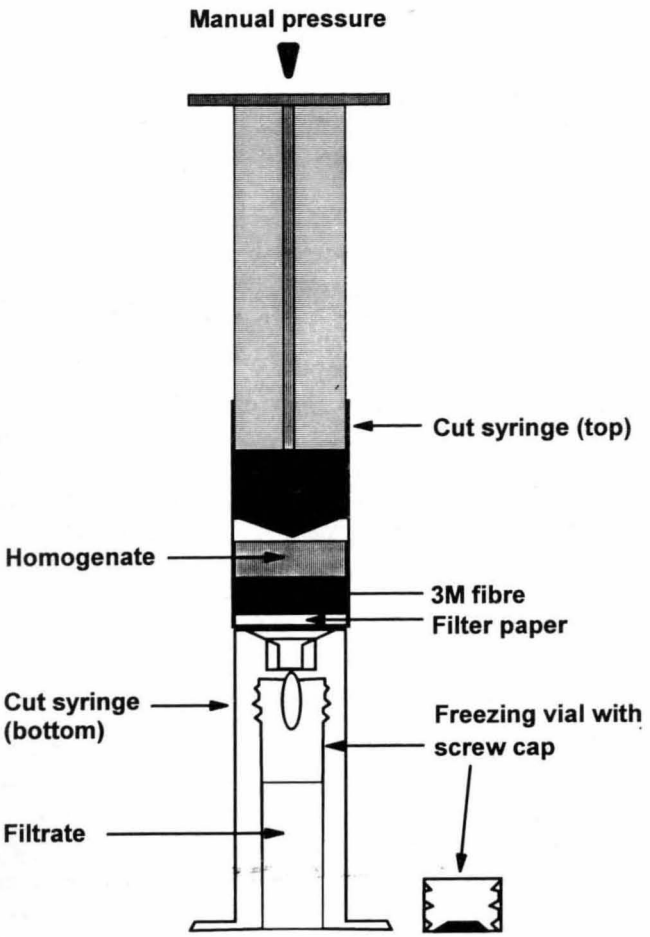

Figure 1. Device used for the rapid filtration of the protein extracts.

Electrophoresis and visualisation.

Proteins were electrophoresed in either an alkaline (Davis, 1964; Ornstein, 1964) or an acidic (Reisfeld *et al.*, 1962) discontinuous buffer system. 7.5 per cent polyacrylamide was used in the separating gels and 13 mA/cm^2 constant current was applied during the run. 100 µl of extract was loaded per lane. After the electrophoresis, the protein patterns were visualized by activity staining of esterases, glutamate dehydrogenases, malate dehydrogenases, peroxidases and acid phosphatases (Arulsekar and Parfitt 1986), endopeptidases (Association of Official Seed Analysts, 1991), and by coomassie blue staining of total proteins (Dunbar, 1987).

Effect of the environment and the leaf development on the protein profiles.

The possibility that developmental events affect the expression of the protein systems analysed was evaluated by comparing their electrophoretic profiles in intermediate pinnae of leaves which show representative stages of their post-emergence development: young (unopened), mature (fully expanded, of middle position in the crown) and senescent (with early symptoms of yellowing). Also, the effect of possible effects of the day time was tested by repeating the analyses at 2 h intervals, in the period from 8:00 a.m. to 6:00 p.m. Other parameter evaluated was the transition from dry to rainy season, for which the leaves were collected at 10:00 a.m. formerly during the dry season (middle of May), and some months later (beginning of September), after one week of daily rains.

Search and genetic interpretation of polymorphisms.

The protein systems chosen for the stability of their expression were screened for the presence of polymorphic bands in the population of mixed genotypes conceived for this study. The polymorphisms detected were classed into basic electrophoretic phenotypes. The variability observed was genetically interpreted, in terms of tentative alleles, based on the genetic determinisms described for a number of enzyme systems from plants (Gottlieb, 1981; Torres and Tisserat, 1980).

Genotyping of cultivars and hybrids.

The genetic interpretation of the polymorphisms was considered to analyze their distribution in the populations with the aims: i) to establish a genotyping scheme that differentiates the cultivars from each other, ii) to compare the distribution of phenotypes in the populations of PB121 and PB111 with that expected from the cultivar genotyping, and iii) to establish a scheme for certifying hybrids derived from the cultivars tested.

Possibility of linkage of the protein polymorphisms with LY resistance.

The possibility that the alleles identified could be linked with LY resistance was evaluated by comparing their distributions in Ca1 and Ca2. Significance of the differences observed was determined by the χ^2 test (P = 0.05).

RAPD ANALYSES.

Genetic material.

Whenever populations were analysed, these came from genotypes present in México. Such genotypes were either subjected to field-selection for LY resistance, or collected from unaffected areas. In the latter case, the resistance level of the genotypes was known from previous evaluations (Been, 1981; Schuiling *et al.,* 1992; Kullaya *et al.,* 1997; Sangare *et al.,* 1992; Dery *et al.,* 1997; Zizumbo *et al.,* in press). Non-selected genotypes comprised the 'West African Tall' (WAT, susceptible) and 'Malayan Yellow Dwarf' (MYD, resistant), whereas the selected plants came from an 'Atlantic Tall' population (AT, susceptible). The AT survivors were in Sabancuy, Campeche, and had been exposed to a severe outbreak of LY which lasted six years (Oropeza and Zizumbo, 1997). They were around 20 years old, and two years before collection their survival had already stabilized in 10 per cent. LY diagnoses performed by I. Córdova (CICY, México) with those plants by a nested polymerase chain reaction (PCR) protocol (N.A. Harrison, unpubl.) gave negative results in all the cases. Analyses of fruit components and five isozyme loci, as well as historical records, indicate that the original AT population was closely related to WAT (Zizumbo, 1997; Zizumbo-Villarreal and Piñero, 1998). The WAT and MYD populations have the same origin as those used for the protein analyses. Alternatively, DNA pools from the MYD or WAT populations were used. Each population comprised 21 individuals.

The evaluation of extraction protocols was performed during a training stay at Rothamsted Experimental Station (U.K.), before routine population analyses were initiated. For these tests, an inflorescence from an unknown genotype was used. It was collected in the germplasm collection of the Coconut Industry Board (Jamaica), freeze-dried, and refrigerated in a plastic bag for about ten years before analysed.

Evaluation of DNA extraction protocols.

Molecular analyses of genetic diversity usually rely on performing routine DNA extractions with large numbers of individuals. For PCR-based markers, this compels to use protocols that both are simple and yield DNA of sufficient purity as concerns to

PCR inhibitors. Previous RAPD analyses in coconut (Ashburner, 1994) were based on the DNA extraction protocol by Rogers and Bendich (1985). However, the greater simplicity of other PCR-compatible protocols (Edwards *et al.*, 1991; Klymyuk *et al.*, 1993) prompted to perform a comparative analysis of their applicability in the RAPD analysis of coconut palms. The protocols were tested for their ability: i) to achieve a satisfactory compromise between the yield and quality of the DNA extracted, and the simplicity of the procedure; ii) to enable PCR amplification with RAPD primers; and iii) to reproduce some previously characterised RAPDs. The RAPDs scored were some of those previously revealed in an analysis involving the 'Malayan Yellow Dwarf' (MYD) and the 'Panama Tall' (PT) (Banks, 1994). Such RAPDs, named here as suggested by Michelmore *et al.* (1991), were found specific to either the MYD ($R13_{1500}$, $W3_{1020}$) or the PT ($W3_{820}$). The DNA extractions were performed in duplicate, and each extract was screened for the RAPDs of interest after Banks *(op. cit.)*. For the electrophoresis, equal volumes of the DNA extracts (2 µl) or the reaction mixtures used for the PCR (10 µl), were loaded in the gel.

RAPD analysis.

DNA was extracted from young pinnae, and its concentration was determined visually by comparison with known amounts of salmon testes DNA after electrophoresis (1 per cent agarose in TAE, 100 V constant, 1 h) and staining (15 min in ethidium bromide at 1µg/ml, 15 min wash). Concentration of each individual extract was brought to approximately 20 ng/µl. RAPDs were generated by 80 Operon Technologies decamers (series A, B, AB and AL), after a protocol (Banks, 1994) modified as next mentioned. The temperature profile comprised 5 min at 85 °C; 43 cycles of: 1 min at 94°C, 40 s ramp to 35 °C, 1 min at 35 °C, 1 min ramp to 72 °C, 1 min at 72 °C, 45 s ramp to 94 °C; 1 min at 94 °C; 40 s ramp to 35 °C; 1 min at 35 °C; 1 min ramp to 72 °C; 6 min at 72 °C; overnight at 4 °C. The PCR reaction mixture was prepared with taq polymerase from BIOSELEC (México), and the $MgCl_2$ concentration ranged between 1.5 and 3.0 mM (see next section). Amplified fragments were electrophoresed and stained as described above, and their sizes relative to the fragments of a 1 kb DNA ladder estimated by regression analysis (Stat Graphics, version 5.0). RAPDs were named as suggested by Michelmore *et al.* (1991).

Optimisation of $MgCl_2$ concentracion in the PCR reaction mixture.

Due to a period of irreproducibility of RAPD profiles, found to be associated with variations in the concentration of the $MgCl_2$ stocks provided by the taq polymerase manufacturer, it was necessary to re-define the working concentration of this salt for each of the decamers under analysis. In that period such decamers included a total of 11 (A11, B1, B4, B7, B11, AB3, AB4, AB5, AB16, AL3, AL7), which were tested with

$MgCl_2$ concentrations ranging from 1.5 mM to 3.0 mM, at 0.025 mM intervals. For these experiments, a molecular biology-tested preparation of $MgCl_2$ (Sigma, catalog No. M 1028) was used.

Genetic subtraction procedure.

For the identification of RAPDs potentially linked with LY resistance, the selected primers were screened consecutively in three sets of genetic stocks: 1) DNA pooled from the MYD or the WAT population, 2) individual DNAs from those populations, 3) individual DNAs from the AT population. Linkage of a RAPD with LY resistance was assumed as likely if it, or its respective null phenotype, apeeared at frequency 0.85 or greater in MYD, 0.15 or lesser in WAT, and 0.5 or greater in AT. Bands difficult to be scored because of faint staining or poor resolution, or which did not reproduce on three separate occasions, were not examined. The experimental design is outlined in Figure 2.

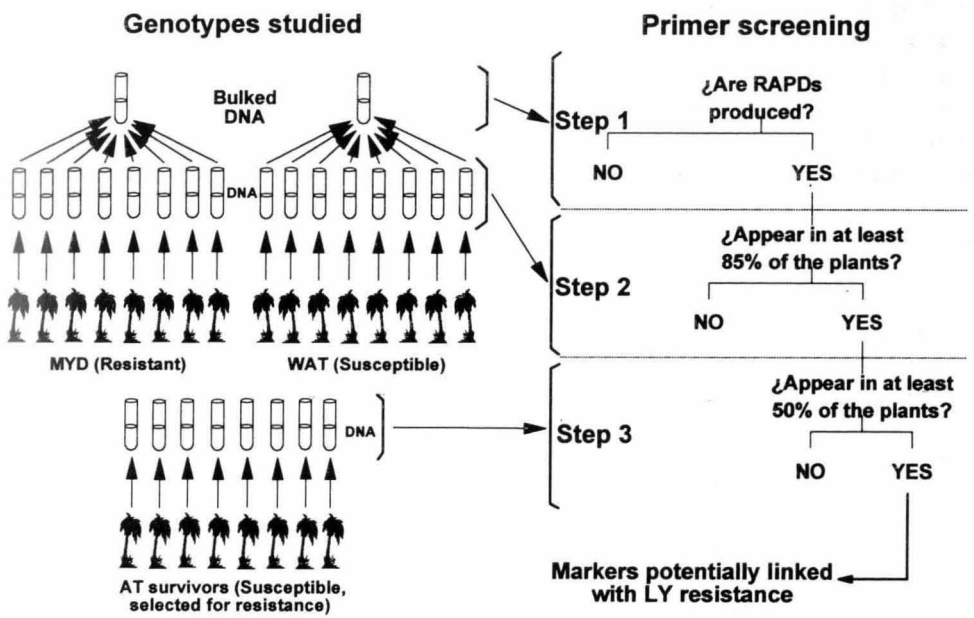

Figure 2. Experimental design for the identification of RAPDs potentially linked with LY resistance.

Results

EVALUATION OF ISOZYME EXTRACTION PROCEDURES.

An extraction protocol (hereafter referred to as coconut protocol) was designed to avoid the oxidation and to keep the enzyme activities of protein extracts from coconut leaves, such that electrophoresis of isozymes could be reliably used in genetic diversity analyses. The effectiveness of such protocol was compared with that of another (hereafter named as date palm protocol) used previously for the same purpose with date palms (Bennaceur *et al.*, 1991). The analysis of fresh extracts from both protocols allowed the visualisation of all the enzyme systems tested. However, except for the anodic peroxidases, all the systems showed greater intensity, sharpness and number of bands with the coconut protocol. The acidic electrophoresis system, aimed to the separation of cathodic proteins and peptides, only permitted the detection of peroxidases. The beneficial effects of the coconut protocol were particularly evident with the malate dehydrogenases and esterases, whereas the banding patterns of the anodic peroxidases were comparable between both protocols (Figure 3). As regards to the colour of the extracts, those from the date palm protocol started to darken as the tissues were still being ground, and the supernatant collected after their centrifugation had developed a marked grey colour. Those from the coconut protocol, on the contrary, retained a light green-yellow shade even after a six month storage at -80 °C. After this period, no deterioration was detected in the visualisation characteristics of the extracts obtained with the coconut protocol, whereas the paterns of those from the date palm protocol became paler and less distinct (esterases, acid phosphatases, anodic peroxidases, coomassie blue-stained proteins), or even disappeared (the rest).

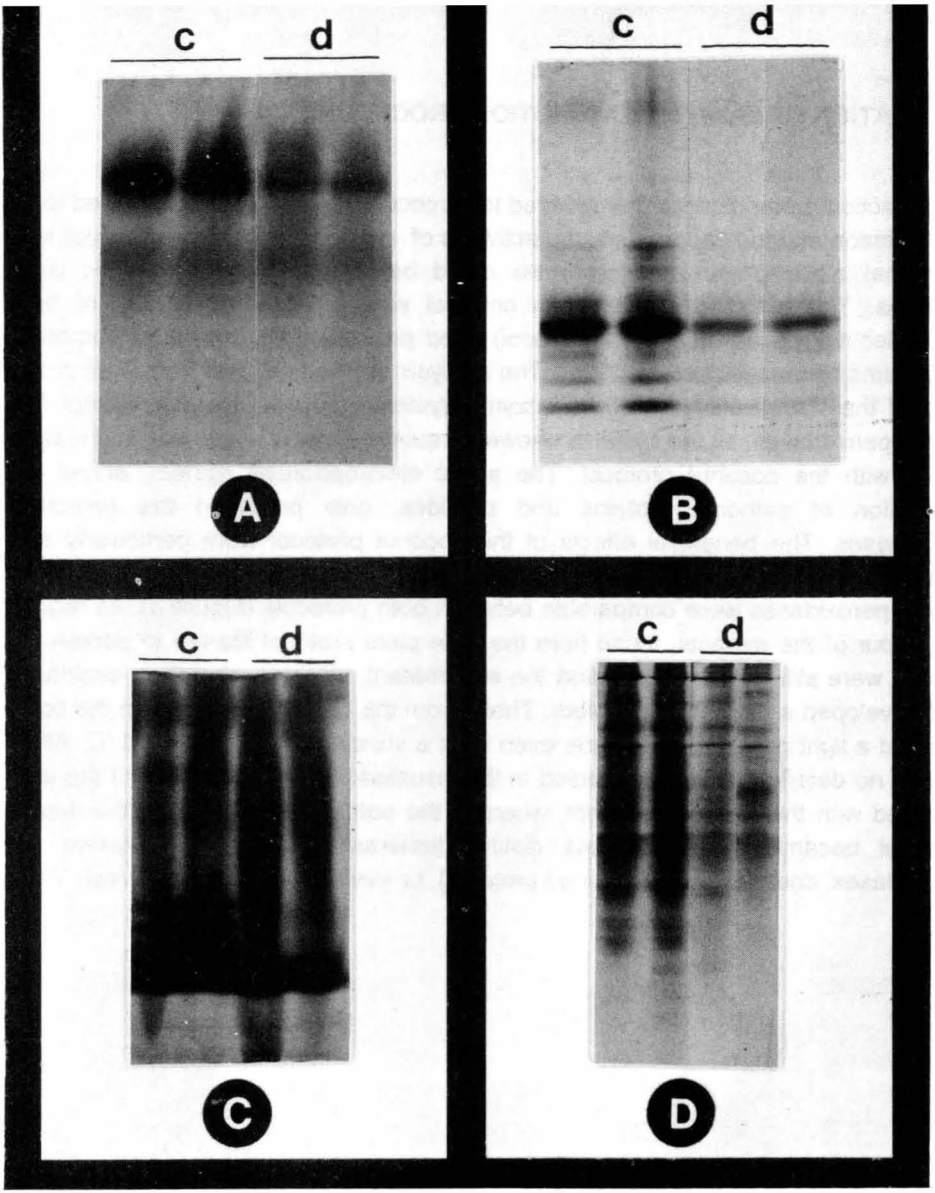

Figure 3. Protein banding profiles obtained with the designed extraction protocol (coconut protocol, C) and with the protocol previously used by Bennaceur *et al.* (1991) (date palm protocol, D). A, anodic peroxidases; B, esterases; C, cathodic peroxidases; D, coomassie blue-stained proteins.

ENVIRONMENTAL AND DEVELOPMENTAL EFFECTS ON THE PROTEIN PROFILES.

In general, the developmental stage of the leaf had a marked effect on the electrophoretic profiles of the protein systems analysed. In the simplest case, the process from expansion to maturity and finally senescence caused all the bands to faint and lose resolution *(e.g.* malate dehydrogenases, Figure 4A). In other cases the main bands remained considerably recognizable, whereas other minor bands fainted gradually until they disappeared in the senescent leaf *(e.g.* anodic peroxidases, Figure 4C). However, in some cases dramatic changes were observed, with some bands appearing and others disappearing, either gradually or abruptly. The systems in which these effects were more evident comprised the acid phosphatases (Figure 4B) and the cathodic peroxidases (Figure 4D).

In the acid phosphatases, the sudden appearance of the activity zone 3 in the senescent leaf occurred simultaneously with an abrupt disappearance of the slower migrating zone 2. As both zones of activity consisted of a single band, these results suggested that some post-translational modification of zone 2 could account for the appearance of zone 3. Contrarily, the activity zone 1 in the cathodic peroxidases from senescent leaf contains the slower-migrating forms of this enzyme system. Thus, their appearance cannot be explained by post-translational modifications of previously active precursos which confer them a greater mobility, such as proteolisis, phosphorilation or glycosilation with acidic sugars. It could be that the two bands of this region appear as result of *de novo* protein synthesis. It was also noted that the expression pattern found in the senescent leaf was practically specific to this developmental stage. The other parameters evaluated (day time, transition from dry to rainy season) were not found to have effects on the electrophoretic patterns of the protein systems analysed.

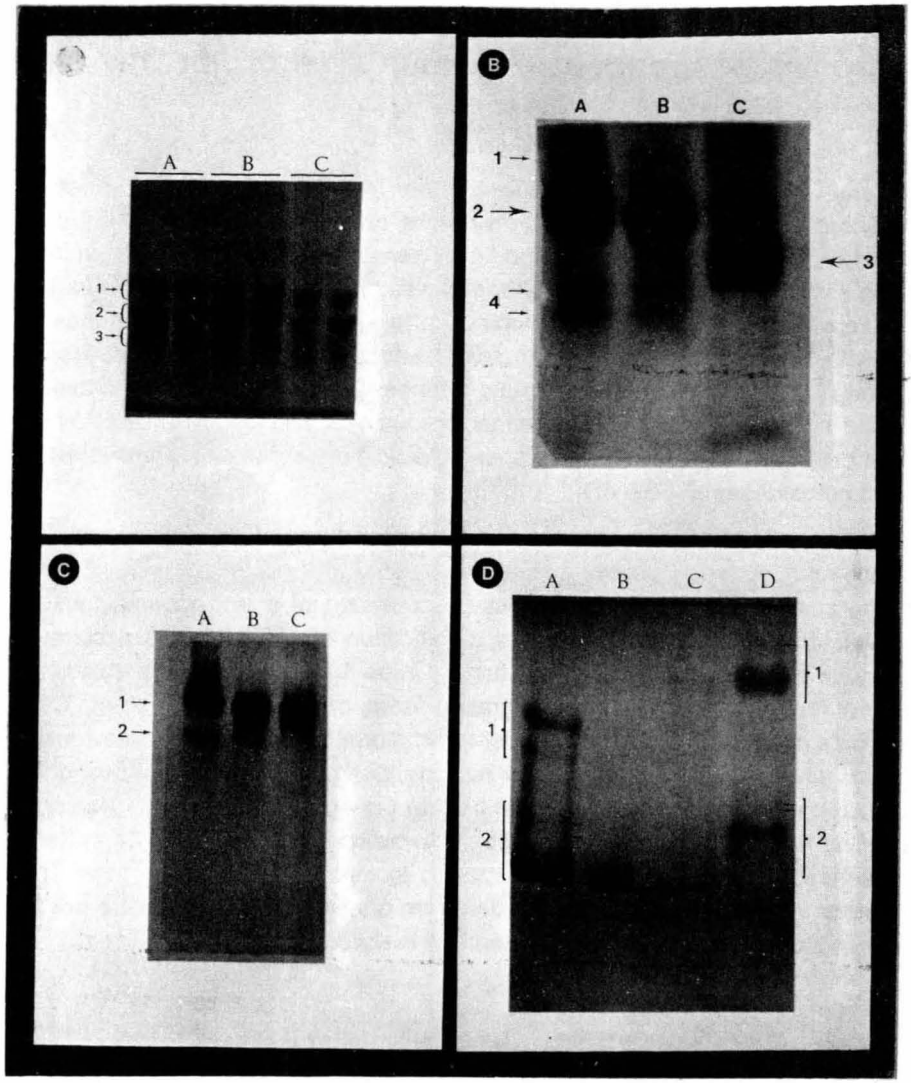

Figure 4. Effect of the leaf developmental stage on the banding profiles of malate dehydrogenases (A), acid phosphatases (B), anodic peroxidases (C) and cathodic peroxidases (D). From left to right, each row represents a consecutive stage in the transition from young to mature leaves.

INTERPRETATION OF THE PROTEIN POLYMORPHISMS DETECTED.

The basic electrophoretic phenotypes recognised appear in Figure 5. Three phenotypes of peroxidase activity (PER) were observed. These consist of either a single, fast (F) or slow (S) band, or a triplet (T) with equidistant bands. The slow and fast bands of the triplet have R_f values equal to those of the single bands in the S and F phenotypes, respectively. A single zone of endopeptidase activity (ENP) was detected that also showed three phenotypes. A slow (S) and a fast (F) band migrating very close to each other, and a doublet (D) which appeared to be composed of the S and F phenotypes. The patterns of coomassie blue stained proteins (CP) showed a fast migrating band that was present (active phenotype, A in Figure 5) or absent (null phenotype, N in Figure 5).

Phenotype variability fits the expression of a diallelic locus per system (Table 1). PER and ENP allozymes would be dimeric and monomeric proteins, respectively. The simplest genetic interpretation for the phenotypes detected with CP implies a pair of active and null alleles. The subunit structure of the protein responsible for the CP A phenotype can not be determined from the variability observed. In accordance with these interpretations, the loci detected with PER, ENP and CP will be referred to as *Per1*, *Enp1* and *CP1*; the slow and fast allozymes of both *Per1* and *Enp1* will be named S and F; A and N will be the denominations used for the active and null alleles of *CP1*.

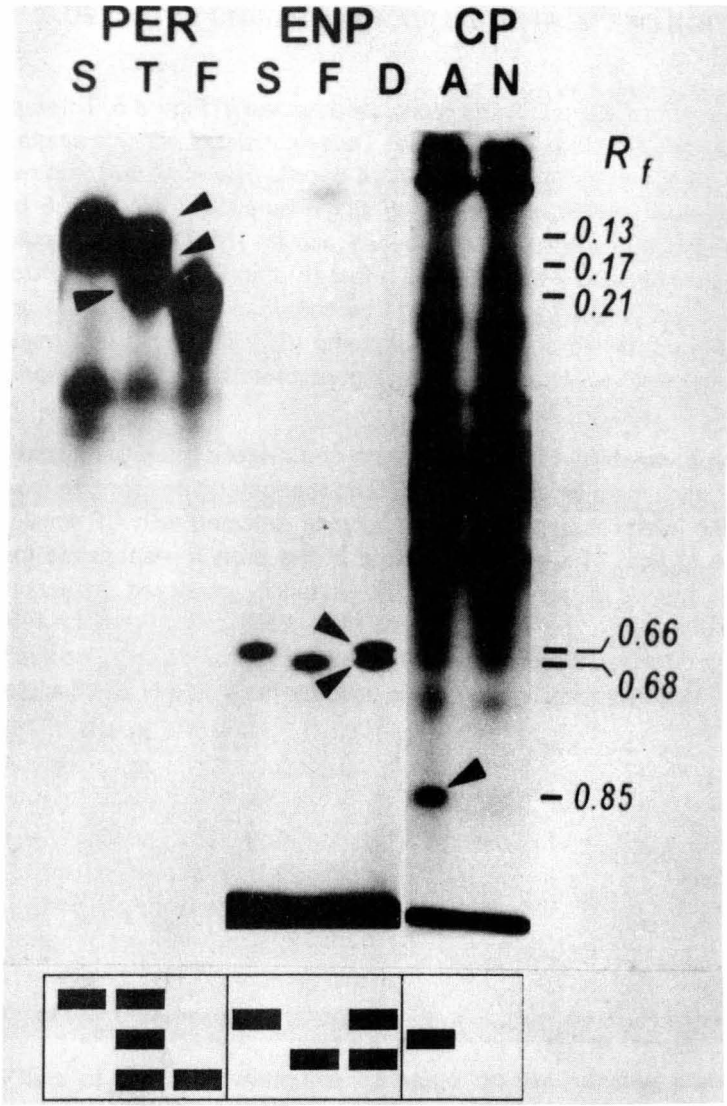

Figure 5. Electrophoretic phenotypes detected. Polymorphic bands are indicated by arrowheads, and appear schematized at the bottom. PER, peroxidases; ENP, endopeptidases; CP, coomassie blue stained proteins; S, slow band; F, fast band; D, doublet; T, triplet; A, active phenotype; N, null phenotype.

Table 1. Genetic interpretation of the electrophoretic phenotypes shown in Figure 5.

System/locus	Electrophoretic phenotype	Genotype	Apparent subunit structure
	S	SS	
PER/*Per1*	F	FF	Dimer
	T	SF	
	S	SS	
ENP/*Enp1*	F	FF	Monomer
	D	SF	
CP/*CP1*	A	AA or AN	Uncertain
	N	NN	

PROTEIN GENOTYPING.

The distribution of electrophoretic phenotypes is shown in Table 2. In ten out of the twelve cultivar/system combinations, fixed phenotypes were detected. With PER and ENP, these consisted of either of their respective S and F phenotypes, whereas with CP either the A or N phenotype was fixed. Two cultivar/system combinations showed, in addition to the above phenotypes, a second phenotype at a low proportion of 9.5 per cent. These were WAT/PER in which both S and T (minor phenotype) were detected, and CRD/CP in which both A and N (minor phenotype) appeared.

With each of the systems, PB121 and PB111 showed combinations of a major and a minor (lesser than 20 per cent) phenotype. The combinations of major/minor phenotypes were, for PER, T/F; for ENP, D/S; and for CP, A/N. According to the genetic interpretations of Table 1, the phenotype distributions shown in Table 2 suggest that each of the cultivars is homozygous in at least two of the loci. WAT would be homozygous for *Enp1*-F and *CP1*-A; RLT for *Per1*-F, *Enp1*-S and *CP1*-A; MYD for *Per1*-F, *Enp1*-S and *CP1*-N; and CRD for *Per1*-F and *Enp1*-S).

Table 2. Number of plants with the electrophoretic phenotypes of Figure 5, in each experimental population.

Population	PER			ENP			C P	
	S	F	T	S	F	D	A	N
WAT	19	0	2	0	21	0	21	0
RLT	0	21	0	21	0	0	21	0
MYD	0	21	0	21	0	0	0	21
CRD	0	21	0	21	0	0	2	19
PB121	0	4	17	3	0	18	19	2
PB111	0	3	18	2	0	19	19	2

LINKAGE OF PROTEIN POLYMORPHISMS WITH LETHAL YELLOWING RESISTANCE.

The genetic structures of Ca1 and Ca2 for the protein loci studied, inferred from their electrophoretic phenotypes, are presented in Figure 6. As the CP A phenotype is dominant, no distinction could be made between the *CP1*-AA and *CP1*-AN genotypes. The genetic structures of Ca1 and Ca2 were comparatively similar. The greatest differences were observed with *Per1,* whereas *CP1* appeared almost identical in both populations.

The corresponding allele frequencies are shown in Figure 7. The impossibility to detect *CP1*-AA separately from *CP1*-AN was accounted for in calculating the frequencies of the *CP1* alleles. Thus, the extreme cases were considered that all the plants with CP A phenotype were either *CP1*-AA, or *CP1*-AN, and ranges, rather than fixed values, were assigned to the frequencies of the *CP1* alleles. Such ranges, and the frequencies of the other four alleles, were practically the same in Ca1 and Ca2.

None of the differences between the genetic structures and allele frequencies of Ca1 and Ca2 were found to be significant (Table 3). As the frequencies of the *CP1* alleles consisted of ranges rather than fixed values, for this locus only the highest estimation of X^2 is presented. It corresponds to the event that the frequency of *CP1*-A changes from 0.29 in Ca2 (lower limit of its range, see Figure 7) to 0.6 in Ca1 (upper limit of its range, adjacent bar in Figure 7).

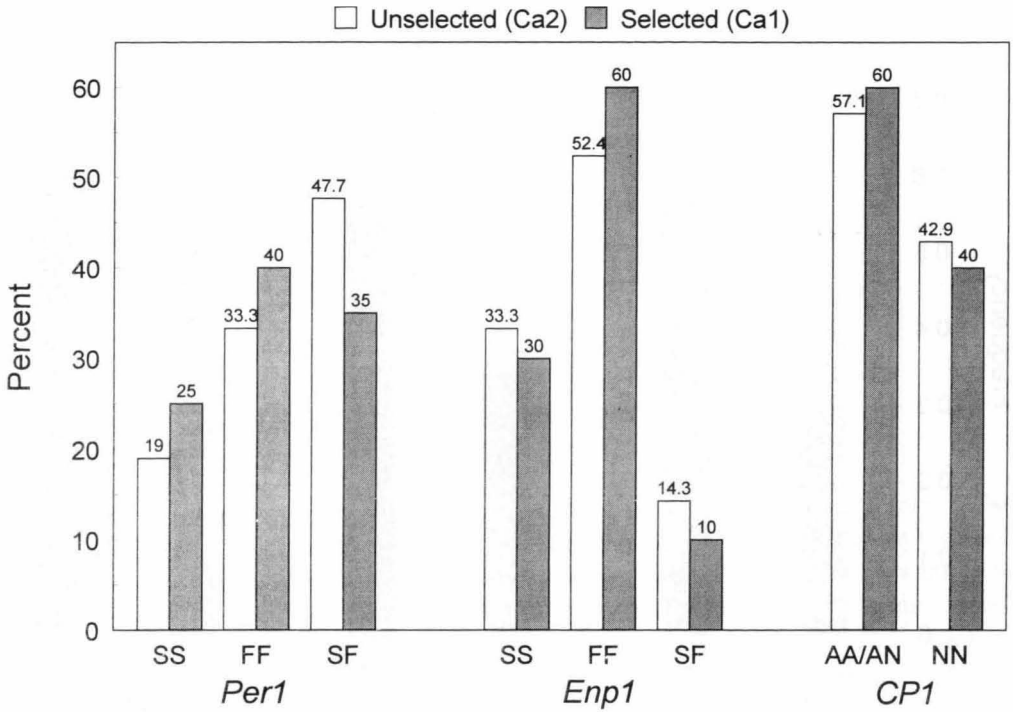

Figure 6. Distribution of the *Per1*, *Enp1* and *CP1* genotypes in a population selected by LY (Ca1, n = 20), and in the unselected progeny of the original population (Ca2, n = 21).

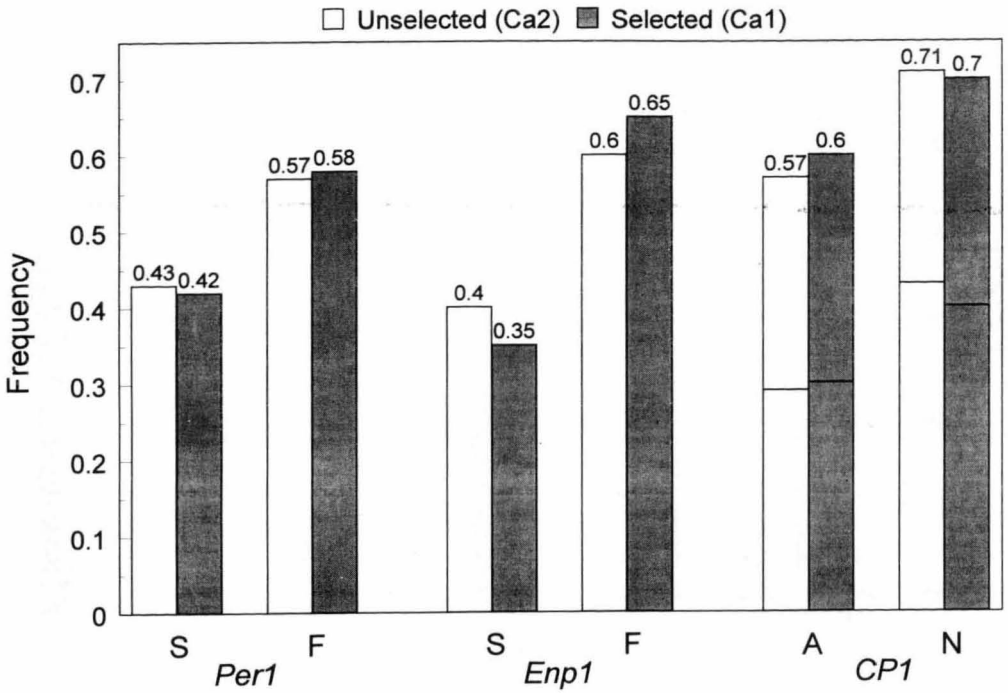

Figure 7. Allele frequencies calculated from the genetic structures shown in Figure 6. The horizontal lines in the *CP1* bars indicate the lower limit of the ranges of possible frequencies.

Table 3. Significance test (P = 0.05) for the differences between the genetic structures and allele frequencies of Ca1 and Ca2.

Locus	Genetic structures		Allele frequencies	
	X2	c2	X2	c2
Per1	0.6832	5.991	0.0205	3.841
Enp1	0.2962	5.991	0.5333	3.841
CP1	0.0345	3.841	0.1945	3.841

EVALUATION OF DNA EXTRACTION PROTOCOLS.

Molecular analyses of genetic diversity usually rely on performing routine DNA extractions with large numbers of individuals. For the analysis of PCR-based markers, this compels to use protocols that both are simple and yield DNA of sufficient purity as concerns to PCR inhibitors. Previous RAPD analyses in coconut (Ashburner, 1994) were based on the DNA extraction protocol by Rogers and Bendich (1985). However, many other PCR-compatible extraction procedures have been published. For their greater simplicity, two of them (Edwards *et al.,* 1991; Klymyuk *et al.,* 1993) were compared with the Roger and Bendich's protocol to determine their applicability in the RAPD analysis of coconut palms.

The efficiency of the three protocols tested is compared in Figure 8. The Klymyuk *et al.'s* protocol gave extensive smearings which extended from the base of the wells, with no distinct bands attributable to the of genomic DNA. With the other protocols, evident DNA accumulations in the region of high molecular size could be observed. Such accumulations diffused into smearings which were much lesser extensive than those of the Klymyuk *et al.'s* protocol. Amongst them, the Rogers and Bendich's protocol gave a sharper genomic DNA band, which was slightly greater in size. These results showed that the Klymyuk *et al.'s* protocol yielded low amounts of extremely fragmented DNA. This somehow could have been suspected, considering the extreme simplicity of the protocol. Indeed, DNA is claimed to be extracted by boiling leaf tissue in alkali, neutralising and boiling again in the presence of detergent. On the

contrary, the Edwards *et al.'s* and Rogers and Bendich's protocols are more elaborate, both of them involving isopropanol precipitation and consequently resulting in greater DNA yield and quality. According to the yield and quality achieved, the Rogers and Bendich's protocol proved to be the best amongst the three that were tested. However, the Edwards *et al.'s* protocol gave comparable results with less steps and could be suitable for subsequent PCR analyses.

Analysis of the RAPD primers R13 and W3 revealed no fragments with the extracts from the Klymyuk *et al.'s* protocol, and nearly identical banding profiles between the extracts from the other two protocols (Figure 9). The amplified fragments were evidently more intense with the extracts from the Rogers and Bendich's protocol. These results agreed with the quality and relative concentration of the extracts, as revealed by the electrophoresis and staining (Figure 8). At the time these tests were done, no MYD or PT samples were available. Thus, their DNA could not be used as positive control for the amplification of the RAPDs subejct to screening ($R13_{1500}$, $W3_{820}$ and $W3_{1020}$). However, amongst the fragments generated by R13, one which co-migrated with the 1.5 kb marker was clearly visible (see arrow in Figure 9). This suggested that the DNA tested was positive for $R13_{1500}$, and consequently that the original plant could be related to MYD.

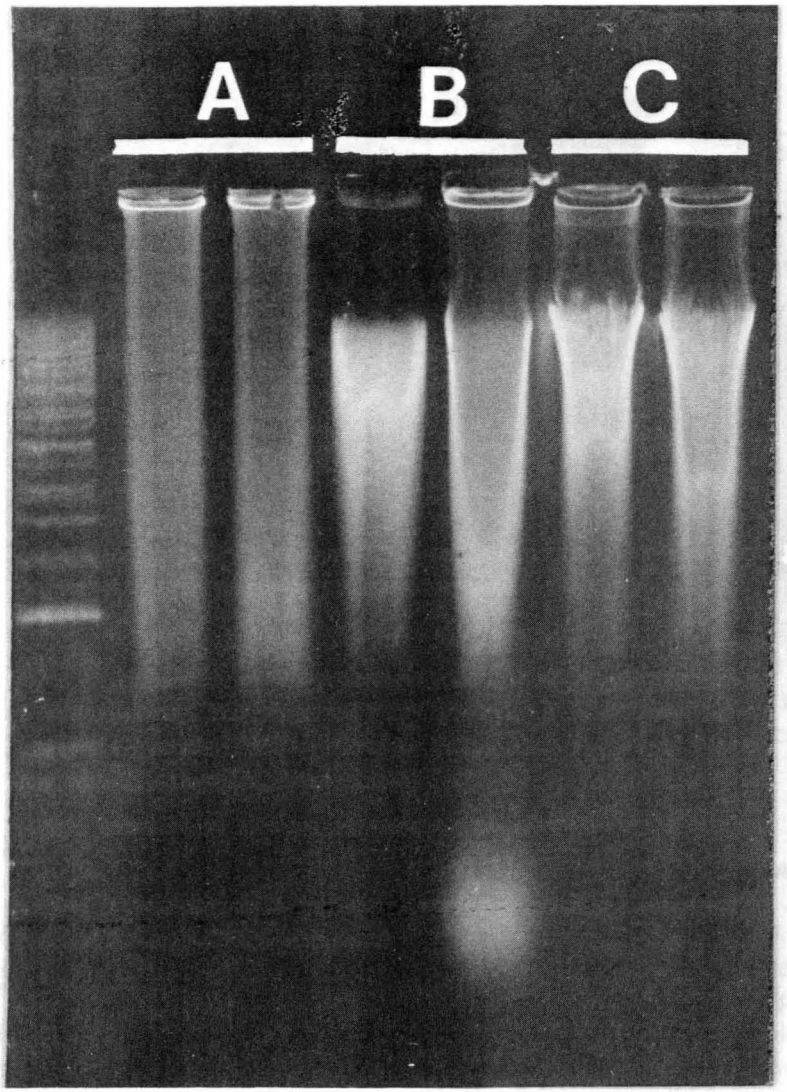

Figura 8. Coconut DNA extracted after the protocols by Klymyuk *et al.* (1993) (A), Edwards *et al.* (1991) (B), and Rogers and Bendich (1985) (C).

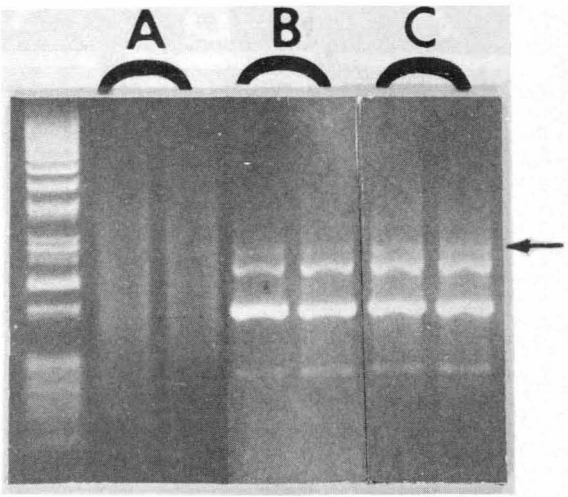

Figure 9. RAPD profiles generated with the decamer R13, and DNA extracted after the protocols by Klymyuk *et al.* (1993) (A), Edwards *et al.* (1991) (B), and Rogers and Bendich (1985) (C). The arrow indicates the position of the fragment putatively considered as $R13_{1500}$.

OPTIMISATION OF $MgCl_2$ CONCENTRATION IN THE PCR.

Figure 10 shows the banding profiles generated by the decamer B4 in response to a gradient of $MgCl_2$ concentration which ranged from 1.5 mM to 2.5 mM. The amplified fragments were barely perceptible at 1.5 mM, and their intensity increased as the $MgCl_2$ concentracion was greater. The RAPD of interest, $B4_{570}$, was clearly detected at the concentrations 2.25 mM and 2.5 mM. However, band sharpness was optimum at 2.5 mM. Similar comparisons performed with the other decamers under analysis indicated that their visualisation was optimum at, or close to, concentration values of 3.0 mM (B1, B11, AB3, AB4, AB16), 2.5 mM (B7, AB5, AL3) or 1.5 mM (A11, AL7). Ulterior screening of such decamers was done at their respective optimum of $MgCl_2$ concentration.

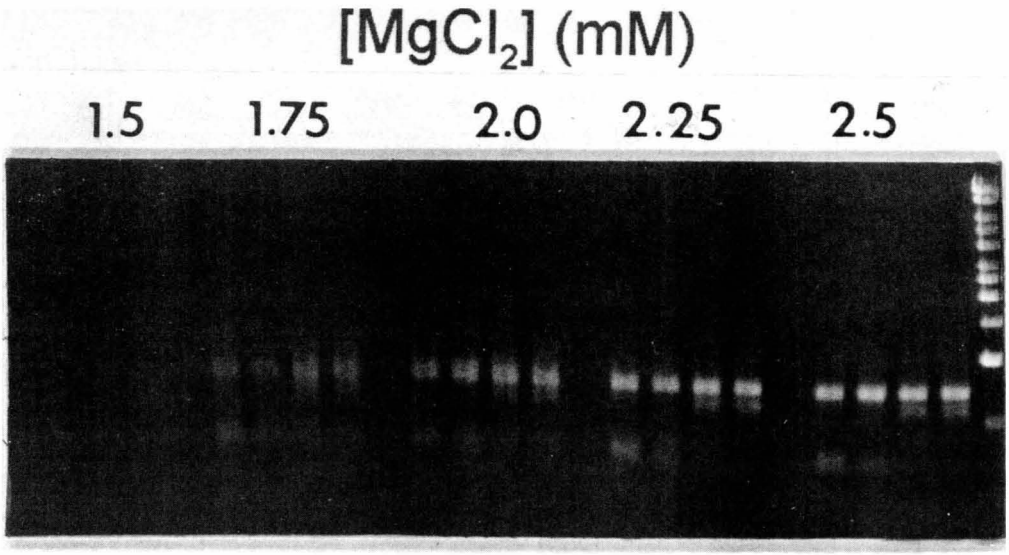

Figure 10. Variation of the RAPD profiles generated by the decamer B4, in response to a gradient of $MgCl_2$ concentrations. The arrow indicates the position of the RAPD $B4_{570}$.

RAPD SCREENING.

By screening the 80 primers chosen for this study in the MYD and WAT DNA pools, 33 were selected for their ability to generate polymorphic banding profiles. Such profiles revealed a total of 82 scorable RAPDs with sizes ranging from 350 to 1710 bp (Table 4, Figure 11). The distribution of such RAPDs in the MYD and WAT populations resulted in a further selection of 12 which appeared at frequencies 0.85 or greater in one of them, and 0.15 or lesser in the other (Table 5, Figure 12). Those RAPDs were scored in the AT survivors, wherein their distributions ranged from 0 to 1.0 (Table 6, Figure 13). The null phenotypes of three of them, $OB4_{570}$, $OB7_{990}$ and $OAL3_{1160}$, appeared at frequencies greater than 0.5, that is 0.80, 0.60 and 1.0, respectively.

Table 4. Size (bp) of the scorable RAPDs generated by the primers analysed, in the DNA pools from the 'Malayan Yellow Dwarf' (MYD) and the 'West African Tall' (WAT).

DNA	A7	A11	A13	B1	B4	B5	B7	B11	B13	B16	B17
MYD		520	410	410	1140 380	1290 550	780 620	570	1710 1310 440	1130 580 950	
WAT	540 470	990	460	610	980 570		990	1140 480	800 610	520	690 420
	B19	B20	AB2	AB3	AB4	AB5	AB13	AB16	AB18	AB19	AB20
MYD	520	830 500	460	410		1310	780 690 490 430	780 600	680		460
WAT	1160	690	510	650	930 640	500	1250	450 990		860 550	1130
	AL3	AL7	AL8	AL9	AL11	AL12	AL14	AL16	AL18	AL19	AL20
MYD			1320 1060 430	570	970	860 500	1370	1080	430	790 660	1080
WAT	1160 930 540	1350 350				1370 430		370		1180	1210 700

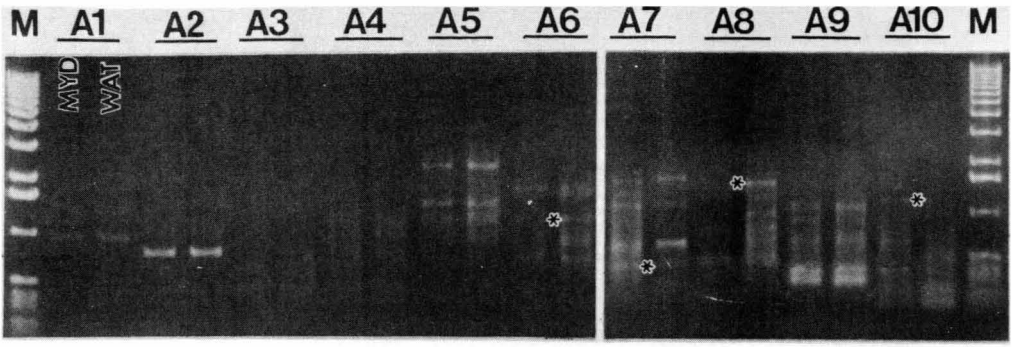

Figure 11. Banding profiles obtained with decamers from the series A. Each decamer was analysed, successively, with the DNA pools from the 'Malayan Yellow Dwarf' (MYD) and the 'West African Tall' (WAT). Lanes labeled as M show the molecular size markers of a 1 kb DNA ladder. The asterisks show the position of detectable polymorphisms.

Table 5. Size (bp) of the RAPDs selected in the analysis of the 'Malayan Yellow Dwarf' (MYD) and the 'West African Tall' (WAT) populations.

	Population	
Decamer	MYD	WAT
A11	990	520
B1		610
B4		570
B7		990
B11		1140
AB3		650
AB4		930
AB5		500
AB16		450
AL3		1160
AL7		350

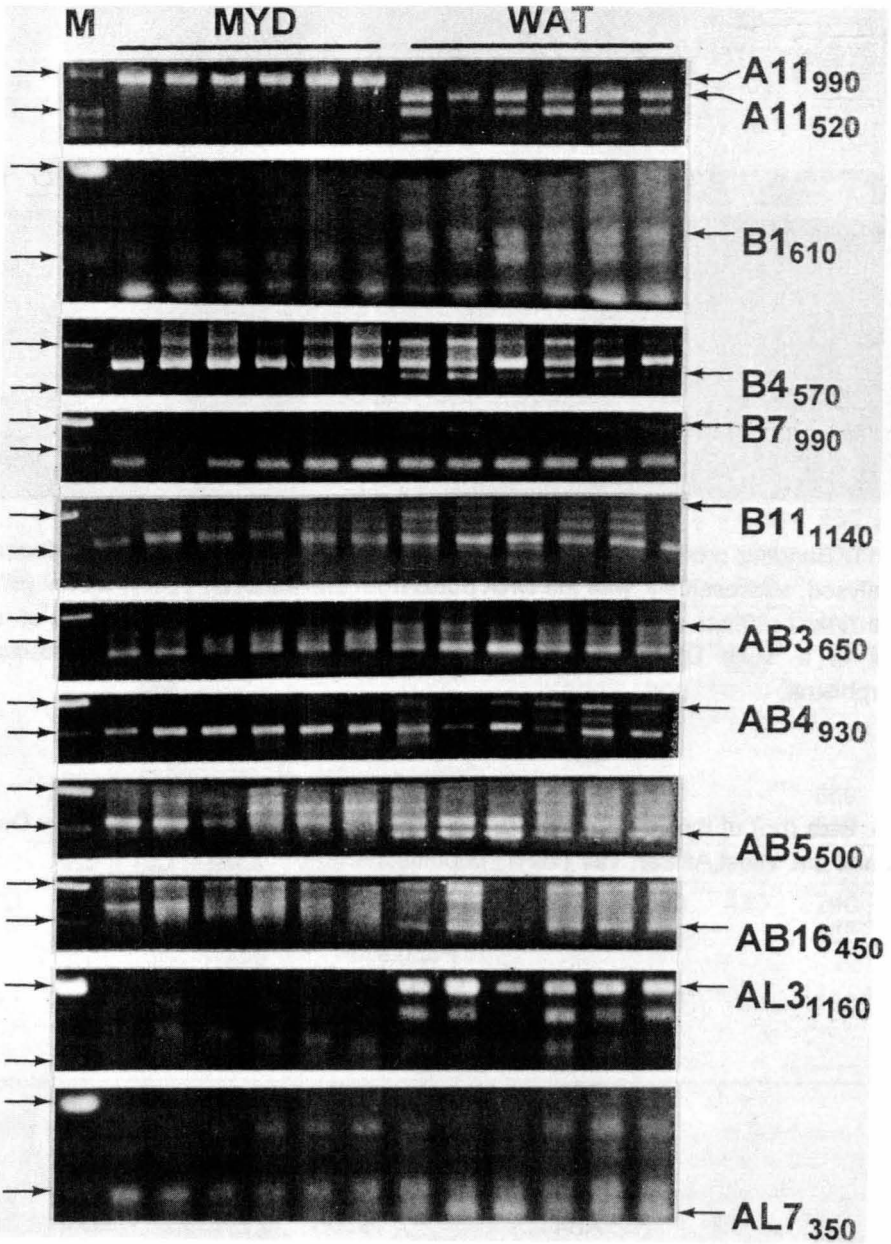

Figure 12. Banding profiles of the RAPDs listed in Table 5. MYD, 'Malayan Yellow Dwarf'; WAT, 'West African Tall'; M, 0.5 and 1 kb fragments (upper and lower arrows, respectively) from a 1 kb DNA ladder.

Table 6. Distribution of the RAPDs shown in Figure 6 within the AT survivors.

Individual	RAPD											
	$A11_{520}$	$A11_{990}$	$B1_{810}$	$B4_{570}$	$B7_{990}$	$B11_{1140}$	$AB3_{850}$	$AB4_{930}$	$AB5_{500}$	$AB16_{450}$	$AL3_{1180}$	$AL7_{350}$
1	1	0	1	1	1	1	1	1	1	0	0	1
2	1	0	1	0	0	1	1	0	1	1	0	1
3	1	0	0	1	1	1	1	1	1	0	0	1
4	0	0	1	0	1	1	1	1	0	1	0	1
5	0	0	1	0	1	1	0	0	1	1	0	1
6	1	0	1	1	0	1	1	0	1	1	0	1
7	1	0	1	0	1	1	1	1	1	0	0	1
8	0	0	0	0	0	1	0	1	0	1	0	1
9	1	0	1	0	1	1	1	0	1	0	0	1
10	0	0	0	0	0	1	0	1	1	0	0	1
11	1	0	1	0	0	1	0	1	1	1	0	1
12	1	0	1	0	0	1	1	0	0	1	0	1
13	1	0	1	0	0	1	0	0	1	1	0	1
14	1	0	1	0	0	1	0	1	0	1	0	1
15	1	0	0	0	0	1	0	1	1	1	0	1
16	1	0	0	0	1	1	1	0	1	1	0	1
17	1	0	0	0	0	1	0	0	1	1	0	1
18	0	0	0	0	0	1	1	1	0	1	0	1
19	1	0	1	1	1	1	1	1	1	0	0	1
20	1	0	1	0	0	1	1	0	1	1	0	1

1, present; 0, absent.

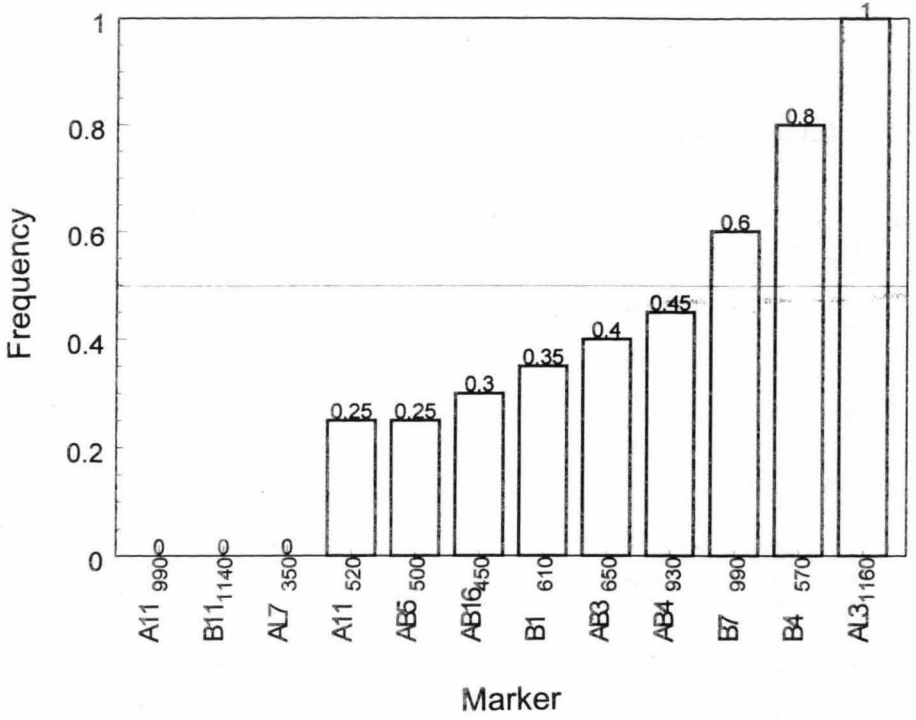

Figure 13. Frequency of the RAPDs shown in Figure 12 within the AT survivors. Except for $OA11_{990}$, names of RAPDs refer to their respective null phenotypes. The horizontal line represents the threshold frequency to consider a marker as potentially linked with LY resistance.

Discussion

The conjunction of several approaches to deal with high phenolic content tissues proved to be a valuable tool in retarding the oxidation and keeping the enzyme activities of protein extracts from coconut leaves, for up to six months under ultrafreezer storage. All the buffers so far used for the extraction of proteins from the coconut and other palms, that are aimed to genetic diversity analyses, include many components. Too, there is agreement on the problems that phenolic compounds impose to the extraction of proteins from these species (Al-Jibouri and Adham, 1990; Bennaceur *et al.,* 1991; Benoit and Ghesquiere, 1984; White et al., 1987). Thus, the extraction protocol devised for this study involved the union of various useful strategies in a fairly simple buffer, which proved to be remarkably convenient for the extraction of active enzymes from coconut leaves. That buffer should be particularly useful for similar purposes in other plants whose tissues are rich in phenolic compunds.

None of the previous isozyme variability studies in the coconut palm sought after the systematic characterisation of non-genetic effects on the expression of the protein systems analysed. The results of this work showed the occurrence of some marked effects of the leaf developmental stage on the electrophoretic profiles of the protein systems tested. This might explain the previous irreproducibility of results achieved when leaves were used as a source for the extraction of isozymes. The lack of recognisable effects of the other parameters tested (day time, transition from dry to rainy season) would be an additional advantage for any genetic polymorphisms that could be eventually identified. The reliability of such polymorphisms as tools for genetic analyses would be greater if they occur in otherwise stable protein systems. In relation to this topic, the results showed the electrophoretic patterns of anodic peroxidases (hereafter named simply as peroxidases), endopeptidases and coomassie blue-stained proteins, as the most feasible candidates for further application in the study of coconut genetic diversity. With regards to the interest in identifying markers for LY resistance, it was particularly interesting that numerous studies with peroxidases had associated this group of enzymes with disease resistance in different plant-pathogen interactions *(e.g.* see Baaziz and Saaidi, 1988).

Polymorphic banding patterns were detected with the three protein systems evaluated. The analysis of those polymorphisms in the cultivars tested showed them to be homozygous for at least two of the three protein loci described in Table 1. The *Per1* heterozygotes detected in WAT (Table 2) could be due to cross-pollination of the mother palms, as this cultivar is known to be allogamous (Rognon, 1976). It is also possible that *Per1*-F is a normal component of the genetic structure of the population analysed. The presence of the CP A phenotype in CRD suggests that *CP1*-A is a normal component of its genetic structure, as this cultivar is autogamous (Rognon, *op.*

cit.). In such a case, the highest frequency of this allele would be 0.095, corresponding to the event that the two palms with A phenotype are homozygotes. In WAT, the allele frequency observed for *Per1*-F is 0.024.

Such low allele frequencies are susceptible to become zero in successive generations by genetic drift effects. However, for the alleles to be reliably applied in the differentiation of the studied cultivars, pure genotypes should be selected by removing the palms with minor alleles. Should this be done, the classification scheme of Table 7 would be applicable. It shows three combinations of homozygous genotypes; one of them corresponds to the dwarf cultivars, and the others correspond one to each of the tall cultivars. Assuming that such genotyping scheme is applicable, the populations of PB121 and PB111 showed both the expected phenotypes (PER T, ENP D, and CP A) and those which would correspond to their respective dwarf progenitor. Depending on the system, the percentages of plants with the expected, hybrid phenotypes, appeared in 81 to 90 per cent of PB121, and in 86 to 90 per cent of PB111. The differences would be a consequence of the sample size used.

The populations of PB111 and PB121 used in this study are non-selected progenies from crosses performed by IRHO's program for hybrid seed production. By using the petiole color marker, Sangare and Rognon (1980) estimated the pollination efficiency of this program to range from 93 to 97 per cent. This estimate was made with progenies from crosses involving yellow and red dwarves as female progenitors, during the nursery stage. The higher than expected percentages of plants with dwarf phenotypes could have occurred as analyses were performed with adult plants, after possible contaminations or unintended selections during transport, quarantine, germination and final planting in the field.

With regard to the possibility of linkage of the protein alleles with LY resistance, the lack of significance for the differences observed between their distributions in Ca1 and Ca2 suggests that none of them is linked with the resistance trait. However, under the assumption that LY resistance is controlled by QTL, the extent and type of selection that would occur over any contributing allele are by no means predictable. Those factors would rely on the relative contribution of that allele to the resistance trait, and on its interactions with other contributing alleles that may be present. That is, although no positive evidence of linkage of the studied alleles with LY resistance was found, the existence of such linkages can not be ruled out.

The comparative analysis of three protocols for DNA extraction, in relation with their applicability in the next steps of this work, indicated that both the Edwards *et al.'s* and the Rogers and Bendich's protocols yielded DNA extracts that were suitable for use in RAPD analysis of coconut palms. However, the Rogers and Bendich's protocol gave the best DNA extracts and amplified more intense bands in the PCR, which determined to prefer it for the routine, subsequent analyses. In addition, evidence was provided in

support of the possibility of using freeze-drying followed by refrigeration as a means for the long-term preservation of coconut samples intended for PCR-based analyses.

Concerning the application of the genetic subtraction strategy, it permitted the selection of three markers which fulfilled the criteria to consider them as potentially linked with loci controlling LY resistance. That the AT plants analysed were indeed LY survivors is strongly suggested by the severity and duration of the outbreak to which they were exposed, and by the stabilization of their survival down to the resistance level expected for similar genotypes. Aditionally, the negative results from the PCR diagnoses suggest that such survival is possibly a consequence of defence mechanisms that impede the acquisition of the pathogen, or hinder their subsequent distribution. These aspects remain to be unveiled by future research, and are of utmost importance in considering the type of resistance to which the markers selected in this work could be linked. Genetic evidence for such a linkage would require to perform segregant analysis, a goal attainable at the expense of many years of concerted efforts between breeders, farmers and molecular geneticists.

Conclusions and perspectives

This work achieved for the first time reproducible results for the electrophoretic analysis of native proteins from coconut leaves. By this approach, genetic markers were identified that can be used in breeding programs, as well as in basic studies on population genetics and reproductive biology. Applications of the markers include:

1) Cultivar differentiation. The genotyping scheme of Table 7 would be applicable if populations that are homozygous in *Per1*, *Enp1* and *CP1*, are selected. Such a scheme does not differentiate MYD and CRD from each other, for which appropriate markers remain to be identified.

2) Progeny legitimacy. Assuming that the genotyping scheme of Table 7 is applicable, eight out of the twelve performable hybrids would have phenotypes different to those of the female progenitor (Table 8). RLT x Dwarf and Dwarf x Dwarf hybrids would have phenotypes identical to those of the mother palms.

3) Estimation of pollen contamination. Distances between seed orchards and neighboring plantations that prevent pollen contamination depend on the environmental conditions and the type of pollinators occurring in the region (Ashburner, 1995b; Free *et al.*, 1975; Hedström, 1986). Such distances need to be experimentally determined, and the protein markers would be of great value in estimating pollen contamination when the above cultivar combinations are involved.

4) Studies on breeding systems. Four breeding systems have been identified in the coconut palm which range from strict allogamy, as in WAT, to strict autogamy as in MYD (Rognon, 1976). Outbreeding rates have been estimated only by the analysis of petiole color in some yellow populations from New Guinea (G.R. Ashburner, unpub.). The protein markers could be of aid in performing this type of study.

Table 7. Genotypes assignable to the studied cultivars.

Cultivar	Locus		
	Per1	*Enp1*	*CP1*
WAT	SS	FF	AA
RLT	FF	SS	AA
MYD	FF	SS	NN
CRD	FF	SS	NN

Table 8. Hybrids with phenotype combinations that would differ from those of the female progenitor.

Hybrid	Phenotype		
	PER	ENP	CP
Dwarf x Tall			
MYD x WAT	T	D	A
CRD x WAT	T	D	A
MYD x RLT	F	S	A
CRD x RLT	F	S	A
Tall x Dwarf			
WAT x MYD	T	D	N
WAT x MYD	T	D	N
Tall x Tall			
WAT x RLT	T	D	A
RLT x WAT	T	D	A

As regards to the RAPD analyses, the screening strategy followed proved to be a quick and useful way to identify markers which can later be subjected to genetic analyses that confirm their applicability as tools in MAS. By starting with such a relatively low number of primers as 80, three were selected as candidates to generate an equal number of RAPDs linked with LY resistance (Table 9). In addition, as many as 82 polymorphisms were identified that can assist in studies on characterisation of coconut germplasm. Those 12 RAPDs which appeared at minimum frequency of 0.85 in either MYD or WAT are valuable tools to differentiate these genotypes, and could be used to assess the production efficiency of their hybrid in breeding programs aimed at this purpose (Bourdeix *et al.*, 1992).

Table 9. Number of primers and RAPDs selected in the successive steps of analysis.

	Initial	MYD and WAT DNA pools	MYD and WAT populations	AT survivors
Primers selected	A1 to A20, B1 to B20, AB1 to AB20, AL1 to AL20	A7, A11, A13, B1, B4, B5, B7, B11, B13, B16, B17, B19, B20, AB2, AB3, AB4, AB5, AB13, AB16, AB18, AB19, AB20, AL3, AL7, AL8, AL9, AL11, AL12, AL14, AL16, AL18, AL19, AL20	A11, B1, B4, B7, B11, AB3, AB4, AB5, AB16, AL3, AL7	B4, B7, AL3
No. of primers	80	33	11	3
No. of RAPDs		82	12	3

Schemes for QTL tagging similar to that undertaken in this study could be applied to other perennial crops which, like the coconut palm, face such problems as long generation times, poor seed production, and/or inefficient methods of vegetative propagation. The use of such schemes would rely on the availability of genotypes expressing contrasting phenotypic scores for the trait of interest. Difficulties to perform formal genetic analyses could mean for those crops that this type of strategy represents the closest approach to identifying markers applicable in the selection of desirable traits.

The current interest on the topic addressed in this work is attested by recent reports dealing with the use of isozymes and different DNA markers in analyses of coconut genetic diversity. Fernando and Gajanayake (1997) tested six enzyme systems for the characterisation of germplasm from Sri Lanka. By using starch gel electrophoresis, they detected only two polymorphic loci, one of peroxidases and the other of endopeptidases. Although no genetic determinism was proposed for the polymorphisms observed, their peroxidase patterns are similar to those of the *Per1* locus described in this study. RAPDs were used by Ashburner *et al.* (1997) to analyse material from the South Pacific region, and a more comprehensive study including further accessions from Asia and the Indian Ocean was performed with RFLPs by

Lebrun *et al.* (1998). RAPDs were also used by Wadt *et al.* (in press) to reveal the genetic diversity between- and within- tall ecotypes from a germplasm collection in Brazil. In another work, RAPDs and other PCR-based markers were tested with germplasm from Africa and the Philippines to compare their applicability in genetic diversity studies, and to construct a linkage map from a Dwarf x Tall cross (Rohde *et al.*, in press). The mapping population used in this study is intended to serve for future attempts to identify markers linked with such characters as dwarfism and early bearing.

For the purpose of germplasm characterisation, the present study showed that RAPDs are more appropriate than isozymes. Indeed, isozyme analysis relies on diverse and sometimes cumbersome techniques of visualization, which demand a great number of chemicals. In contrast, new RAPDs can be generated by simply changing a single reagent, an oligonucleotide, in a basic protocol. It should be noticed, however, that RAPD analysis is demanding in terms of the equipment, training and skills required to obtain reproducible results. For some routine applications of MAS, as is the case for cultivar differentiation and progeny legitimacy, breeders might find protein analysis easier to perform.

Concerning the future of DNA marker applications in coconut, current trends for other crops indicate an increasing preference for PCR-based markers like RAPDs, amplified fragment length polymorphisms (AFLPs), and sequence tagged microsatellite (SSMS) markers. RFLPs are renowned for their reproducibility and, as mentioned above, have also been used in coconut. However, in comparison with PCR-based markers, their analysis is expensive, cumbersome and time-consuming. Recent research has resulted in the identification of 41 coconut SSRs which are being tested for their usefulness to detect polymorphisms in populations from diverse geographic regions (A. Karp, upubl.). This work is currently continued as a British-French collaboration which intends, as a final output, to implement workshops for the transfer of a technology package that includes kits of primers, and standardised protocols for the PCR analyses. In any case, the protein and RAPD markers described in this work, with any other coconut genetic markers that may be described in the future, will broaden the alternatives available to breeders of this perennial crop.

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