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IMPROVED PROTOCOL FOR AFLP ANALYSIS AS A BASE FOR TAGGING ROOT (WILT) RESISTANCE GENES IN COCONUT

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ABSTRACT

The root (wilt) disease of coconut is endemic in Southern districts of Kerala and the adjoining areas of Tamilnadu causing enormous loss of nuts. Integrated management practice is the only suggested method to reduce its effect to some extent while development of resistant varieties will offer permanent solution, for which concrete breeding efforts are required. Considering the long life cycle of coconut, selection of resistant varieties through conventional methods will be time consuming and laborious. Molecular markers offer numerous advantages over markers traditionally used in plant mapping and selective breeding.

AFLP (Amplified Fragment Length Polymorphism) is a versatile DNA fingerprinting technique for marker assisted selection which combines universal applicability with high powers of discrimination and reproducibility. In this preliminary study for tagging root(wilt) resistance genes in coconut, AFLP parameters, gel running and silver staining conditions were optimized. Also, primer pairs detecting variations between resistant and susceptible palms were selected.

INTRODUCTION

The coconut root (wilt) disease is a debilitating disease which was reported over a century ago in Kerala. It is contiguously prevalent in eight Southern districts of Kerala and sparsely in isolated tracts in a few Northern districts of Kerala and in areas of Tamilnadu adjoining Kerala. The disease is characterized by abnormal bending or ribbing (*'flaccidity'*) of the leaflets, foliar necrosis and marginal necrosis (Fig.1). The annual loss due to this disease is estimated to be about 968 million nuts. This disease has no definite control measures. Although integrated management practices has been recommended to increase the productivity of palms in severely affected areas, the permanent solution can be possible only if a resistant/tolerant variety is available. To improve the yield of coconut, it is therefore necessary to (i) screen available germplasm for disease resistance/ tolerance and other important traits and identify gene(s) responsible, (ii) introgress these gene(s) into high yielding varieties to get disease resistant/ tolerant high yielding varieties/hybrids and (iii) large scale propagation of these resistant/tolerant high yielding palms.

At CPCRI, a large collection of coconut germplasm is being maintained and evaluated for resistance to root (wilt) disease. None of the so far tested accessions are found to be completely resistant/tolerant to root(wilt) disease. However, some disease free high yielding West Coast Tall (WCT) and CGD (Chowghat Green Dwarf) palms have been located in the heavily diseased areas (*hot spots*) in districts of Southern Kerala. A comprehensive breeding programme for evolving resistant/tolerant coconut varieties is being implementerd at CPCRI from 1988 (Nair *et al.*, 1996). The disease-free, high yielding palms located in *hot spot* areas are being used in crossing programmes (Fig.2).

Fig. 1. Coconut palm showing symptoms of root (wilt) disease

Molecular markers offer numerous advantages over morphological markers traditionally used in plant mapping. Considering the long life cycle of coconut, the task of examining

myriad individual palms to identify the presence or absence of the marker is arduous and time-consuming. Consequently, breeders are abandoning this traditional approach in favour of the much faster, more highly discriminating and less costly approach of using molecular DNA markers such as RFLPs, RAPDs, SSRs and AFLPs.

Fig. 2. Healthy and high yielding CGD mother palm

Identification and tagging of genomic region(s) to the expression of resistance/tolerance against the root(wilt) disease using molecular markers in these palms will help not only in better understanding of the inheritance of resistance but also future crop breeding programmes to develop resistant/tolerant palms either by conventional methods or through biotechnological means. Also, breeders can select for the desirable traits at the seedling stage itself rather than at the adult plant stage. This early selection of trait can save a lot of time in a long duration crop like coconut.

AFLP (Amplified Fragment Length Polymorphism) produces highly complex DNA profiles by arbitrary amplification of restriction fragments ligated to double stranded adaptors with hemi-specific primers harboring adaptor complementary 5′ termini (Vos *et al.*, 1995). The most straightforward applications of AFLP technique in marker-assisted breeding include genetic distance analysis, variety identification, isolation of markers tightly linked to specific genes and marker assisted backcrossing. The most efficient method for visualizing single-strand DNA in polyacrylamide gels uses radioactive or fluorescent labeling of nucleotides. These procedures require special facilities which are expensive and time consuming, rendering them impractical in most tropical countries where sophisticated infrastructures are lacking.

In this study, AFLP parameters were optimized for coconut. Gel running and silver staining conditions were also standardized. Primer pairs detecting variations between resistant and susceptible palms were also identified.

MATERIALS AND METHODS

Collection of leaf samples from resistant/ susceptible palms

Field trips were undertaken in the root (wilt) *hot spot* areas of Kollam, Allapuzha, Pathanamthitta and Kottayam districts of Kerala. Leaf samples were collected from resistant mother palms already identified by plant breeders and also susceptible palms. The criteria used for the selection of resistant mother palms (Nair *et al.*, 2001) is given in Table 1. A total of 130 samples were collected, the details of which are given in Table 2.

Table 1 : Selection criteria for resistant parental palms

Sl. No.	Character
1	Palms should be more than 30 years old (for talls) and 20 years old (dwarfs)
2	Palms should be regular bearers and high yielding.
3	Palms should be free from all pests and diseases and surrounded by palms of which at least 80% are affected by root (wilt) disease in an endemic area.
4	Mother palms should show typical cultivar characters.
5	Palms should be serologically negative and the serological tests are to be repeated every year.

Table 2 : Details of coconut palms from which leaf samples were collected

Sl. No.	Details of materials	No. of samples
1	Resistant palms (Talls)	37
2	Resistant palms (Dwarfs)	45
3	Susceptible palms (Talls)	43
4	Susceptible palms (Dwarfs)	5
	Total	**130**

DNA extraction, purification and quantification

DNA was extracted from the leaf samples using the CPCRI protocol (Upadhyay *et al.*,1999) and quantified. The purity of DNA was also tested on a 0.8% agarose gel as well as spectrophotometrically. The absorbance ratio A260/A280 was more than 1.8 and the molecular weight was more than 25 Kb indicating good quality of DNA. The average yield of DNA was 200 ìg/g FW.

AFLP parameters

The AFLP reaction was performed as described by Vos *et al.*, 1995, but with half the quantity of enzymes and primers provided in the manufacturer's (GIBCO-BRL, Life technologies) kit. 64 primer combinations were initially used for AFLP analysis of 'bulked' DNA of root(wilt) diseased and susceptible palms (*Talls*).

125 ng DNA was digested with EcoRI/MseI in 12.5 ìl of 1X reaction buffer. After restriction, the reaction mix was ligated to EcoRI/MseI adaptor solution in 25 ìl reaction volume. The ligated mixture was diluted 1:10 times. 2.5 ìl diluted DNA was used as template for preamplification. To this 20 ìl preamplification mix, 2.5 ìl of 10X PCR buffer and 0.5 U of Taq DNA polymerase were added. PCR was 20 cycles at 94°C for 30s, 56°C for 60s and 72°C for 60s. The reaction was diluted 1: 50 with sterile water. 2.5 ìl was selectively amplified in a 10 ìl reaction. PCR was one cycle at 94°C for 30s, 65°C for 30s, 72°C for 60s, annealing temperature was lowered each cycle by 0.7°C during next 12 cycles. Lastly, 23 cycles at 94°C for 30s, 56°C for 30s, 72°C for 60s were carried out.

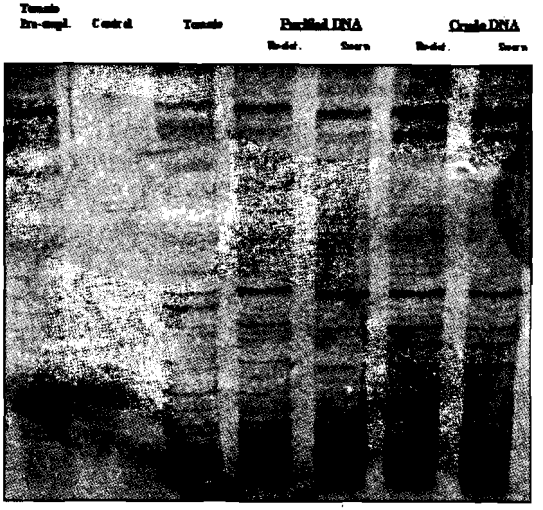

Fig. 3. AFLP analysis of bulked coconut DNA samples (resistant and susceptible) using E-ACC/M-CAA primer combination

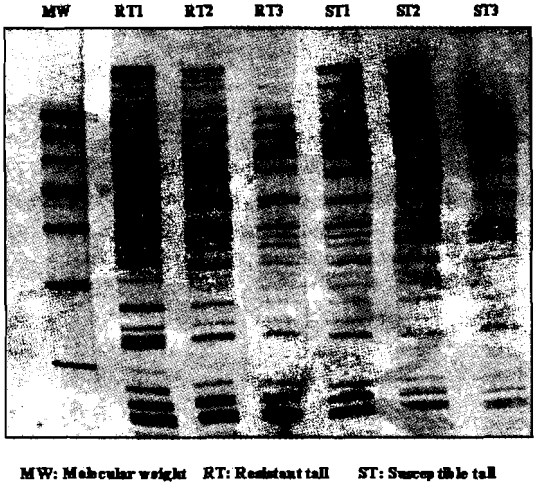

Fig. 4. AFLP analysis of individual palms using E-AAC/M-CTT primer combination

Electrophoresis on acrylamide gels

After selective amplification, the amplified products were diluted with an equal volume of dye (40% urea, 3% Ficoll, 0.02% xylene cyanol FF, 0.02% bromophenol blue in EDTA buffer) and were denatured at 90°C for 3 minutes and immediately placed on ice. 3ml of the products was then resolved on either a 5% or 6% denaturing

polyacrylamide gel (with 7M urea) and run at a constant power of 55W until xylene cyanol (slower dye) was two-thirds down the length of the gel.

Silver staining of gels

The acrylamide gels were silver stained using one of the two protocols: Bassam *et al.*, (1991) or the improved protocols of Beidler *et al.*, (1982) and Creste *et al.*, (2001). After staining, the gels were preserved by drying at room temperature.

Scoring of bands

The number of scorable bands, clarity of banding pattern and the number of polymorphic bands were recorded for each primer pair. Primer pair detecting variations between resistant and susceptible palms (bulked samples) were used for screening variations between individual palms.

RESULTS AND DISCUSSION

The use of reduced quantities for AFLP reactions allowed a savings of at least 10 units of enzymes (polymerase, ligase, kinase) per complete reaction compared to the quantities used in the original protocol described by Vos *et al.*, 1995. A total of 64 EcoRI/MseI primer pairs were screened to detect polymorphism between resistant and susceptible palms. Better separation of the amplified products was obtained on a 5% denaturing acrylamide gel compared to a 6% gel. Both the silver staining protocols yielded comparable results.

Out of the primers tested, 61 primer pairs gave good amplification. The amplification profiles contained on an average 40-80 scorable bands. This indicates that AFLPs have a clear advantage over other molecular markers in terms of number of amplicons amplified per reaction. Eleven primer pairs could detect polymorphism between resistant and susceptible palms (bulked samples) (Fig.1). The details of the primer pairs detecting polymorphism are given in Table 3. These primers were then used to detect variations between individual resistant and susceptible palms (Fig.2) and will be used to screen samples from segregating populations.

The understanding of mechanism of root (wilt) resistance/tolerance and any information on the coconut genetic organisation and genetic inter-relationship will definitely provide a fillip to ongoing programmes to develop resisitant/ tolerant cultivars. Therefore, the preliminary results obtained in this study gain importance in the context of current status of coconut genetic research as they will provide procedures and data, for identification of genomic regions conferring resistance/tolerance to root (wilt) disease and elucidation of genetic organisation with the help of recent molecular biology techniques. Also, these markers will be useful for developing efficient and reliable screening procedures to select desirable progenies of a cross at an early stage.

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Table 3 : AFLP primer combinations detecting polymorphism between resistant and susceptible palms

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	#	#	#					#
E-AAG							#	
E-ACA	#	#						
E-ACC	#				#		#	
E-ACG			#					
E-ACT								
E-AGC								
E-AGG								

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