DNA amplification fingerprinting in coconut: protocol optimization and analysis of resistance to root (wilt) disease

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Abstract

Root (wilt) disease is a serious malady in coconut causing a loss of approximately 968 million nuts a year in India. Integrated management practices are the only suggested methods to reduce its effect to some extent, while the development of resistant varieties will offer a 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. Here, we present the optimization of PCR conditions for DNA amplification fingerprinting (DAF) of coconut using arbitrary oligonucleotide primers. Three of the primers could detect variations between root (wilt) resistant and susceptible coconut palms. This study constitutes the basis for future efforts to tag the root (wilt) resistant gene(s) in coconut.

Key words: Root (wilt) disease, DNA amplification fingerprinting, oligonucleotide primers, coconut, host plant resistance.

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Introduction

Root (wilt) disease is one of the most serious maladies affecting the coconut palm in India. This disease was reported for the first time from Erattupetta area of Meenachil Taluk in Kottayam district of Kerala State of India in 1884 (Butler, 1908; Pillai, 1911). It has since then spread from the original foci of infection and currently occupies a contiguous area covering eight districts of Kerala and the adjoining areas of Tamil Nadu State. The diagnostic symptoms of the disease are the characteristic bending of the leaflets termed as 'flaccidity', yellowing and necrosis (Radha and Lal, 1972). The disease is debilitating in nature, but not lethal, and gradually reduces the yield of nuts, ultimately rendering the palms nearly barren. The annual loss due to this disease is estimated to be 968 million nuts (Anonymous, 1985). Leaf rot occurs superimposed over >60% of the root (wilt) affected palms (Sreenivasan, 1991). Integrated management practice has been recommended to increase the productivity of palms in severely affected areas (Muralidharan *et al*., 1986; Mathewkutty, 1998). As the disease cannot be controlled by conventional methods, development of resistant varieties is the only practical solution.

To develop resistant varieties, it is necessary to (i) screen available germplasm for disease resistance / tolerance, along with other important yield contributing traits and identify gene(s) responsible, (ii) introgress these gene(s) into high yielding varieties to get disease resistant/ tolerant high yielding varieties and hybrids and (iii) large-scale propagation of these resistant /tolerant high yielding palms.

Earlier studies have shown that none of the so far tested coconut accessions and hybrids are completely resistant / tolerant to root (wilt) disease (Jacob *et al*., 1998). As an initial step towards breeding for resistance / tolerance to root (wilt) disease, an intensive survey covering the heavily root (wilt) affected areas ('hot spots') of Kottayam, Alappuzha, Pathanamthitta and Kollam districts of Kerala State was initiated during 1985 (Anonymous, 1986). Some disease-free, high yielding cultivars such as West Coast Tall and Chowghat Green Dwarf were identified by plant breeders in these areas and these palms are being used in breeding programmes since 1988 (Nair *et al*., 1996).

Considering the long life cycle of coconut palms and their low multiplication rate, the task of examining myriad individual palms to identify the presence or absence of morphological markers for resistance is an arduous and time-consuming job. Molecular markers (RFLP, RAPD, AFLP, SSR and DAF) offer numerous advantages over morphological markers traditionally used in plant mapping, as they are much faster, more highly discriminating and less costly. They have become powerful tools for marker-assisted selection of resistant genotypes in many crop species.

DNA amplification fingerprinting (DAF) employs single arbitrary primers as short as 5 nucleotides in length to produce characteristic and highly informative DNA patterns (Caetano-Anollès *et al*., 1992). These patterns are adequately resolved by polyacrylamide gel electrophoresis and silver stained. DAF can be distinguished from other genome scanning techniques by high primers to template ratio, simplicity, and excellent reproducibility. DAF has been used for identity testing phylogenetic relationships, population and pedigree analysis, molecular characterization, high density mapping, tagging useful genes and marker assisted selection (He *et al*., 1995; Yazdi-Samadi *et al*., 1996; Caetano-Anollès *et al*., 1995; Shahnejat *et al*., 1999; Richard-Molard *et al*., 1999; Assefa *et al*., 1999).

A typical DAF reaction involves the use of a single primer usually composed of 8 to 12 nucleotides, a length that approaches the minimum configuration for DNA amplification (Caetano-Anollès *et al*., 1992; Vincent *et al*., 1994). Amplification conditions discriminate bonafide amplicons from those of artifactual origin, and a non-stringent reaction environment ensures the reproducible targeting of multiple

sites (Caetano-Anollès *et al*., 1991). To guarantee optimal performance and reproducibility, a good understanding of primer design and a careful optimization exercise of amplification parameters are required. The specificity, efficiency and fidelity of DNA amplification are strongly influenced by the different components of the reaction (such as primer, magnesium and deoxynucleotide triphosphate concentrations) and are modulated by thermocycling conditions (such as annealing temperature). These conditions need to be optimized to avoid non-specific amplification products such as primer-dimers. Similarly conditions to detect DNA on gel need to be optimized to improve the efficiency of DAF analysis.

The present study was undertaken to optimize PCR conditions for DAF analysis in coconut and screening of primers for detecting variations between root (wilt) field resistant and susceptible coconut palms.

Materials and methods

Plant materials

The leaf samples were collected from 37 root (wilt) field resistant and 43 susceptible West Coast Tall palms already identified by a team of plant breeders and pathologists in the disease endemic areas of the four southern districts of Kerala state, *viz*., Kottayam, Alappuzha, Kollam and Pathanamthitta. The criteria followed for the selection of the field resistant/tolerant mother palms are: (i) the palms should yield 80 or more nuts per year; (ii) they should be regular bearers and be absolutely free from diseases and pests; (iii) the palms should be more than 45 years old and be surrounded by palms of which at least 80% are affected by the root (wilt) disease in an endemic area; (iv) they should have typical WCT characters and (v) the palms should be negative in their reaction to the root (wilt) antiserum and the serological tests are to be repeated every year (Nair *et al*., 1996).

DNA extraction and quantification

Total genomic DNA was extracted from the spindle leaves of the palms using protocol developed at CPCRI (Upadhyay *et al.*, 1999). Following ethanol precipitation, DNA was vacuum dried, resuspended in sterile TE buffer and stored at -20°C. The concentration of DNA samples was determined by spectrophotometric measurement at 260nm and by comparison with known amounts of DNA ladder (M/s Bangalore Genei, India) on a 0.8% agarose gel stained with ethidium bromide. For PCR amplification DNA concentration was adjusted to the desired concentration by diluting with sterile water.

Amplification conditions

The different PCR parameters tested are as follows:

- Template DNA: 10, 20, 30 ng
- Taq polymerase (M/s Bangalore Genei, India): 1,2,3,4 Units
- Magnesium chloride: 1.5, 4, 6.5, 9 mM
- Primer concentration (M/s GIBCOBRL Life Technologies, India): 1, 5, 10, 15, 20, 25, 50µM
- Annealing temperature: 45^{o}C, 50^{o}C, 55^{o}C
- dNTPs: 200, 250, 500, 750µM

DNA amplification was performed in a 10µl volume. To determine the effect of a single parameter, the given parameter was varied, keeping the rest constant. A negative control (blank) containing all components of typical PCR reaction except the template DNA was used in every experiment. The PCR amplification of DNA was performed on Eppendorf Gradient Master Cycler programmed for 3 minutes initial denaturation at 94^{o}C followed by 5 seconds of denaturation at 94^{o}C, 20 seconds annealing at 45^{o}C/50^{o}C/55^{o}C, 30 seconds of primer extension at 72^{o}C, and 5 minutes final extension at 72^{o}C for a total of 35 cycles. The PCR products were stored at -2^{o}C until electrophoresis. All the experiments were repeated thrice to confirm reproducibility of the results.

Primer screening

Sixteen arbitrary sequence decamers from the University of British Columbia (UBC) were screened for DAF analysis of coconut for detecting polymorphism between root (wilt) field resistant and susceptible palms. The details of the primers are given in Table 1.

Visualization of amplification products

Amplification products were separated by electrophoresis in 0.5 mm thick 5% denaturing polyacrylamide gel containing 8M urea. The gel was pre-run for 10 minutes and the wells were loaded with 6µl of a 1:2 dilution of each amplification reaction with loading buffer (40% urea, 3% Ficoll, 0.2% xylene cyanol, 0.2% bromophenol blue in 1X TBE buffer). The samples were denatured at 90^{o}C for 3 min and immediately placed in ice prior to loading. Electrophoresis was run at 18W constant power for 55 minutes. Molecular weights of bands were approximated using a 100bp DNA ladder (M/s Bangalore Genei, India). After electrophoresis, gels were fixed and silver stained using a slightly modified protocol of Bassam and Caetano-Anollès (1993). The gels, after staining, were lifted off on a Whatman paper (No. 1) and preserved by drying at room temperature.

Gel analysis

Silver stained gels were scanned using an image scanner (M/s Amersham Pharmacia Biotech) and MAGICSCAN software (M/s UMAX Data Systems Inc.) and the acquired images were analyzed with the programme TOTALLAB, version 2.00 (M/s Nonlinear Dynamics Ltd.). The number of bands was calculated for each primer.

Results and discussion

Annealing temperature

The selection of the annealing temperature is possibly the most critical component for optimizing the specificity of a PCR reaction. The annealing temperature is a function of the length and base composition of the primer as well as the ionic strength of the reaction buffer. Three annealing temperatures were tested 45, 50 and 55ºC. An annealing temperature of 45ºC gave more non-specific bands. Amplification at 55ºC gave good scorable bands compared to 45ºC and 50ºC (Fig. 1).

Template DNA

All the three levels of template DNA tested, viz., 10ng, 20ng and 30ng were amplified, of which DNA concentration of 20ng gave good scorable bands (Fig. 1).

Primer concentration

Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR. Of the seven levels of primer concentrations tested, viz., 1,5,10, 15, 20, 25 and 50µM, all the levels gave amplification Primer concentration of 1µM gave very less amplification products compared to 10 µM and 15µM, but 15µm gave optimum amplification products. Primer concentrations of 20, 25, 50µM gave higher number of amplification products (Fig. 2). Higher primer concentration may promote mis-priming and accumulation of non-specific products and increase the probability of generating primer-dimer artifacts (Innis and Gelfand, 1990).

Concentration of Taq polymerase

The amount of Taq polymerase, which gave optimum amplification products was 2 Units per reaction (Fig. 3). When the Taq polymerase concentration was reduced, insufficient amounts of desired products were produced. Non-specific products were formed and the resolutions of the bands were decreased at higher enzyme levels.

Concentration of $MgCl_2$

Lower $MgCl_2$ concentration (1.5mM) yielded more number of amplification products while higher $MgCl_2$ concentrations (9.0mM)

failed to yield visible bands. The optimum $MgCl_2$ concentration in the reaction mix was 4.0mM (Fig. 4). The $MgCl_2$ concentration may affect one or all of the following: primer annealing, strand association temperature of both template and PCR product, product specific formation of primer-dimer artifacts and enzyme activity and fidelity (Innis and Gelfand, 1990).

Concentration of dNTPs

Of the four levels of dNTPs concentration tested, 200µM gave good and scorable bands (Fig. 5).

Primer screening

Sixteen primers were initially screened for amplification of coconut DNA (Fig. 6). Those primers, which gave optimum amplification, were then used for detecting variations between field resistant and susceptible palms. The number of scorable bands ranged from 2-29 (Table 1). Primers UBC66, UBC84 and UBC 729 could detect variations between root (wilt) field resistant and susceptible palms. A putative marker for resistance around 260 bp was detected using UBC 84 (Fig. 7) and around 254 bp using UBC 729 (Fig. 8). UBC 66 could detect a potential marker for resistance at around 460bp (Fig. 9).

Presently, the cultivation of coconut palms in southern Kerala region is dependent on planting material that is resistant / tolerant to root (wilt) disease. None of the coconut accessions or hybrids tested so far at CPCRI have exhibited complete resistance to the disease. Little is known about the action and inheritance of genes for resistance to root (wilt) disease of coconut. The preponderance of susceptible palms when compared to disease-free palms in the natural population and almost half of the palms in the segregating population taking up the disease in the juvenile stage itself suggests the possibility of the disease being governed by recessive genes (Nair *et al*., 2003). Cardena *et al*. (2003) have reported identification of RAPD markers associated with resistance to lethal yellowing disease of coconut palm in resistant Malayan Yellow Dwarf and Atlantic Tall populations. The identification of West Coast Tall and Chowghat Green Dwarf field resistant palms in the disease endemic areas of southern Kerala offers hope for marker-assisted selection (MAS) strategies for resistance to root (wilt) disease in coconut. This is the first study involving identification of molecular markers linked to root (wilt) resistance / tolerance and constitutes the basis for future efforts to tag the root (wilt) resistant gene(s) in coconut. The primers identified in the present study could be valuable for identification of resistant palms from among segregating populations at the seedling stage itself.

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Table 1. Details of UBC primers used in present study, their sequences and the number of scorable bands

Primer	Sequence 5'-3'	GC/AT	Number of scorable bands
UBC2	CCTGGGCTTA	6:4	21
UBC8	CCTGGGGGTA	7:3	4
UBC52	TTCCCGGAGC	7:3	20
UBC66	GAGGGCGTGA	7:3	17
UBC72	CAGCACGGGA	7:3	19
UBC83	GGGCTCGTGG	8:2	21
UBC84	GGGCGCGAGT	8:2	24
UBC87	GGGGGGAAGC	8:2	29
UBC88	CGGGGGATGG	8:2	18
UBC100	ATCGGGTCCG	7:3	19
UBC321	ATCTAGGGAC	5:5	2
UBC351	CTCCCGGTGG	8:2	12
UBC356	GCGGCCCTCT	8:2	20
UBC701	CCCACAACCC	7:3	8
UBC729	CCCAACCCAC	7:3	26
UBC800	TCTCCCTCCT	6:4	2

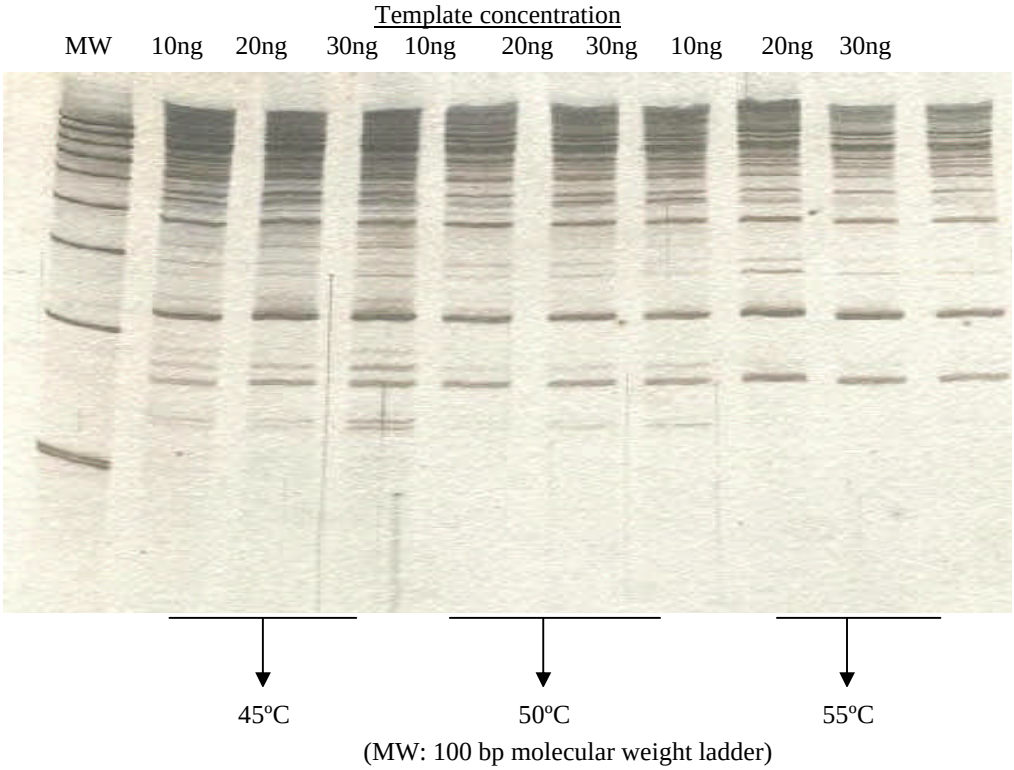
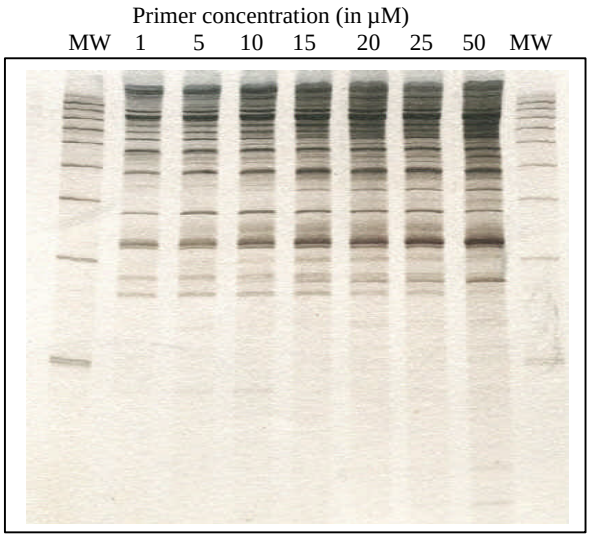
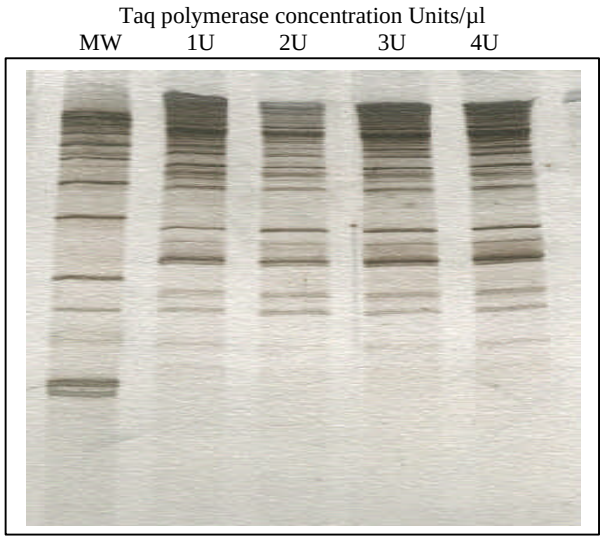


Fig. 1. DAF analysis in coconut with different annealing temperatures and template concentrations



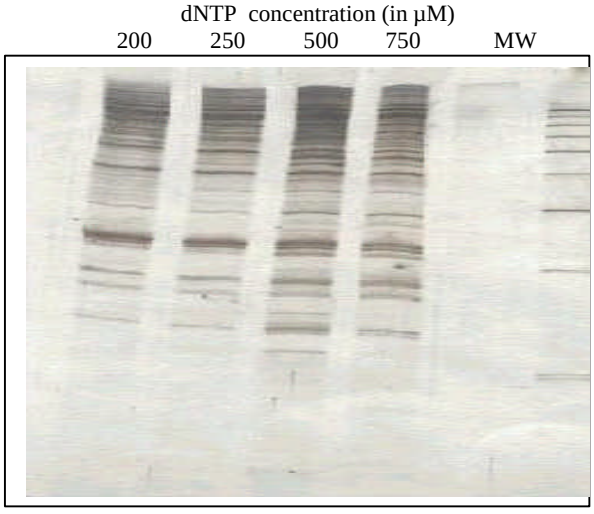
(MW: 100 bp molecular weight ladder)

Fig. 2. DAF analysis in coconut with different primer concentrations



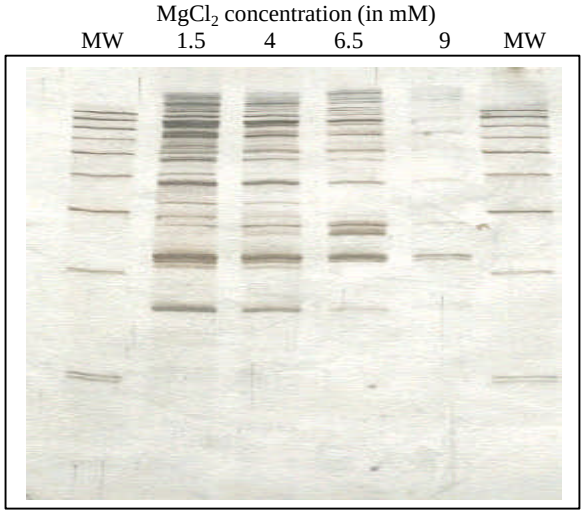
(MW: 100 bp molecular weight ladder)

Fig. 3. DAF analysis in coconut with different Taq Polymerase concentrations



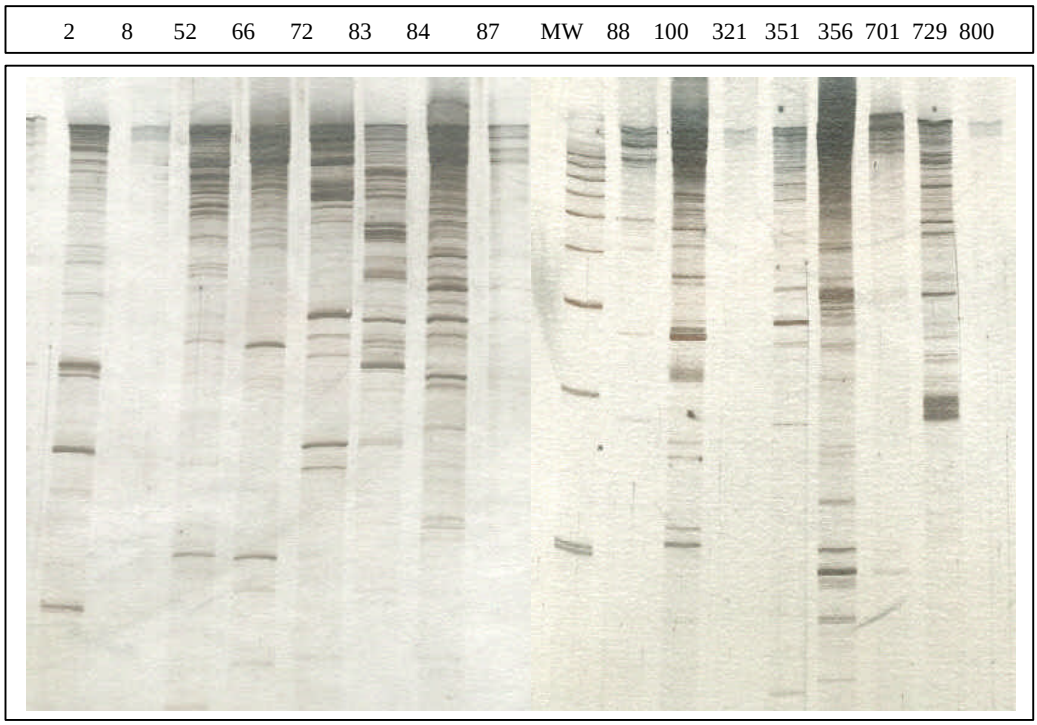
(MW: 100 bp molecular weight ladder)

Fig. 4 DAF analysis with different dNTP concentrations



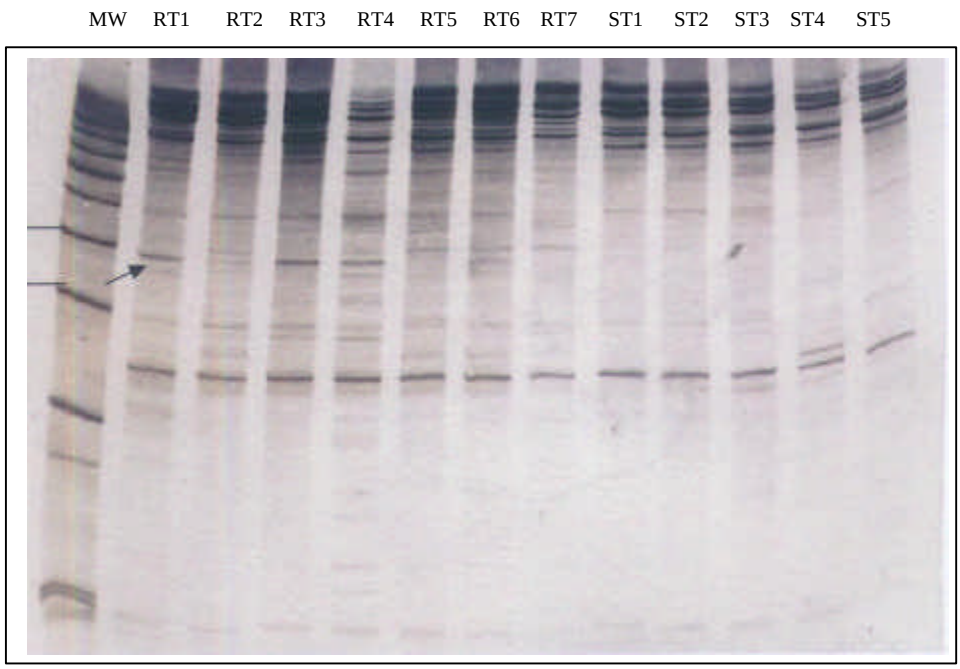
(MW: 100 bp molecular weight ladder)

Fig. 5. DAF analysis with different $MgCl_2$ concentrations

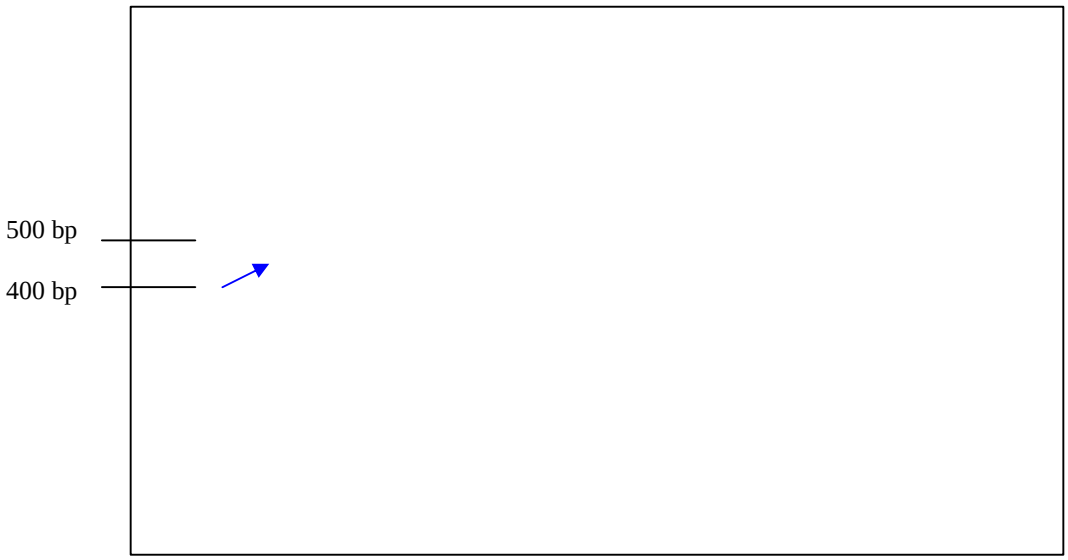


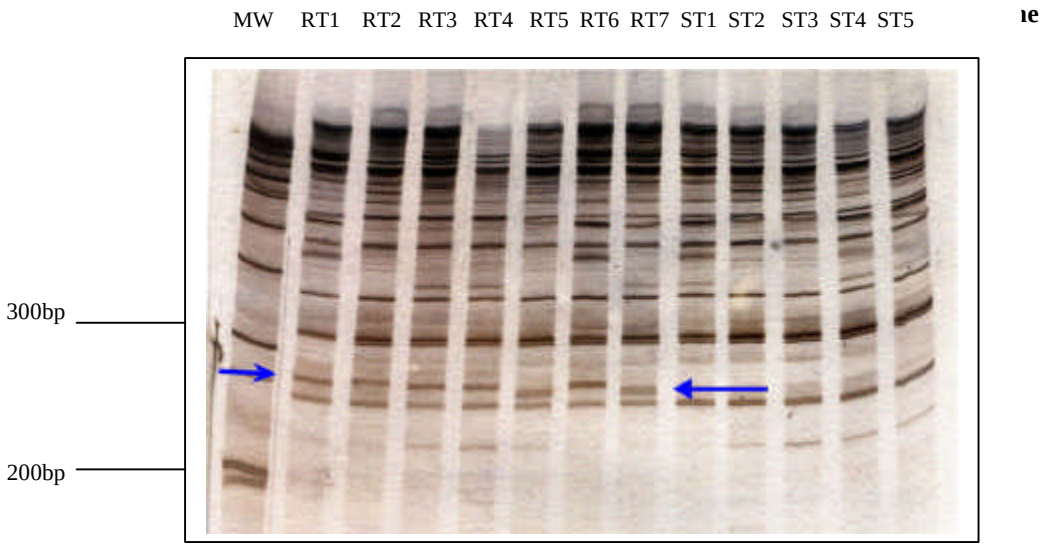
(MW: 100 bp molecular weight ladder)

Fig. 6. Screening of UBC primers for DAF analysis in coconut



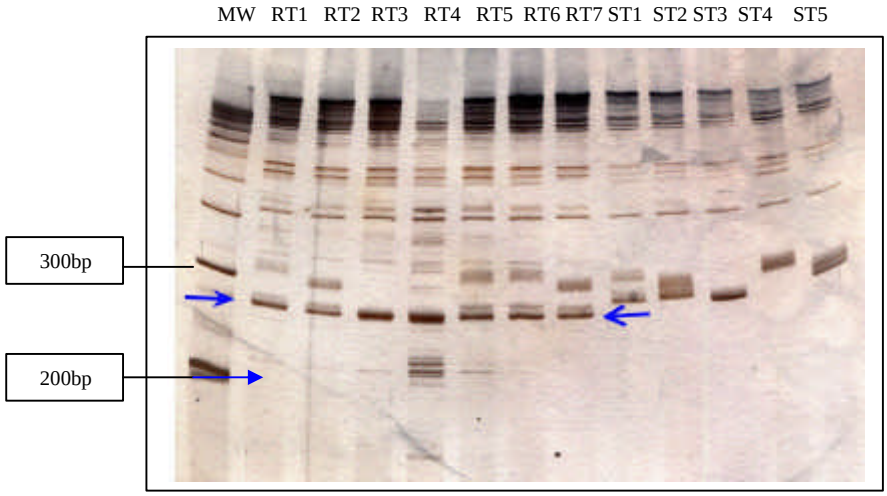
(MW - 100bp molecular marker; RT1 to RT7 Resistant Talls; ST1to ST5 - Susceptible Talls; Arrowhead indicates putative marker for resistance around 460 bp)





DAF analysis of UBC 66 primer: MW - 100bp Molecular marker
DAF analysis of UBC 66 primer: MW - 100bp Molecular marker
RT1 to RT7 Resistant Tall, ST1to ST5 - Susceptible Tall RT1 to RT7 Resistant Tall, ST1to ST5 - Susceptible Tall
(MW - 100bp Molecular marker; RT1 to RT7 Resistant Talls; ST1to ST5 - Susceptible Talls; Arrowhead indicates putative marker for resistance around 260bp)

Fig. 8. DAF analysis of root (wilt) field resistant and susceptible palms using the primer UBC 84



(MW: 100 bp molecular weight ladder; RT 1 to RT7: resistant mother palms; ST1 to ST5: susceptible palms; Arrowhead indicates putative marker for resistance around 254 bp)

Fig. 9. DAF analysis of root (wilt) field resistant and susceptible palms using the primer UBC 729

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