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Molecular marker-based genetic variability among Yellow Leaf Disease (YLD) resistant and susceptible arecanut (*Areca catechu.* L.) genotypes

R. Manimekalai*, K.P. Deeshma, K.P. Manju, V.P. Soumya, M. Sunaiba, Smitha Nair and K.S. Ananda**

Central Plantation Crops Research Institute, Kasaragod 671 124, Kerala

ABSTRACT

Yellow Leaf Disease (YLD) is a lethal disease affecting the arecanut palms of south India causing heavy yield loss. Presently, there is no cure for this disease and breeding for resistance is the only solution. Disease-free palms identified in hotspot areas (*i.e.*, heavily disease affected regions), may harbour special gene family. The present study aimed to differentiate YLD resistant areca palms with susceptible palms using three different marker systems, *i.e.*, RAPD, ISSR and resistant gene based markers. Amplification of arecanut genomic DNA using these marker systems yielded a total of 248 fragments, that could be scored, of which 130 were polymorphic between YLD resistant and susceptible individuals. Among these markers, RAPD generated greater polymorphic fragments (61.6%) than ISSR (34.9%) and resistant gene specific markers (40.7%). The primers, UBC 321, OPAF 15, and OPE13 produced distinct banding patterns for resistant and susceptible palms. The average genetic similarity coefficient for pair-wise comparison of individuals ranged from 0.73 to 0.88. The average similarity between the YLD resistant palms was found to be 0.79 and that of susceptible was 0.80. The highest similarity coefficient, 0.88 was observed between the YLD resistant individuals (R1 and R2). It has been found that the resistant and susceptible areca palms show relatively less genetic diversity and are skewed towards their phenotype. The results of this molecular characterization may provide starting points for map-based cloning of the YLD resistant genes.

Key words: Arecanut, Yellow Leaf Disease, ISSR, RAPD, resistant gene marker.

INTRODUCTION

Arecanut palm (*Areca catechu*) or betel nut palm is one of the most important commercial crops in India. Its cultivation is threatened by a number of diseases during different stages of its growth and development. Among these, the Yellow Leaf Disease (YLD) is the main problem faced by the arecanut cultivators of Kerala and Karnataka. Foliar yellowing is the most conspicuous symptom of YLD. Yellowing of leaves begin in the inner whorl, gradually spreading to the outer parts of the crown. Chlorosis is finally observed on almost all leaves in the whorl from the edges of the individual leaflet to the mid-rib region. Stem of the affected palms becomes spongy and friable, the conducting strands get destroyed. In advanced stages, the stem breaks off at the top. Rotting of the roots is also observed. Nuts are reduced in size and kernel turns into black (Rawther, 1). Phytoplasmal etiology of arecanut YLD in India was proved by electron microscopy (Nampoothiri *et al*., 2) and simple staining techniques (Ponnamma and Solomon, 3). Furthermore, Ponnamma *et al*. (4) identified the vector of the disease as *Proutista moesta*. The phytoplasma associated with the YLD was identified as 16s rDNA group XI through molecular methods (Manimekalai *et al.*, 5). Considering the long life cycles of areca palm, selection and characterization of resistant and susceptible varieties through conventional method will be time consuming and laborious. For genetic variability analysis, molecular markers are advantageous over other morphological markers in terms of unlimited number, ease, fast assay and accuracy (Garg *et al*., 6). Many individual and combined assays of RAPD and ISSRs were conducted and could prove the genetic discrimination among genotypes (Goswami *et al*., 7). Genetic diversity studies in *V. umbellate* suggested the equal effectiveness of RAPD and ISSR markers in detecting polymorphism (Muthusamy *et al*., 8).

Disease resistance often results from the presence of a specific resistance gene (R-genes) in the plant and a corresponding avirulence (*avr*) gene in the pathogen and many complex clusters of R-genes are common in plant genomes (Mutlu *et al.*, 9). Many different disease R-genes have been cloned from a variety of plant species. Polymerase chain reaction (PCR) analysis using gene specific primers are helpful to monitor the expression of individual genes in different tissues and developmental stages (Michelle *et al*., 10). Panwar *et al*. (11) could discriminate resistant from susceptible bulks using resistant gene primers and

*Corresponding author's E-mail: rmanimekalai@rediffmail.com

**Central Plantation Crops Research Institute, Regional Station, Vittal, Karnataka

they could clearly cluster the resistant genotypes from susceptible genotypes. Various successful genetic variability studies between resistant and susceptible plant varieties using molecular markers like ISSR, RAPD and AFLP are reported in other plants (Datta *et al*., 12; Wingbermuehle *et al*., 13; Claudia Ferreira *et al*., 14). In our study, two dominant markers, ISSR and RAPD and a gene specific marker were used to detect the molecular variability between YLD resistant and susceptible areca palms. No such reports on molecular based genetic variability were available in areca palms. Hence, the study presents the genetic variability among areca palm genotypes with reference to YLD resistance.

MATERIALS AND METHODS

Immature leaf samples were collected from the South Kanara local genotype at the diseased endemic area of Sullia, Karnataka. In this hotspot area, only 18 YLD resistant arecanut palms were identified, of which maximum 12 resistant samples were collected. Thus, the plant materials used in this study consisted of 24 arecanut palms, 12 each from YLD resistant (R1-R12) and susceptible (S1-S12) palms. Resistant gene primers were designed based on various classes of 'R'genes and their conserved motifs using softwares like Fast PCR and Primer 3 (Table 1). Class members of different plant resistant gene sequences with specificities against plant pathogens retrieved from National Center for Biotechnology (NCBI). DNA was extracted from the spindle leaves of arecanut using the SDS buffer (100 mM Tris pH 8, 1.4 M NaCl, 50 mM EDTA). The leaf sample was frozen in liquid nitrogen and ground into fine powder. To that 0.5 g of poly vinyl (poly) pyrolidone (PVPP) was added, mixed well and transferred to a centrifuge tube containing pre-heated (65°C) extraction buffer wtth β-mercaptoethanol. Mixture was incubated at 65°C for one hour with intermittent mixing. After incubation, chloroform: isoamyl alcohol mixture (24:1 v/v) was added and homogenized by gentle inversion for 15 min. and centrifuged at 8,000 rpm for 20 min. at 4°C. The clear aqueous phase was then transferred into a fresh centrifuge tube and 2/3rd volume of ice-cold isopropanol was added. The DNA pellets then generated was collected in 1.5 ml microfuge tubes, washed thrice with 70% alcohol, air dried and pellet dissolved in TE buffer. The DNA were purified by RNase treatment and quantified using spectrophotometer. The quality was checked in 0.8% agarose gel and the samples diluted to 20 ng/µl were used for the molecular marker analysis.

Initial screening of 134 RAPD (Operon series) and 41 ISSR primers (UBC series) was performed. 21 RAPD and 9 ISSR primers were selected based on amplification. Also 8 out of 10 resistant gene primer combinations were used generating fingerprints. The amplification was carried out in 15 µl cocktail containing 20 ng DNA, 200µM each dNTPs, 20 picomoles primers, 1X assay buffer, *Taq* polymerase (0.5 units) and 2.5 mM $MgCl_2$. The thermal cycler (Biorad Gradient thermo cycler) was programmed as follows: initial denaturation at 94°C for 5 min followed by 39 cycles of 1 min. denaturation at 94°C, 1 min. annealing at 42°C and 1 min. and 30 sec. primer extension at 72°C followed by a final extension at 72°C for 10 min. But for RAPD primers, annealing were kept at 38°C for 45 sec. While for resistant gene combinations, each primer had given different annealing temperatures (Table 1).

The amplified fragments were separated by electrophoresis in 1.5% agarose gel. The banding patterns generated in all different primers were scored as presence (1) and absence (0) of band of a particular molecular size to compile a binary matrix, which was then subjected to cluster analysis. For resistant gene primers, all the amplified bands which are clear, unambiguous and reproducible were scored apart from the regions specific fragment. Cluster analysis performed based on the Jaccard's (Jaccard, 8) similarity coefficient using NTSYS pc package

Table 1. Details of disease resistant gene specific primers used in the present study.

Res. gene primer	Forward sequence	Reverse sequence	T_m
RGP 1	5'-GCATTGGAACAAGGTGAA-3'	5'-AGGGGGACCACCACGTAG-3'	45.0
RGP 2	5'-TAGTTCGGACGTTTACAT-3'	5'-AGTGTCTTGTAGGGTATC-3'	45.4
RGP 3	5'-ACAGAACTGCATCAGCATCG-3'	5'-AGGCAGTCTCACCATGATCC-3'	45.4
RGP 4	5'-TGCAAAGCAGGTGCAGTATC-3'	5'-GTTCTTGCGGACGTCTTCTC-3'	45.0
RGP 5	5'-TGCGAGCAGCTACAGACACT-3'	5'-GGGAGGCCAGAAGCATAAAT-3'	47.9
RGP 6	5'-GTTGGGAAGACAACGTTGC-3'	5'-CAACTCAACATTCAACCGAGG-3'	46.7
RGP 7	5'-TGGGTGGAGTTGGTAAGACC-3'	5'-TGGTGAGGAGAGAGGCAAGT-3'	46.7
RGP 8	5'-GGTGGGGTTGGGAAGACAACG-3'	5'-CCACGCTAGTGGACCTCC-3'	46.7

Annealing temperature (T_m) during PCR cycle of all the resistant gene primers are given

(Exeter, New York). Similarity coefficients were used to construct a dendrogram using UPGMA (Unweighted Pair Group Method with arithmetic Average) routine in the NTSYS program.

RESULTS AND DISCUSSION

Different fingerprints generated with each marker technique can throw light upon the region specific genetic variations in the resistant/susceptible individuals. But differences in the resolution of all the three marker systems were observed as they targets different portions of the genome. All the chosen 8 resistant gene primers amplified the arecanut genomic DNA with number of amplified fragments ranging from 1 (RGP 1) to 11 (RGP 8) and yielded 54 clear and scorable amplifications with an average of 6.75 fragments per primer. Out of the 54 fragments, 22 were polymorphic (Table 2). The range of polymorphic bands was 1 (RGP 3, RGP 4) to 10 (RGP 6) with an average of as 2.75 fragments per primer. Percentage polymorphism ranged from 0 (RGP 1, RGP 2) to 100% (RGP 6) with an average of 40.7 % polymorphism. The highest similarity index of 1, observed between the YLD resistant arecanut palms R4 and R5 showed the identical genetic composition of these two candidates. The average similarities between the YLD resistant palms were 0.81 and between the YLD susceptible palms was 0.75. The lowest similarity coefficient of 0.41 was observed between the resistant and susceptible palms, R10 and S6 and thus the similarity coefficients ranged from 0.41 to 1.00.

Nine selected ISSR oligonucleotides produced a total of 43 scorable amplicons, out of which 15 were polymorphic (Table 3). The total number of amplified fragments ranged from 3 (UBC 2, UBC 351) to 7 (UBC 701) with an average of 4.78 bands per primer. But the number of polymorphic bands ranged from 1 (UBC 351) to 3 (UBC 52, UBC 356) with an average of 1.67 fingerprints per primer producing bands with 34.88% of polymorphism. The genetic similarity coefficient for pair-wise comparison of accession ranged from 0.50 to 0.86. The average similarity between the YLD resistant palms

Table 2. Details of markers produced using resistant gene specific primers in YLD resistant and susceptible palms.

Primer No.	Total bands (Nos.)	Polymorphic bands (Nos.)	Polymorphism (%)
RGP 1	1	0	0.0
RGP 2	5	0	0.0
RGP 3	6	1	16.7
RGP 4	5	1	20.0
RGP 5	9	3	33.3
RGP 6	10	10	100.0
RGP 7	7	4	57.1
RGP 8	11	3	27.3
Total	54	22	
Av.	6.75	2.75	40.7

Table 3. Details of ISSR markers produced with YLD resistant and susceptible palms.

Primer	Total bands (Nos.)	Polymorphic bands (Nos.)	Polymorphism (%)
UBC 2	3	0	00.0
UBC 52	6	3	50.0
UBC 72	4	2	50.0
UBC 84	5	0	00.0
UBC 100	4	2	50.0
UBC 321	5	2	40.0
UBC 351	3	1	33.3
UBC 356	6	3	50.0
UBC 701	7	2	28.6
Total	43	15	
Av.	4.78	1.67	34.9

was 0.71 and between the YLD susceptible was 0.72. The highest similarity coefficient, 0.86 was observed between the YLD resistant arecanut palms R5-R2, R5-R4 and R5-R6, while the lowest similarity coefficient, 0.50 observed between R6 and S7. With the primer UBC 321, the band number 1 having 1000 bp was present in 9 resistant samples numbered as R1, R2, R3, R4, R5, R6, R7, R10, and R12 (approximately 75%) and this band was present only in three susceptible samples (S1, S3, & S9), *i.e.*, 25%.

The twenty one RAPD primers generated a total of 151 bands out of which 93 were polymorphic (61.9%). The number of amplified fragments ranged from 1 (OPC 05) to 12 (OPE 01). The number of polymorphic bands ranged from 1 (OPC 05) to 6 (OPAB 16) (Table 4) with an average of 4.43 bands/primer. The highest percentage of polymorphism (61.6%) was detected for RAPD, than ISSR (34.9%) and resistant gene assay (40.7%). This result is in accordance with the earlier studies (Gupta *et al.*, 11) showing the better efficiency of RAPD markers than ISSR assay with regard to the polymorphism detection. The highest similarity index, 0.88 was observed between the YLD susceptible arecanut palms, S3 and S7, while the lowest similarity coefficient of 0.66 was found between the resistant and susceptible palms, R4-R8, R8-S4 and R8-S6. Also the average similarity between the YLD resistant was 0.79 and that of susceptible palms was 0.85 and thereby the similarity coefficient ranged from 0.66 to 0.88.

With the primer OPE 13, an amplicon having 1000 bp was present in four resistant samples numbered as R1, R2, R8 and R12, approximately 33.3% and this amplicon was absent in all susceptible samples (Fig. 1). With the primer OPAF 15, an amplicon of 1450 bp was absent in all resistant samples, and this was present in approximately 66.7% susceptible samples including S1, S2, S3, S5, S6, S7, S11 and S12.

Since ISSR and RAPD targets different portions in the genome, the amalgamated analysis of this two dominant markers in the genetic diversity studies found to be more effective for disease- resistance discrimination. Also the use of more than one molecular marker type will always increase the accuracy of diversity analysis and also the merging of different scored data can pave path to more precise resolution.

Table 4. Details of RAPD markers produced with YLD resistant and susceptible palms.

Primer	Total bands (No.)	Polymorphic bands (No.)	Polymorphism (%)
C 05	5	1	16.6
OPC 07	6	4	66.6
OPC 09	6	5	83.3
OPAH 01	6	5	83.3
OPAH 15	6	5	83.3
OPAH 18	6	3	50.0
OPAH 20	6	4	66.7
OPAF 06	7	5	71.4
OPAF 14	7	4	57.1
OPAF 15	6	5	83.3
OPAB 02	6	3	50.0
OPAB 16	7	6	85.7
OPBA 02	8	5	62.5
OPBA 03	8	5	62.5
OPBA 10	7	4	57.1
OPBA 20	7	4	57.1
OPE 01	12	7	58.3
OPE 11	7	4	57.1
OPE 12	9	5	55.6
OPE 13	10	6	60.0
OPA 08	9	3	33.3
Total	151	93	
Av.	7.2	4.4	61.6

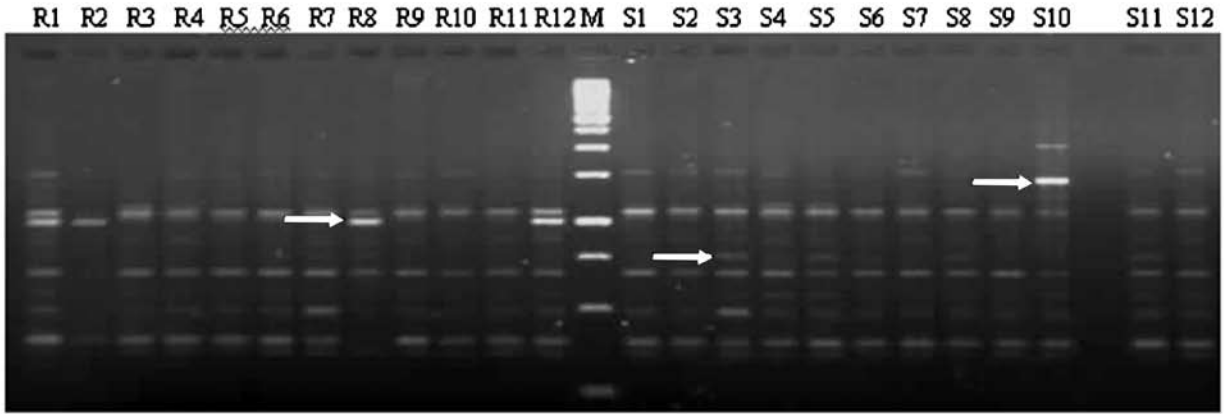

Fig. 1. Resistant (R1- R12) and susceptible (S1-S12) palm samples are marked over each lane in the 1.5% gel. Polymorphic bands are marked with arrows. 1 kb ladder is marked as M.

In the present study, the productivities of the prominent dominant markers, ISSR and RAPD were incorporated with the gene specific markers.

In total, 38 primers produced 248 amplicons among YLD resistance/susceptible areca genotypes. The average number of bands and polymorphic bands per primer were 6.53 and 3.42, respectively thereby generated 54.42% of polymorphism among the genotypes. The comparative list of the 3 marker details are shown in Table 5. The genetic similarity coefficient for pair-wise comparison of accession ranged from 0.73 to 0.88. The average similarity between the YLD resistant palms was found to be 0.79 and that of susceptible was 0.80. The highest similarity coefficient, 0.88 was observed between the YLD resistant arecanut palms R1 and R2, while the lowest similarity coefficient, 0.73 was observed between R8 and R12.

Cluster analysis performed on three sets of combined marker data and the resultant dendrogram (Fig. 2) showed that the majority of resistant genotypes placed together possessing high level of genetic relatedness and likewise the susceptible genotypes clustered among themselves. Three major clusters were deduced in which the resistant group cluster includes 9 of the resistant genotype candidates such as R1, R2, R3, R4, R5, R6, R9, R10, and R11. Similarly susceptible genotypes are clubbed together within the two adjacent clusters with minimum divergence. Cluster I includes R1, R2 and R3., in which R1 and R2 were observed as less diverged, having similarity co-efficient value of 0.88, which is the maximum similarity in the entire pairwise comparison. Second cluster was subdivided into two sub-clusters, cluster IIa (R4, R5, R6, R9, R10, R11 and S3); cluster IIb (S1, S2, S4, S5, S6, S7, S8, S9, S10, S11, S12) and finally cluster III includes only the resistant candidates R7, R8 and R12. In cluster IIa, the two sub-clusters, R4-R9 and R5-R6 are highly similar having least divergence with R10-S3 and all were having the same similarity co-efficient value of 0.87. This showed that the unique susceptible candidate S3 yielded the major composition of resistance genotypes and was having minimum region specific variation from the resistant candidates. The genotype R11 was clubbed to cluster IIa with a nearby similarity value of 0.85. The cluster IIb and cluster IIc consisted exclusively of susceptible candidates. Thus the dendrogram supports the similarity in the genetic composition of susceptible candidates by plotting

Table 5. Comparitive list showing different marker details (RAPD, ISSR and gene specific primers).

Particulars	RAPD	ISSR	Resistant gene	Combined marker data
No. of primers used	21	9	8	38
Total No. of polymorphic bands	93	15	22	130
Total No. of monomorphic bands	58	28	32	118
Total No. of bands	151	43	54	248
Polymorphism (%)	61.6	34.9	40.7	52.4
Av. No. of bands/ primer	7.19	4.77	6.75	6.53
Av. No. of polymorphic bands/ primer	4.43	1.67	2.75	3.42

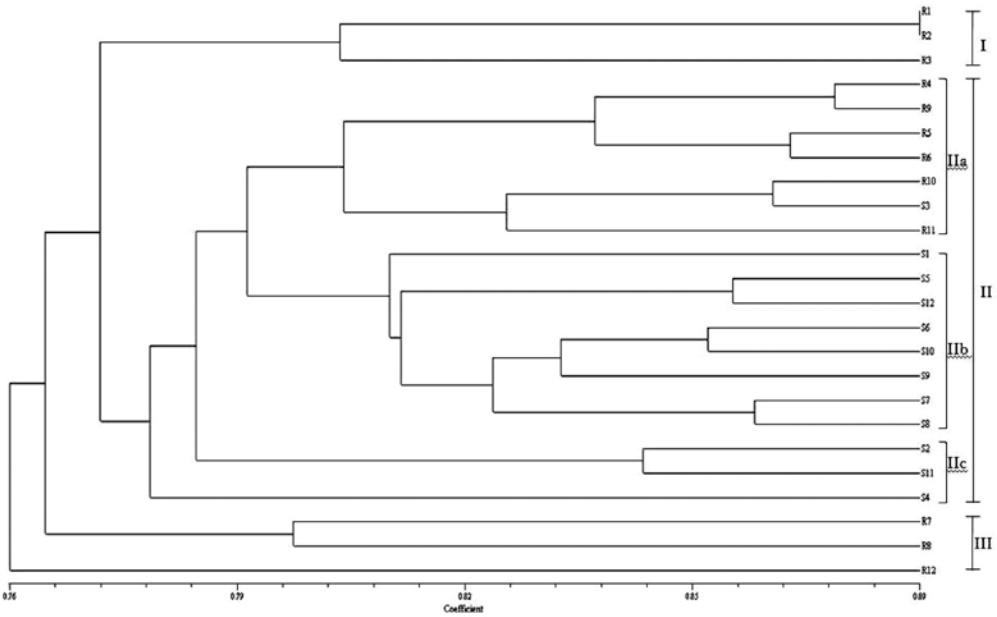

Fig. 2. Dendrogram of combined marker assay (RAPD + ISSR + Resistant gene primers). Cluster analysis performed was based on the Jaccard's similarity coefficient using NTSYS pc package. Dendrogram classified into 3 main clusters, I, II and III are labelled on the right side of the fig. Subclusters of cluster II was again labelled as IIa, IIb and IIc.

the susceptible candidates solely in the same or near region of convergence and these two clusters was unique as it contains only susceptible candidates. The 3D representation of clustering showed the exact discrimination by the distinct clustering of resistant types from the susceptible varieties as two sections on the plane among the 24 arecanut palms.

The polymorphic fragments obtained can be sequenced for the characterization of genomic region associated with the resistant genes. UPGMA cluster analysis showed that closely related cultivars collected from same geographical area can be differentiated and the genetic difference between them could clearly establish. The genetic diversity analysis revealed low similarity between resistant types than susceptible palms in general with some palms showing exceptions. Even though the YLD resistant and susceptible palms have the same genetic origin, they had clustered according to their disease phenotype. The resistant palms, *viz*., R1, R2, R3, R4, R5, R6, and R9 were distinct and could be further studied by sequencing the polymorphic region for generating specific primers for the isolation and cloning of resistant genes.

In this study we have used ISSR, RAPD and gene specific markers to characterize the genetic variability among YLD resistant and susceptible areca nut palms. The results obtained will help in the future research to rely on the merging of markers for the complete discrimination of resistant and susceptible diversity assays and to facilitate the design of new region-specific markers more effective in genetic discrimination among genotypes and to generate distinct alleles that could be cloned and sequenced. Moreover, these will pave path to the breeding of disease resistance, through marker-assisted selection.

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