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DETECTION PROCEDURE FOR PAPAYA PHYTOPLASMA

C. RANASINGHE [1], S. DISANAYAKA [2], M. THAMMITIGE [3] AND R. EKANAYKA [1],

[1] *Plant Virus Indexing Centre, Department of Agriculture, Homagama, Sri Lanka*
[2] *Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya, Sri Lanka*
[3] *Medical Research Institute, Borella.*

INTRODUCTION

Papaya (*Carica papaya* L.) is a very popular perennial tropical fruit crop belongs to the family Caricaceae. In Sri Lanka, papaya is grown primarily as a homegarden crop and there is limited extent under commercial cultivation. Demand for papaya fruit is high because of its high nutritive and medicinal value. Total extent under papaya cultivation at present in Sri Lanka is 8,058 ha and production is 32,240 t/year (Agstat, 2011). Because of the extensive mono culturing and the narrow genepool of papaya, a virus disease caused by Papaya Ring Spot Virus (PRSV) and several bacterial diseases remain as constrains for papaya cultivation. Recently a fast spreading disease for papaya cultivation was observed in southern regions of Sri Lanka, with initial symptoms of yellowing and necrosis of lower leaves, stunting and collapse of the plant. Studies have revealed that the causal organism as phytoplasma (Anonymous, 2011). Papaya phytoplasma has been observed as an emerging threat to Sri Lanka due to higher incidence reported. Moreover, mixed infection of phytoplasma with PRSV has been identified (Anonymous, 2011). The detection of phytoplasma by Polymerase Chain Reaction (PCR) techniques is efficient but it is costly for routine laboratory testing of large number of samples. On the other hand, techniques like Immunoassays used to diagnose pathogens and quantization of microorganisms are highly specific and advantageous on present situation. Highly purified antigens are needed to raise specific polyclonal antibodies for immunoassays. This study was based on adopting differential filtration technique for the purification of phytoplasma; production of antiserum and assaying detection efficacy by using Enzyme Linked Immunosorbent Assay (ELISA).

MATERIALS AND METHODS

Sample collection

Papaya leaf samples which showing typical phytoplasma symptoms were collected from diseased plants of field at the Plant Virus Indexing Centre (PVIC), Homagama. Apparently healthy papaya leaf samples were also collected from the field at district training centre, Homagama. Infection was confirmed by PCR using universal specific primers for phytoplasma.

Purification of papaya *Phytoplasma* by differential filtration

Diseased tissues of papaya taken from the interveinal regions of leaves (25 g) were sliced out with clean surgical blade. Tissues were washed with running tap water for 10 minutes, treated with a detergent for 3 minutes and again thoroughly washed with running tap water. Sliced tissues were homogenized using 0.3M glycine-sodium hydroxide buffer, pH 8.0 containing 0.02M magnesium chloride (25 g fresh weight leaves/100 ml buffer). The extract was passed through three layers of cheesecloth. Filtrate was again homogenized and filtered through new three-layers of cheesecloth followed by filtration through Whatman number 1 and 5 filter papers. The clear extract was then passed through 0.45 µm pore size Millipore filter (Millipore, USA), and centrifuged at 45,000 g (rpm) for 45 minutes (Sorvol Ultra Centrifuge). The yellow pellet was dissolved in the same buffer. After law speed centrifugation at 4,700 g for 20 minutes the pellet was discarded and the supernatant was centrifuged at 65,000 g for 90 minutes. The pale yellow pellet was re-suspended in 1 ml of 0.3M glycine-sodium hydroxide buffer without magnesium chloride.

Raising of antibodies against purified papaya *Phytoplasma*

The purified Phytopasma was diluted 1:3 ratio using 0.3M glycine buffer without magnesium chloride and sonicated thrice for 30 seconds each time, with an interval of 2 minutes on ice. New Zealand White rabbit was injected at two sites with 750 µl of above preparation mixed with an equal volume of Freund's incomplete adjuvant. Initial injection was given to ear veins and subsequent injections were given to hind legs in two-week intervals. Blood was collected from the ear vein after $6^{th}$and 12^{th}week of the first injection.

Separation and Preservation of Antiserum

Blood samples were collected from ear vein under aseptic conditions and kept in room temperature for 2-3 hours to coagulate. It was centrifuged at 6,000 rpm for 5 minutes to separate the serum from blood cells. Supernatant was separated carefully, mixed with an equal volume of glycerol, 0.025 % Sodium azide and refrigerated at -20 °C.

Testing the papaya phytoplasma antiserum by indirect ELISA, cross absorption and threshold value

Protocol developed to detect Weligama Coconut Leaf Wilt phytoplasma disease (Ranasinghe *et al.*, 2010) was followed to test locally produced papaya phytoplasma antiserum.

Leaf tissues obtained from healthy papaya plant were crushed without buffer and filtered through muslin cloth. Antiserum of the 2nd bleed was mixed with different ratios of healthy sap i.e., 1:1, 1:5, 1:10, 1:15 and 1:20 (v/v). The mixture was incubated at room temperature for 4 hours and centrifuged at 5,000 rpm for 5 minutes. Supernatant was collected as absorbed antiserum which is more efficient than crude antiserum. Threshold value (THV) was calculated according to the Sutula *et al.* (1986) for crude sap extracts. Sample absorbance values, which gave higher than THV was considered as positive for the test as and lower than THV was considered as negative for the test.

Data analysis

The colourimetric readings taken on the two treatment levels were analyzed with Complete Randomized Design to check whether there is a significant difference between the diseased and healthy groups. Since the significant difference is not enough the turkeys mean separation was performed to separate the two groups with significant difference in mean values. The statistical analysis was done using the software Statistical Analytical Systems (SAS) Portable V9.0. In the Analysis of Variance the Coefficient of Variation was measured for precision of the test trials.

RESULTS AND DISCUSSION

PCR conformation of papaya Phytoplasma disease

Conformation of diseased and healthy papaya plants was done by PCR amplification techniques with phytoplasma universal primers. The sequences of primers were oligonucleotide of forward ACGAAAGCGTGGGGAGCAAA and reverse GAAGTCGAGTTGCAGACTTC. The papaya plants possessing symptoms of papaya phytoplasma were conformed as diseased (Figure 1). The healthy plants that was pre-selected with no visible symptoms of phytoplasma, was conformed as negative for phytoplasma infection in the same PCR test (Figure 1).

Production of antiserum and testing of the prepared antiserum

Two bleedings were done and serum was separated by centrifugation. Final antiserum volume, 8 ml of 1st bleed and 15 ml of 2nd bleed was produced. According to the results, tested antiserum was unable to give positive results for diseased samples producing lower absorbance values when compared to threshold values. This was further confirmed by Turkey's studentized analysis. The computed F ratios were insignificant on the treatment levels. The numerical ranking between treatment means behaved similarly of the grouping of Turkey's mean separation.

Cross absorption

The optimum ratio of cross absorption was carried out with a series of pre-defined concentration (1:1, 1:5, 1:10, 1:15 and 1:20 (v/v)). Only the cross absorption of

1:1 (v/v) ratio, displayed a significant difference and a correct mean separation of diseased samples, when individually analyzed (F ratio 31.22; Pr> F 0.0014).

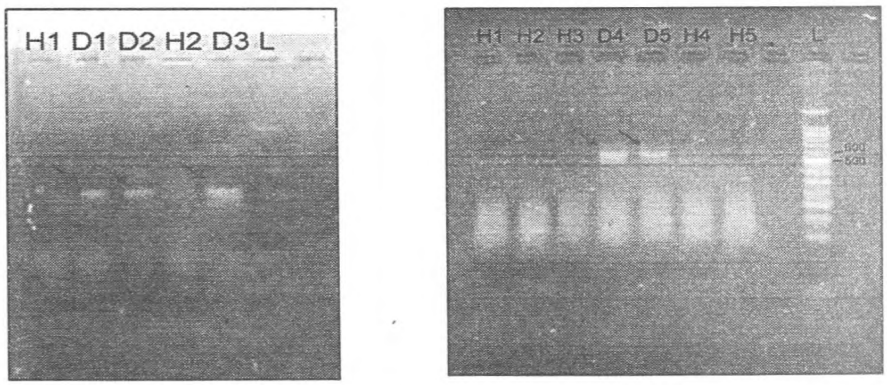

Figure 1. **Agarose gel image of PCR products obtained with universal phytoplasma specific primers.** Note: D and H represent diseased and healthy papaya samples where only diseased samples produced band; L=100 bp DNA Ladder).

ELISA test results for cross absorbed antiserum

Papaya phytoplasma infected and non infected samples were tested by indirect ELISA using cross absorbed antiserum. Table 1 shows the ELISA test results of the cross absorbed antiserum. All diseased samples gave higher values than the threshold values whereas, all healthy samples gave less than threshold value proving the absence of the disease.

Table 1. **Results of ELISA test of cross absorbed papaya phytoplsma antiserum 18 hours after substrate addition at 4 ^{0}C.**

Sample	Absorbance values	Results	Sample	Absorbance values	Results
Healthy	0.075	-	Diseased	0.292	+
Healthy	0.122	-	Diseased	0.258	+
Healthy	0.104	-	Diseased	0.539	+
Healthy	0.121	-	Diseased	0.562	+
Healthy	0.055	-	Diseased	0.629	+
THV1	0.188		THV2	0.190	

Introduction of cross absorption to antisera may help to differentiate diseased from healthy. In the analysis of variance, the treatment differences were highly significant with the F ratio of 94.91. The turkey's mean separation has ranked the mean of diseased papaya group (0.220 ±) higher than the healthy papaya group (0.150) to the confident level of 95% with valued Coefficient of Variation was 8.7.

CONCLUSIONS

The attempt of obtaining a purified phytoplasma using glycine based differential filtration technique was successful. The sensitivity of the rabbit antiserum was enhanced by the

cross absorption of antiserum with healthy papaya leaf extract at the ratio of 1:1 (v/v). These modifications of the ELISA test method was proved highly significant in ANOVA for the difference of diseased and healthy samples. Locally developed antiserum for papaya phytoplasma can be used for the identification of phytoplasma infected plants that help to manage this disease spread. Further, optimization of the protocol is needed in order to increase the sensitivity, specificity and for efficient use of antiserum.

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