Research Article

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# Modified protocol of ELISA for rapid detection of coconut root (wilt) disease

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## Abstract

Root (wilt) disease of coconut is a phytoplasmal malady causing heavy economic loss to the coconut farmers in Kerala and Tamil Nadu. The disease is generally recognized based on foliar symptoms. But the development of foliar symptoms in coconut palms is very slow and there is a time lag between infection and symptom expression. To identify such latent infected palms, Direct Antigen Coated (DAC) indirect ELISA had been standardized earlier. But this ELISA system was not rapid. Though subsequently it was refined, 24 h was required for the completion of the test. Hence, a modified protocol was developed using leaf disc as test antigen. The modified protocol is simple, rapid and results could be obtained within 7 h. The test yielded very high sensitivity of 98 % with respect to visual observations. The modified protocol is being used for the routine screening of coconut palms for selection of disease-free mother palms for developing root (wilt) resistant/tolerant varieties. Besides this, the test is being used for confirmation of root (wilt) disease in coconut palms seen in mildly disease affected and disease outbreaks in new areas.

*Keywords:* Coconut, ELISA, horse radish peroxidase, leaf disc, Phytoplasma, root (wilt) disease

## Introduction

Root(wilt) disease of coconut is a phytoplasmal malady (Solomon, 1991) causing heavy economic loss to the coconut growers in Kerala and Tamil Nadu. The annual loss due to the disease in Kerala was valued to Rs. 3,000 million on the basis of 1984 price index (CPCRI, 1985). Apart from Kerala and Tamil Nadu, the disease has been reported from Goa and Karnataka (Koshy *et al.*, 2001; Sasikala *et al.*, 2005a). It is a non-lethal but slow declining disease and affecting palms of all age groups. Root (wilt) disease is generally recognized based on foliar symptoms. Flaccidity or inward bending of the leaf lets of leaves in the middle and outer whorl is the characteristic visual diagnostic symptom of the disease (Radha and Lal, 1972). Yellowing and marginal necrosis of the leaflets are the other associated foliar symptoms. However, the development of foliar symptoms in coconut palm is very slow and there is a time lag between infection and symptom expression. Healthy looking palms in the disease-endemic areas may be harbouring the pathogen/ incipient infection without expressing symptoms. To identify such latent infected palms early diagnostic tests viz. agar gel double diffusion test (Solomon *et al.*, 1983) and DAC indirect ELISA (Sasikala *et al.*, 2001) had been developed.

Since root (wilt) is a phytoplasmal malady, no therapeutic measures are available for controlling the disease. The lasting solution for maintaining the production and productivity of coconut palms in the disease-prevalent tracts is to evolve high yielding and root (wilt) resistant/tolerant varieties through systematic sustained breeding programmes. Serological screening of elite palms is a basic requirement for the selection of healthy mother palms for breeding programme.

The DAC indirect ELISA standardized earlier as well as the test subsequently refined for mass screening of coconut samples are time consuming, as it needs a minimum of 24 h to complete the test (Sasikala *et al.*, 2005b). Moreover, the sample preparation is laborious and time consuming. Hence, investigations were carried out to develop a simple and rapid detection technique by suitably modifying the existing ELISA procedures.

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## Materials and Methods

### Preparation of phytoplasma specific antiserum

Phytoplasma specific polyclonal antiserum was prepared in New Zealand white rabbits using purified preparation of phytoplasma (Sasikala *et al.*, 2002). Rabbits were immunized intramuscularly with one ml of purified phytoplasma emulsified with equal volume of Freund's incomplete adjuvant. The emulsion was introduced into the animals through the thigh muscles at seven-day intervals. After a series of four such injections, the rabbits were bled from the marginal vein of the ear on 35th day of the start of immunization. Antiserum recovered from the blood was stored in small vials at –40 °C till use after addition of a small pinch of biocide. Bleeding was continued at weekly intervals till the titre of the antiserum dropped.

### Preparation of samples

Non chlorophyllous tender leaflets were collected from the spear leaves of diseased and healthy coconut palms. Leaf discs of 10 mg were taken and cut into small pieces and used as test antigen. Carbonate bicarbonate buffer(0.05 M, pH 9.6) was used as antigen coating buffer.

### DAC Indirect ELISA

Protocol developed earlier by Sasikala *et al.* (2005 b) was modified as follows. Instead of leaf grindates being used as antigen, leaf discs were punched out from the spear leaves of diseased and healthy palms and added to each well of Nunc immunological microtitre plate (Nunc, Denmark) preloaded with 50µL 0.05 M carbonate bicarbonate buffer pH 9.6. After loading the antigen, 50µL of the same buffer was added. Besides the carbonate bicarbonate buffer, buffer with additives like gelatin 1 % and ovalbumin 0.2 % were used as antigen coating buffer. In addition to the samples taken from healthy palms, 0.05M carbonate bicarbonate buffer was also included as negative control. After the addition of leaf discs, the wells were sequentially loaded with blocking agent (Bovine Albumin Fraction V; Hi Media, Bombay), root (wilt) phytoplasma specific antiserum (unfractionated), Enzyme conjugate (Goat antirabbit IgG conjugated with horseradish peroxidase; Genei, Bangalore) and substrate (Tetramethyl benzidine/ hydrogen peroxidase). To get better adsorption of antigen molecules to the wells of ELISA plate, after loading the antigen, the plate was incubated for 90 min at 37 °C. In a parallel experiment after loading the antigen the plate was incubated at 37 °C for 90 min followed by overnight incubation at 4 °C. Subsequently, the plates in both experiments were washed 3 to 5 times with wash buffer (PBS T pH 7.4). In between the remaining steps, the plates were incubated for 60 min at 37 °C and washed thrice with wash buffer. After the addition of substrate the plates were kept at ambient temperature till a bright blue colour got visualized in positive control. Further reaction was arrested using 1 N $H_2SO_4$ and absorbance measured using 450nm filter in VERSA max microplate reader. The cut off value (COV) of negative controls was calculated as mean of negative controls + 2SD.

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n}}$$

Where x = absorbance value of each negative control

$\bar{x}$ = the mean absorbance value of negative controls

n = total no. of samples used as negative controls

A comparative study was also carried out using leaf disc and leaf grindate as test antigen to find out the difference if any, in the absorbance values between healthy and diseased samples and to assess the sensitivity of both the tests.

### Screening of samples

Spear leaf samples collected from apparently healthy elite coconut palms grown in the disease endemic areas (Hot spots) of Kollam, Alappuzha, Kottayam, Pathanamthitta and Ernakulam districts of Kerala state were employed for the test. Leaf samples were provided by the Officials of Crop Improvement Division of CPCRI and Kerala State Agricultural Department. Apart from this, for the confirmation of the disease leaf samples collected from disease suspected coconut palms grown in various locations were also screened.

## Results and Discussion

### ELISA

In this modified assay bright blue colour appeared in infected samples when the plate was incubated for 5 to 10 min at ambient temperature after the addition of substrate. Negative controls remained as colourless or expressed very faint blue colour as in the earlier procedure. When the reaction was arrested, the colour of the infected samples turned to bright yellow. Healthy samples remained as colourless or showed very faint yellow colour. However, infected samples invariably recorded higher absorbance values over the healthy (Table 1). The samples which expressed high absorbance values over the cut off value of negative controls were considered as infected. However, better resolution was noticed between healthy

and diseased samples when the plate was incubated at 37 °C for 90 min after loading the antigen than overnight incubation (Fig. 1). Similarly, significant difference was observed between healthy and infected samples, when antigen was coated in plain buffer rather than coated in buffer with additives (Fig. 2). In the comparative study using leaf discs and leaf grindates as test antigen it has been found that absorbance values of leaf discs were as same as that of leaf grindates (Fig. 3).

**Table 1. Absorbance value of the healthy and infected coconut samples (Mean absorbance of three replications)**

| Absorbance | |
|---|---|
| Infected | Healthy |
| 0.721 | 0.308 |
| 0.860 | 0.432 |
| 0.740 | 0.379 |
| 0.695 | 0.314 |
| 0.729 | 0.325 |
| 0.589 | 0.236 |
| 0.647 | 0.283 |
| 0.831 | 0.490 |
| 0.753 | 0.327 |
| 0.717 | 0.363 |
| 0.812 | 0.454 |
| 0.763 | 0.330 |
| 0.643 | 0.284 |
| 0.647 | 0.284 |
| 0.675 | 0.303 |

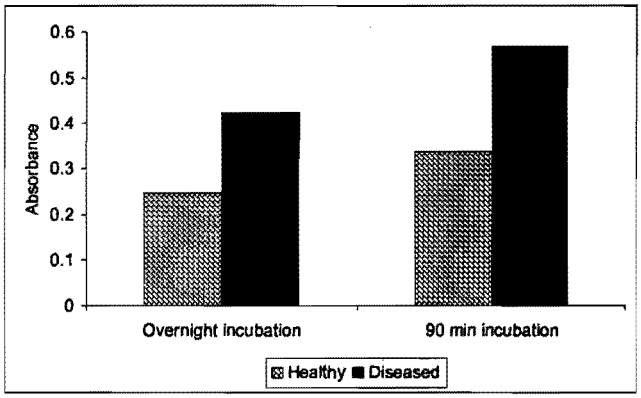


**Fig. 1. Effect of incubation period after coating the antigen**

Due to the fibrous nature of coconut leaf tissues, extraction of antigen was laborious and time consuming especially when large number of samples were to be processed. Moreover, the leaf extract contains lot of tannins and other polyphenols that generally interact with serum proteins and produce non-specific reactions (Shanta *et al.*, 1975). To neutralize the deleterious effects of compounds like tannins and polyphenol oxidase and thereby stabilizing the antigen, 1 % gelatin and 0.2 %

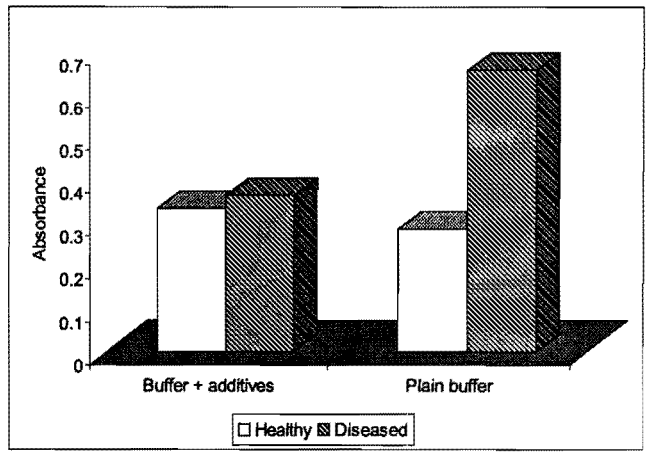


**Fig. 2. Effect of additives on intensity of ELISA**

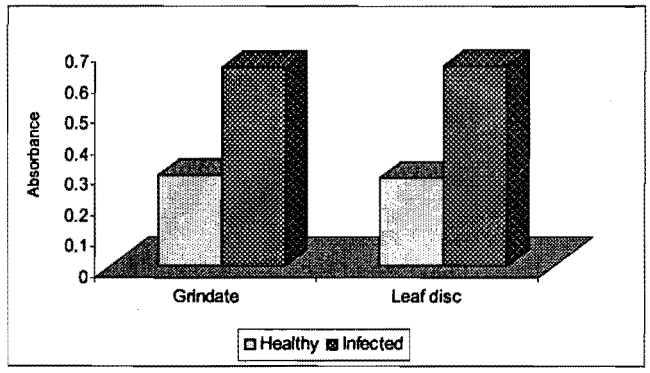


**Fig. 3. ELISA performed using grindate and leaf disc**

ovalbumin were incorporated to the extraction medium at the time of sample extraction (Sasikala *et al.*, 2001). ART-MICCRA D-8 tissue homogenizer was used for sample grinding. The extract was filtered through muslin cloth and centrifuged at 5000 rpm to remove large cell debris. Thus, antigen extraction was a laborious and time consuming process in the previous procedure. In the present modified method, leaf discs of small size were used as test antigen instead of leaf grindates. While using leaf discs it has been found that additives were not required to stabilize the antigen. However, when additives were incorporated to the extraction medium the differences between healthy and infected samples were narrowed down.

Sensitivity of the modified protocol was also evaluated. Out of 60 known diseased samples in the early stage of disease screened, positive reaction was noticed in 59 samples thereby, expressing very high sensitivity of 98 %.

## Screening of samples

Spear leaf samples collected from 445 apparently healthy elite coconut palms were screened with modified protocol of ELISA for identifying healthy mother palms. The data revealed that only 45 % of apparently healthy

palms in the disease endemic areas were free of infection and the remaining 55 % of palms were under latent stage of infection (Table 2). The healthy palms are being used as mother palms for producing quality seedlings either by crossing programmes or using open pollinated nuts.

**Table 2. Serological screening of elite mother palms from various districts of Kerala**

| District | No. of samples | | | % of healthy palms |
|---|---|---|---|---|
| | Tested | Diseased | Healthy | |
| Kollam | 95 | 59 | 36 | 37.8 |
| Alappuzha | 150 | 87 | 63 | 42 |
| Kottayam | 100 | 47 | 53 | 53 |
| Pathanamthitta | 30 | 11 | 19 | 63 |
| Ernakulam | 70 | 40 | 30 | 42.8 |
| **Total** | **445** | **244** | **201** | **45.16** |

Similarly, when 43 spear leaf samples collected from disease suspected palms seen in mildly disease affected areas and 10 samples from new areas were subjected to modified procedure of ELISA, root(wilt) disease was confirmed in 28 samples and 8 samples, respectively. Samples collected from Lakshadweep did not react positively to ELISA (Table 3). Root (wilt) disease has not been so far reported from Lakshawdeep islands.

**Table 3. Screening of disease suspects from mildly disease affected and new areas**

| State/Union territory | Place | No. of samples | | |
|---|---|---|---|---|
| | | Tested | Diseased | Healthy |
| Kerala | Malappuram | 9 | 7 | 2 |
| | Palaghat | 6 | 6 | 0 |
| | Kasaragod | 19 | 8 | 11 |
| Tamil Nadu | *Meenakshipuram | 2 | 2 | 0 |
| | Theni | 6 | 4 | 2 |
| Karnataka | *Sullia | 6 | 6 | 0 |
| Goa | Goa | 3 | 1 | 2 |
| Lakshadweep | *Minicoy | 2 | 0 | 2 |

** New areas*

In the modified procedure, sample preparation was very simple as only 90 min was required for coating the antigen and additives were not needed to stabilize the antigen. Hence, the modified protocol was simple, less time consuming and the results could be obtained with in 7 h. Moreover, the test was very economic because 1200 samples with 3 replications could be screened with one ml primary antibody. The test could be used to diagnose root (wilt) disease of coconut palms even before the appearance of visual symptoms. The modified ELISA is being used for routine screening of coconut samples for selecting healthy elite mother palms for producing quality seedlings and confirmation of root(wilt) disease in coconut palms grown in mildly disease affected and new areas. The test can also be used for selecting disease free seedlings in the nursery.

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