DEVELOPMENT OF DAC INDIRECT ELISA FOR THE RAPID DETECTION OF COCONUT ROOT (WILT) DISEASE

By

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Abstract

Identification of root(wilt) disease-free palm is a basic requirement for evolving disease resistant/tolerant planting materials for the management of this phytoplasma induced malady. For this purpose, various types of ELISA were performed with different enzymes and their respective substrates to standardize the most suitable and sensitive one. Indirect ELISA, Protein A indirect ELISA and $F(ab')_2$ indirect ELISA using Alkaline Phosphatase conjugate and DAC indirect ELISA using Horse raddish peroxidase conjugate were carried out. Of the various types of ELISA tried, DAC indirect ELISA has been found to be the best for the rapid detection of coconut root(wilt) disease. In this assay, crude leaf extracts and unfractionated polyclonal antiserum were employed as test antigen and specific antibody respectively. Higher specificity was observed with the addition of gelatin and ovalbumin in the extraction medium and overnight incubation of ELISA plate at 5°C after substrate addition. Antigen titre was found to be very high in spear leaves followed by the next outer leaf. Similarly, maximum antigen titre was observed during the early stage of the diseased palm. The test could be completed within 44h and in a single ELISA plate, 20 samples with three replications could be screened using microlitre quantities of the specific antibody.

INTRODUCTION

Coconut root (wilt) disease poses a serious threat to coconut cultivation in Kerala and Tamil Nadu. It was first reported from Erattupetta of Kottayam district (Butler, 1908; Pillai, 1911) and has now spread to all the 14 districts of Kerala (CPCRI, 2000) and to some of the districts in the adjoining state of Tamil Nadu (Srinivasan *et al*., 2000) and Goa (Koshy, Personal communication). The disease is non lethal but debilitating and causes an annual loss of 968 million nuts (CPCRI, 1985).

Flaccidity or inward bending of the leaflets of the leaves in the middle and outer whorl is the most consistent visual diagnostic symptom of the disease (Radha and Lal, 1972). The symptom is evident only in the middle and outer whorl of leaves of the diseased palm even though it might have contracted the disease 6-24 months earlier. Similarly, palms growing in shaded conditions may show etiolation of leaves and bending of leaflets which resembles flaccid condition. Hence a reliable test that could detect the disease status with certainty and one which could detect the disease before the expression of foliar symptoms was felt necessary. The disease being caused by phytoplasma (Solomon *et al.,* 1983a) no curative or prophylactic measures are available and breeding for disease resistance is the most desirable alternative. Hence, for the selection of healthy elite parental palms growing in the disease endemic areas, a reliable test was needed. Solomon *et al*., (1983b) developed an agar gel double diffusion test (Ouchterlony test) for the early detection of root(wilt) disease. At present, this is being used for the identification of healthy palms. With this test, only limited number of samples could be screened. Hence, an enzyme-linked immunosorbent assay (ELISA) in which large number of samples could be screened at a time has been developed. This paper describes various types of ELISA performed with different enzymes and their substrates using root (wilt) antigen and specific antibody.

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MATERIALS AND METHODS

Preparation of plant extracts

Leaf samples collected from the unopened spindles of diseased palms were used for sample extraction. After de-ribbing, the leaflets were chopped into small pieces and homogenized in extraction buffer with glass pestle and mortar. Since coconut leaf contains copious amount of tannins and polyphenols that interact with antibodies and reduce the specificity of the test, additives such as antioxidants, tannin inhibitors and blocking agents to block the unbound site of the microplate, were incorporated into the extraction medium. The extracts were centrifuged at 5000rpm for 10 min to remove large cell debris.

Production of polyclonal antiserum

Polyclonal antiserum to root (wilt) antigen was prepared in Newzealand variety rabbits (Solomon *et al*., 1983b). Disease-specific antigen was fractionated and purified from the spear leaves of coconut palms in the early stage of disease by PEG precipitation followed by differential centrifugation method. Purity and concentration of the final pellet was determined by colorimeter and was used for immunization. Rabbits were administered four intramuscular injections at weekly intervals with the partially purified preparation of antigen emulsified with equal volumes of Freund's incomplete adjuvant. Ten days after the last injection (4th injection) the animals were bled and antiserum separated.

Purification of IgG and preparation of $F(ab')_2$ fragments

From the antiserum IgG (Immunoglobulin G) was prepared by the method of Clark and Adams,1977. $F(ab')_2$ fragments of IgG was prepared by pepsin digestion (Barbara *et al*., 1982).

Optimization of reactants

A. Extraction buffer

Following buffers with or without additives were tried for extracting leaf sap.

i. Phosphate buffered saline (PBS) pH 7.4

ii. PBS + 0.05% tween-20 pH 7.4

iii. PBS + 0.05% tween-20 + 1% BSA

iv. PBS + 0.05% tween-20 + 2% Polyvinylpyrrolidone 44,000 + 0.2% Ovalbumin

v. 0.05M Tris-HCl + 0.005M EDTA + 2% Polyvinylpyrrolidone 44,000 + 0.05% tween 20 – pH 8.0

vi. 0.05M Tris-HCl + 0.06M Sodium sulphite pH 8.5

vii. 0.05M Tris-HCl + 0.06M Sodium sulphite + 0.1% 2-mercaptoethanol pH 8.5

viii. 0.05M Carbonate buffer pH 9.6

ix. 0.05M Carbonate buffer + 2% Polyvinylpyrrolidone 44,000 + 1% Gelatin + 0.2% Ovalbumin

x. 0.05M Carbonate buffer + 2% Polyvinylpyrrolidone 44,000 pH 9.6

xi. 0.05M Carbonate buffer + 1% Gelatin pH 9.6

xii. 0.05M Carbonate buffer + 0.2% Ovalbumin

xiii 0.05M Carbonate buffer + 1%Gelatin + 0.2% Ovalbumin

B. Sample dilutions

1:10, 1:100 and 1:500 were tried.

C. Specific antibody dilution

1:200, 1:400 and 1:800

D. Incubation period

After the addition of substrate, plates were incubated at 10,20,30,60 min at room temperature and overnight incubation at 5°C.

Format of ELISA with Alkaline Phosphatase (ALP) conjugate

In all the tests, predetermined dilution of test antigen, blocking agent, specific antibody and enzyme conjugates were tried using Nunc immunol ELISA plate.

Indirect ELISA

Indirect ELISA was performed with the method described by Koeing (1981) with some modifications. Nunc Immunological 1 Microtitre plate (Nunc, Denmark) was sequentially coated with specific antiserum diluted in 1:400 in PBS T + 1% BSA (Dilution buffer), test antigen diluted (1:10) in dilution buffer and diluted (1:2000)ALP conjugate (Genei,Bangalore). In between each addition incubation at 37°C and washing the plate several times with PBS-T (Wash buffer) was invariably needed. Finally substrate (P-nitrophenyl phosphate, Genei, Bangalore) 1mg/ml diluted in 10% diethanolamine buffer (pH 9.8) was added to each well of the plate and incubated at room temperature. After satisfactory colour development, reaction was arrested by the addition of 3M NaOH to each well. Absorbance values were read using 405 nm filter in an Organon Technika ELISA reader.

Indirect ELISA using $F(ab')_2$ fragments coating method

The protocol used was that of Barbara *et al.*, (1982). $F(ab')_2$ fragments of specific antibody were diluted (1:200) in 0.05M Carbonate buffer pH 9.6 and 200µl aliquots were added to each well of the ELISA plate. The plate was covered and incubated for 3h at 30°C. Subsequently leaf extracts of healthy and diseased palms diluted in PBS-TPO were loaded to the plate and incubated overnight at 4°C, addition of polyclonal antibodies diluted in PBS-TPO and incubated for 3h at 30°C. There after wells were loaded with Alkaline phosphatase conjugated protein A (Sigma) diluted (1:1000) in PBS-TPO and incubated for 3h at 30°C. Finally the substrate (P-nitrophenyl phosphate) was added as described before and incubated at 30°C. In between each step washing the plate thrice with PBS-T is essential. After colour development reaction was terminated with the addition of 50µl of 3M NaOH and the absorbance was recorded as before.

Protein A indirect ELISA

The method followed was as described by Lommel *et al*., (1982). Wells of the ELISA plate was coated with 200µl of crude leaf extracts of healthy and root(wilt) affected coconut palms extracted and diluted in carbonate buffer and incubated for 15 min at 37°C. After washing the plate thrice with PBS-T the wells were loaded with specific antiserum diluted as before. After incubating

the plate at 37°C for 30 min, the plate was washed and filled with 200µl of protein A ALP conjugate (Sigma) diluted in 1:1,000 in PBS-TPO. Subsequent incubation at 37°C for 30 min and washing, 200µl of substrate P-nitrophenyl phosphate was added to each well and incubated at room temperature until the colour reaches the desired intensity. Reaction was stopped with the addition of 3M NaOH and the absorbance measured as described earlier.

DAC indirect ELISA using Horse radish peroxidase (HRP) conjugate

The procedure followed was that described by Voller *et al*., (1977) with some modifications. ELISA plate was bound with extracts of leaf, homogenized in carbonate buffer and the buffer with additives such as Polyvinylpyrrolidone 44,000 2%, gelatin 1%, Ovalbumin 0.2% in 1:10 dilution. These additives were incorporated into the buffer singly or in various combinations. The plate was covered and incubated for 90 min at 37°C followed by overnight incubation at 5°C. After washing the plate thrice with wash buffer, the wells were loaded with 150µl of 4% BSA dissolved in PBS-T to bind the unbound site of the wells. After incubation at 37°C for 60 min washed the plate once with wash buffer. Then the wells were filled with 100µl of specific antibody diluted in carbonate buffer + clarified healthy leaf grindates and incubated for 90 min at 37°C. After washing the plate thrice with wash buffer, the wells were coated with 100µl HRP conjugate (Genei, Bangalore) diluted (1:2000) in PBS T + 1% BSA, incubated for 60 min at 37°C, washed the plate five times with wash buffer, added 100µl substrate 3,3',5,5'-tetramethyl benzidine (TMB) and incubated the plate in room temperature till colour developed in the samples to the desired level. The reaction was arrested by the addition of 50µl 1N H_2SO_4. Absorbance was measured with 450nm filter. After taking 3 or 4 readings, the plate was incubated overnight at 5°C and again the absorbance recorded.

RESULTS AND DISCUSSION

Standardization of ELISA

Different types of ELISA were performed with Alkaline phosphatase conjugate and its substrate P-nitrophenyl phosphate and Horse radish peroxidase conjugate and its substrate 3,3',5,5'-tetramethyl benzidine to standardize an ideal one. While in variants of ELISA tried with ALP conjugate only marginal difference was observed between healthy and diseased samples, better resolution was noticed with HRP conjugate (Fig.I).To further enhance the specificity of the test, experiments were carried out with the addition of different additives viz. Polyvinylpyrrolidone (PVP) 44,000 - 2%, gelatin 1% and ovalbumin 0.2% to the extraction medium singly or in different combinations. Incorporation of these additives independently did not yield significant difference between healthy and diseased samples. However, when gelatin and ovalbumin were added together, wide difference in absorbance values between healthy and diseased samples was noticed (Fig.II). Similarly, five fold difference in absorbance values between healthy and infected samples was evident when the plates were incubated overnight at 5ºC after the addition of substrate (Fig.III).

Screening of coconut samples

Leaf samples collected from healthy coconut palms growing in disease-free area (Thirunelveli, Tamil Nadu) and apparently healthy and diseased samples (early and middle stages of disease) from disease prevalent areas in Kerala and Tamil Nadu were employed for the test. Prior to ELISA, samples collected from apparently healthy palms were subjected to immuno-diffusion test to determine whether these samples were actually free from disease or in the early stage of infection. According to the test, apparently healthy samples were categorized as serologically and visually negative and serologically positive but visually negative (symptomless). Higher absorbance values were recorded in samples from symptomless as well as palms in the early stage of disease indicating maximum antigen titre (Fig.IV). Similarly, in an attempt to find out the ideal leaf with maximum

antigen titre, leaflets representing the different leaves of a diseased palm was tested and maximum antigen titre was observed in the spear leaf followed by the next outer leaf.

For the detection of plant pathogens, ALP has been widely used and HRP to a limited extent only (Clark, 1981). In the present study with RWD samples, the HRP system was found to be functioning well. As indicated earlier, 5 fold difference was observed in diseased samples whereas only marginal difference was noticed with ALP conjugate. Polak *et al*.,(1988) recommended HRP labeled antibody for the detection of plant viruses. In this study, crude antigen and unfractionated root(wilt) antiserum have been used and has the advantage of simplicity and convenience of application. Moreover, compared to ALP, HRP is rather inexpensive also. Addition of gelatin and ovalbumin in the extraction buffer enhanced the specificity of the test. Compared to immuno-diffusion test which requires 96h, ELISA is more rapid and could be completed within 44h and at a time 30 samples with replication could be tested using microlitre quantity of reactants especially the disease specific antiserum.

CONCLUSION

DAC indirect ELISA system using HRP conjugate and its substrate 3,3',5,5' -tetramethyl benzidine, crude extract of leaves and unfractionated polyclonal antibodies has been developed for the rapid detection of coconut root(wilt) disease. Diseased samples recorded five times higher absorbance value over healthy samples. This is the first report on which ELISA has been successfully used for the detection of coconut root (wilt) disease.

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REFERENCES

BARBARA,D.J. AND CLARK,M.F.1982. A simple indirect ELISA using F(ab')$_2$ fragments of Immunoglobulin.. *J.Gen. Virol*.,**58** : 315-322.

BUTLER, E.J.1908. Report on coconut palm disease in Travancore. *Agri.Res.Inst. Pusa Bull*., **9**:23.

CENTRAL PLANTATION CROPS RESEARCH INSTITUTE, 1985. Coconut root(wilt) disease: Intensity, production loss and future strategy. CPCRI, Kasaragod, 45p.

CENTRAL PLANTATION CROPS RESEARCH INSTITUTE, 2000. Annual Report for 1999. CPCRI, Kasaragod, pp.239.

CLARK, M.F. and ADAMS, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J.Gen. Virol*., **34** : 475-483.

CLARK, M.F.1981. Immunosorbent assays in Plant Pathology. *Ann. Rev. Phytopathol*., 1**9**: 83-106.

KOENIG, R. 1981. Indirect ELISA methods for the broad specificity detection of plant viruses. *J.Gen. Virology*, **48** : 257-264.

LOMMEL, S.A., McCAIN, A.H. and MORRIS, T.J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for the detection of plant viruses. *Phytopathology*, **72**: 1018-1022.

PILLAI, N.K.1911. “Nalikeram”. Vidyabhivardhini Press, Quilon, Kerala. pp.112.

POLAK,J. and KRISTEK, J. 1988. Use of Horse radish-peroxidase labelled antibodies in ELISA for plant virus detection. *J.Phytopathol*., **122** : 200-207.

RADHA, K. and LAL, S.B. 1972. Diagnostic symptoms of root(wilt) disease of coconut. *Indian J. Agric. Sci*., **42** : 410-413.

SOLOMON, J.J., GOVINDANKUTTY, M.P.and NIENHAUS, F. 1983a. Association of mycoplasma-like organisms with the coconut root(wilt) disease in India. *Z.pflkrankh.pflschutz*., **90**:295-297.

SOLOMON, J.J., SASIKALA, M. and SHANTA, P. 1983b. In: Coconut Research and Development (Ed.) N.M.Nayar; Wiley Eastern Ltd., New Delhi. p 401-405.

SRINIVASAN, N., KOSHY, P.K., KAMALAKSHIAMMA, P.G., SASIKALA, M., GUNASEKARAN, M. and SOLOMON, J.J. 2000. Appraisal of the distribution of coconut root(wilt) and heavy incidence of the disease in Cumbum valley of Tamil Nadu. *Indian Coconut J*., **31**(1) : 1-5.

VOLLER, A. and BIDWELL, D.E. 1977. Enzyme immunoassays and their potential in diagnostic virology. In: Comparative Diagnosis of Viral Diseases. Vol.II (E.Kurstak and C.Kurstak, eds.) pp.449-457. Academic Press, New York.

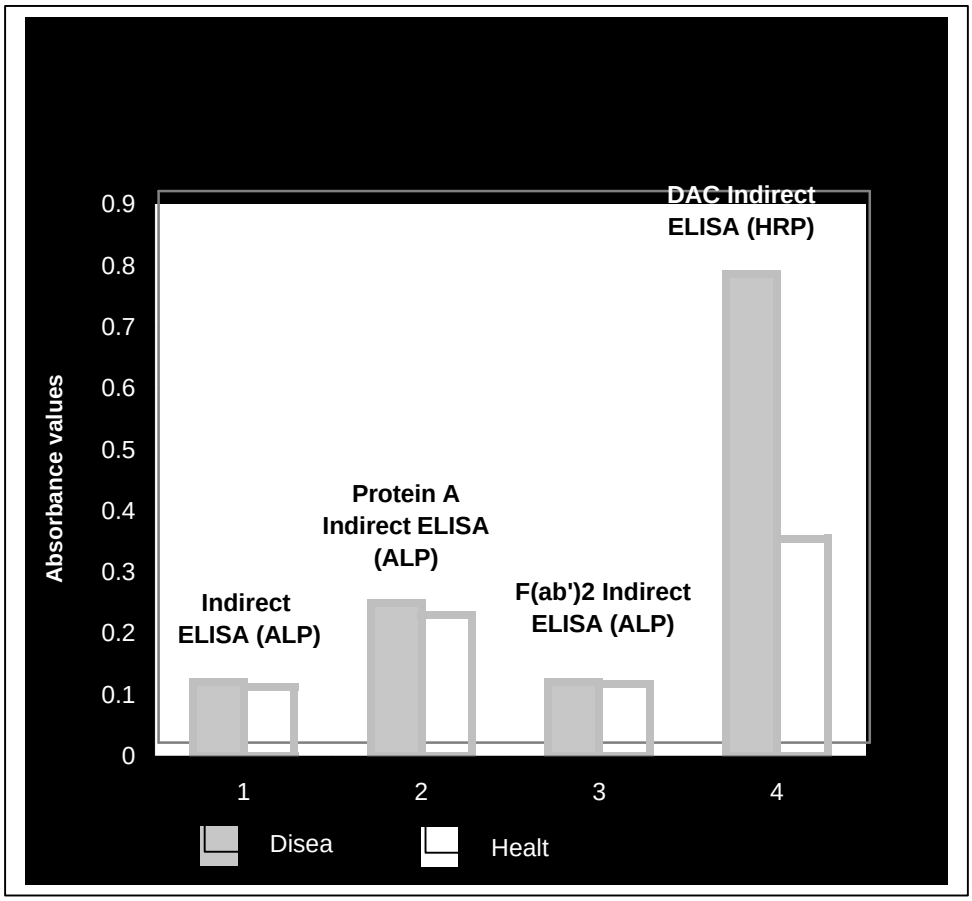

Fig.2 : Absorbance Values after the incorporation of additives

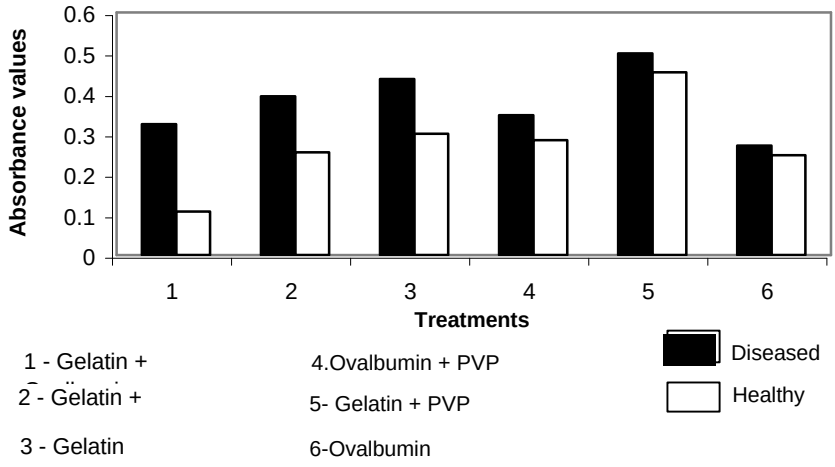

Fig.3 : Effect of incubation time after substrate addition

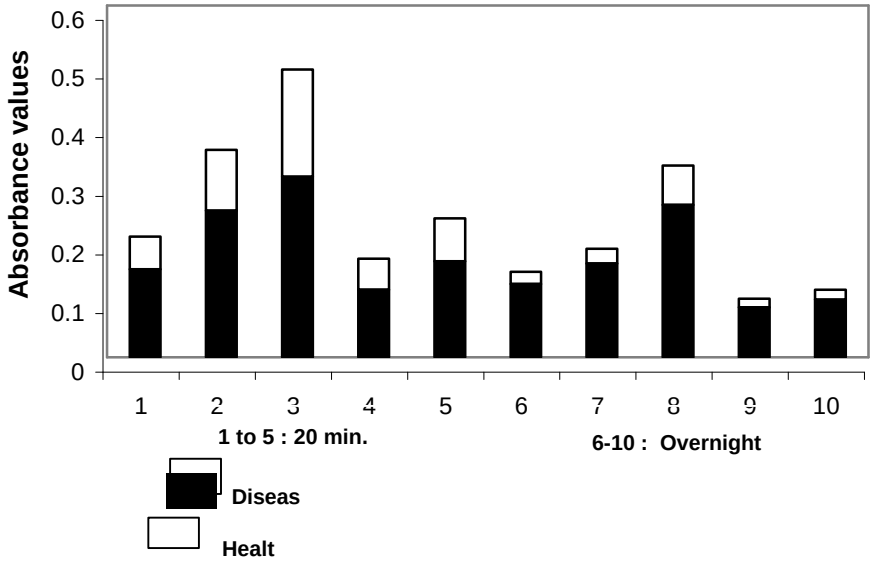

Fig.4 : Absorbance values of healthy, apparently healthy and diseased palms