Weligama Coconut Leaf Wilt Disease (WCLWD) causing phytoplasma

Chamini Kanatiwela[1], Sachini Amarasekara[1], Rohana Wijesekara[2], Preethi Udagama[1]

[1]*Department of Zoology, Faculty of Science*

[2]*Crop Protection Division, Coconut Research Institute, Lunuwila*

Introduction

Coconut is one of the most important plantation crops in Sri Lanka, providing livelihood for nearly 0.5 million people (Ministry of plantation industries, 2008). Sri Lanka has earned about $ 120 million of export revenue by exporting coconut products. The Weligama Coconut Leaf Wilt Disease (WCLWD), the major threat to the crop was first reported in Weligama area in 2006. The disease has so far affected trees in the Galle, Matara, and Hambanthota districts. Although non-lethal, this disease could result in permanent injuries to the plant reducing its productivity. There would be dire consequences if this disease spreads to the coconut triangle, which includes an area of more than 70% of the total coconut cultivated land in the country.

The early symptoms of the disease are difficult to be identified and also symptom development is very slow (Sasikala *et al*, 2005). As no reliable therapeutic agent is yet discovered for this phytoplasmic disease, the only prevention method is to remove and dispose of all affected individual trees. Up to now, the Coconut Research Institute was compelled to cut down and destroy nearly 100,000 coconut palms in the Matara district due to this disease.

Phytoplasma disease diagnosis is difficult due to the inability of phytoplasma cultivation in pure cultures *in vitro,* their low concentration and uneven distribution in the host plant (Fránová *et al*., 2007). A sensitive, specific and rapid diagnostic test would be highly desirable for routine detection, mainly to avoid using infected planting material (Heinrich et al., 2001). A serological diagnostic technique based on monoclonal antibodies to the phytoplasma would provide an excellent diagnostic tool. Thus, this research aims to raise diagnostic monoclonal antibodies to WCLW phytoplasma, where initially polyclonal serum raised in experimental animals to purified phytoplasma was used to establish a specific ELISA for subsequent screening of specific hybridomas. Attempts to validate the in house established WCLWD phytoplasma specific indirect ELISA (Kanatiwela *et al*., 2012), using 110 coconut palms including 80 disease positive and 30 disease negative palms is reported.

Materials and Methods

The total number of samples to be screened was determined using the formula, n= {[4 x ds x (1-ds)]/e} where ds is the diagnosed sensitivity that is sought (95%) and e is the amount of error allowed in the estimate of diagnostic sensitivity (5%) (Crowther, 2009).

Infected coconut palms were collected from the plantations of the Matara district, and apparently healthy palms were collected from home gardens of Moratuwa and Horana areas in the Colombo district. Sugarcane plants infected with White Leaf Disease (WLD), caused by an unrelated phytoplasma, (kindly provided by the Sugarcane Research Institute) were used as disease control.

DNA extracted from all coconut palms and sugarcane plants were subjected to a nested polymerase chain reaction using phytoplasma specific universal primers (P1, P7/R16F2n, R16R2). The coconut samples yielding amplicons that produced a characteristic band of 1.2 kb on 1% agarose gels were considered disease positive (DP) samples (N=80) while the rest that lacked this band were considered to be disease negative (DN) samples (N=30). Five sugarcane samples that produced this characteristic band on the gel were used as disease controls.

The antigen extracted from the leaf samples by grinding 1 g of spear leaves in 10 ml of PBS + 2% Polyvinylpyrolidone was subjected to indirect ELISA. The cut-off value separating positive (True Positive- TP) from negative (True Negative- TN) samples was calculated as 2 standard deviations above the mean OD value of the disease negative (DN) values (DN values + 2SD) obtained from 30 healthy coconut palms.

In the validation of this ELISA, Diagnostic Sensitivity (D-SN= [TP/ (TP +FN)] x 100), Diagnostic Specificity (D-SP = [TN/ (TN+FP)] x 100), False Positivity (FP = 1- D-SP) and False Negativity (FN = 1-D-SN) were determined (Crowther, 2009).

Plotting the receiver operating characteristic (ROC) curve is a common approach of displaying the discriminatory power of a diagnostic test (Williams *et al*, 2011). ROC analysis was carried out using SPSS 15.0 for Windows Evaluation Version to determine the accuracy of the test in distinguishing healthy and infected palms by this established ELISA.

Results

It was calculated that a total of 360 coconut palms should be screened in the validation process.

The OD cut-off value separating positive (True Positive- TP) from negative (True Negative- TN) samples was calculated to be 0.27 (Fig 1).

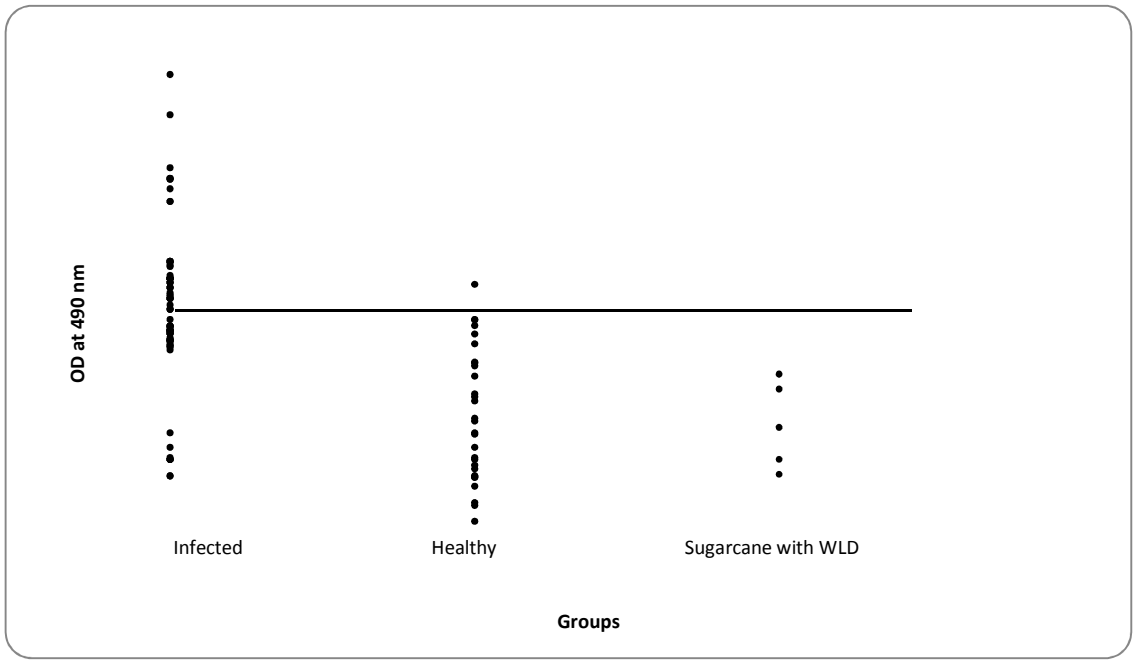

Figure 1: Optical density (OD) responses of WCLWD phytoplasma infected and healthy coconut leaf samples and of sugarcane leaf samples infected with WLD.

Each closed circle represents the mean value of duplicate absorbance values of each antigen sample. Horizontal line indicates the cutoff value (0.27) calculated to distinguish infected and healthy coconut palms.

The following parameters were calculated using data obtained from screening 110 coconut palms (N=80 PCR positive and N=30 PCR negatives) by the established indirect ELISA for WCLWD pyhtoplasma, for the initial validation of this assay: diagnostic sensitivity of 88.75%, specificity of 80%, while false negativity and false positivity were 11.25% and 20%, respectively.

The area under the curve (AUC) of a ROC plot of a perfect diagnostic test with 100% accuracy, that fully discriminates between positives and negative samples equals one. When the ROC curve analysis was performed, the AUC was calculated to be 0.868 (accuracy of 87%) which indicated that this in house established Indirect ELISA for WCLWD phytoplama performs well at distinguishing between phytoplasma infected and healthy palms (Fig 2).

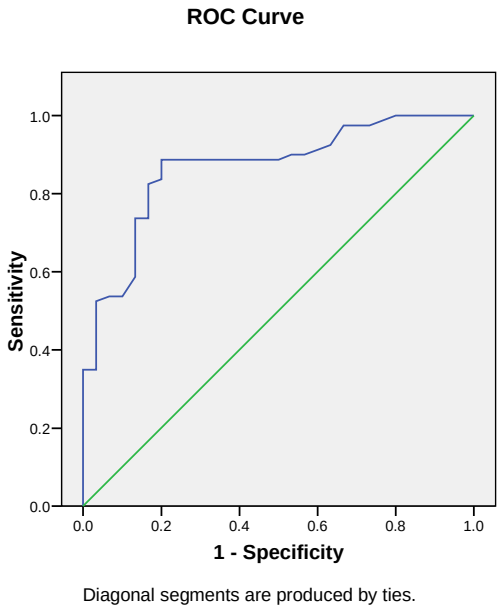

Figure 2: Receiver-operating characteristic curve for the assessment of absorbance values of phytoplasma infected and healthy coconut samples

Many more WCLWD phytoplasma positive and negative coconut palm samples (N=250) as well as several types of disease control samples must be screened by this ELISA in order to further validate this assay.

Conclusions

The in house established Indirect ELISA for WCLWD phytoplasma, using crude plant extract and specific polyclonal serum, seems to have satisfactory sensitivity (89%) and specificity (80%) values and the area under the curve in ROC curve analysis confirms that this assay performs with good accuracy (87%) to distinguish healthy and infected palms. These parameters may be improved by screening 250 more coconut palms as statistically required. It is encouraging that this indirect ELISA does not recognize sugarcane leaf extracts that are infected with White Leaf Disease caused by an unrelated phytoplasma.

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Financial assistance by the Coconut Research Institute is acknowledged.