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IMMUNOSENSOR-BASED DETECTION OF TUNGRO DISEASE IN RICE PLANT

MUHAMMAD NUR AIMAN BIN UDA

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IMMUNOSENSOR-BASED DETECTION OF TUNGRO DISEASE IN RICE PLANT

By

MUHAMMAD NUR AIMAN BIN UDA

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfillment of the requirement for the Degree of Master of Science

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Chair : Associate Professor Hasfalina Che Man, PhD
Faculty: Engineering

Rice tungro disease is the major constraint, caused by a combination of rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV). Major outbreaks of tungro have been occurred in countries of South and Southeast Asia, mediated by a viral carrier green leafhopper. To prevent serious outbreaks, detection of Tungro disease requires a fast, simple and sensitive method. The current study was initiated by transferring viruses from the green leafhopper to susceptible plant host varieties (Y1286 and MR81 for RTSV and RTBV, respectively). After inoculation process, both viruses were purified and spectrophotometrically measured as 1.363 and 1.6075 mg mL-1 for RTBV, 1.227 and 1.6075 mg mL-1 for RTSV. Viral particles were observed by transmission electron microscopy, where RTBV was observed as 168 mm in length with a bacilliform, whereas RTSV appeared with 35 mm in diameter, showed rod-shaped rounded ends. Next, pure viruses were immunized in White New Zealand rabbits and antibody was analyzed on enzyme-linked immunoassay (ELISA) surface. Analysis with RTBV showed that the second bleed has the highest titer, whereas for RTSV bleed 1 has the highest titer (1.6960 mg mL-1 and 2.3251 mg mL-1). Analysis on screen-printed carbon electrode (SPCE) was incorporated with 0.075 M pyrolle monomer where electro-polymerization process occurred at 0.9 V amperometrically for 20 min. Chronoamperometry measurements showed the best potential to be used is located at 0.2 V, for both RTBV and RTSV. In addition, the antibody immobilized surfaces of SPCE were analyzed by scanning electron microscope. A linear standard curve for each virus obtained based on current measurement (μA), where R^2 values were 0.9755 and 0.967, indicates a higher sensitivity of immunosensor developed. Cross reactivity studies, showed the specificity of the antibodies with a low cross-reactivity, although RTBV and RTSV it-self manifested strongest serological cross-reactivity.

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IMMUNOSENSOR-BERASASKAN PENGESANAN PENYAKIT MERAH VIRUS DI POKOK PADI

Oleh

MUHAMMAD NUR AIMAN BIN UDA

Disember 2015

Pengerusi : Professor Madya Hasfalina Che Man, PhD
Fakulti : Kejuruteraan

Penyakit merah virus (PMV) adalah kekangan utama, yang disebabkan oleh gabungan dua virus iaitu virus berbentuk sfera (RTSV) dan virus berbentuk basiliform (RTBV). wabak utama PMV telah berlaku di negara-negara Asia Selatan dan Asia Tenggara dengan pengantara oleh bena hijau pembawa virus. Untuk mengelakkan wabak yang serius, pengesanan penyakit PMV memerlukan kaedah yang cepat, mudah dan sensitif. Kajian ini telah dimulakan dengan proses pemindahan virus dari bena hijau kepada benih khusus (Y1286 dan MR81 untuk RTSV dan RTBV). Selepas proses inokulasi, kedua-dua virus ditulenkan dan kepekatan virus ini telah diperiksa menggunakan UV-spektrofotometer iaitu 1,363 dan 1,6075 mg mL^{-1} untuk RTBV, 1,227 dan 1,6075 mg mL^{-1} untuk RTSV. Zarah virus diperhatikan oleh mikroskop elektron penghantaran, dimana RTBV diperhatikan sebagai 168 mm panjang dengan bacilliform, manakala RTSV muncul dengan 35 mm diameter, menunjukkan rod berbentuk hujung bulat. Seterusnya, virus tulen telah imunisasi menggunakan arnab White New Zealand dan antibodi dianalisis pada permukaan immunoassay enzim berkaitan (ELISA). Analisis dengan RTBV menunjukkan bahawa pengambilan darah kedua mempunyai titer tertinggi, sebaliknya bagi RTSV pengambilan pendarahan pertama mempunyai titer tertinggi (1,6960 mg mL-1 dan 2,3251 mg mL-1). Analisis pada skrin bercetak karbon elektrod (SPCE) dipandankn 0.075 M pyrolle monomer di mana proses elektro-pempolimeran berlaku pada 0.9 V amperometrically selama 20 min. ukuran Chronoamperometry menunjukkan potensi yang terbaik untuk digunakan terletak pada 0.2 V, untuk kedua-dua RTBV dan RTSV. Di samping itu, permukaan antibodi bergerak daripada SPCE dianalisis dengan mengimbas mikroskop elektron. Keluk standard linear bagi setiap virus yang diperoleh berdasarkan ukuran semasa (A) jika, di mana nilai-nilai R^2 adalah 0,9755 dan 0,967, menunjukkan sensitiviti yang lebih tinggi immunosensor yang dibangunkan. kajian tindak balas silang, menunjukkan pengkhususan antibodi dengan tindak balas silang yang rendah, walaupun RTBV dan RTSV ia diri ditunjukkan kuat serologi tindak balas silang.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Hasfalina Che Man, PhD
Associate Professor
Faculty of Engineering
Universiti Putra Malaysia
(Chairman)

Samsuzana Abd Aziz, PhD
Senior Lecturer, Ir
Faculty of Engineering
Universiti Putra Malaysia
(Member)

Faridah Salam, PhD
Senior Research Officer
Malaysia Agriculture Research Development Institute (MARDI)
(Member)

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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LIST OF ABBREVIATIONS

Bsa	Bovine Serum Albumin
Cm	Choro-Amperometric
Cv	Cyclic Voltammetry
Cmv	Cucumber Mosaic Virus
Cg	Colletotrichum Gloeosporioides
Dna	Deoxyribonucleic Acid
Elisa	Enzyme-Linked Immunosorbent Assay
Hrp	Horseradish Peroxidase
Ig	Immunoglobulines
Kda	Kilo Dalton
Lod	Limit Of Detection
Nkea	National Key Economic Area
Pcr	Polymerase Chain Reaction
Prsv	Papaya Ring-Spot Virus
Ppy	Polypyrrole
Re	Reference Electrode
Rtbv	Rice Tungro Bacilliform Virus
Rtsv	Rice Tungro Spherical Virus
Rna	Ribonucleic Acid
Spce	Screen Printed Carbon Electrode
Spr	Surface Plasmon Resonance
Std	Standard
Uv	Ultra Violet
We	Working Electrode

LIST OF SYMBOLS

G	Gram
Kg	Kilogram
%	Percentage
H	Hours
M	Molar
Min	Minutes
Ml	Milliliter
%	Percentage
Rpm	Revolutions Per Minute
°C	Celsius

CHAPTER 1

INTRODUCTION

1.1 Background

The agriculture sector plays an important role in Malaysia's economic development in providing rural employment, uplifting rural incomes and ensuring national food security. Under the Economic Transformation Programme (ETP), (NKEA Agricultural, 2014) the Malaysian government wants to ensure that food security objectives are achieved. There is a need for up-scaling and increasing the productivity of agro food production in Malaysia to increase self-sufficiency, due to the growing population. However, plant disease out-break continues to be one of the most important issues globally especially in the agriculture based countries.

Lately, plant diseases became a serious problem which significantly affects both the quality and quantity of agricultural products (Sankaran *et al*., 2010) The infection of disease to the plant area will reduce the quality of agricultural product (Meunkaewjinda *et al*., 2008) as well as cause a significant agronomic impact (López *et al*., 2003). Besides, plant diseases can cause periodic or catastrophes in large agricultural fields which can lead to famine (Arivazhagan *et al*., 2013)

In 2008, the government announced a Food Security Policy (FSP) as a measure to guarantee adequate supplies of food, especially rice .Rice is the staple food for most Malaysian, it is the most consumed crop where 26,041000 tonne matrix in the year 2013 paddy production with 78.8 kg/years per capital consumption. In addition to that, paddy is the third most widely planted crop in Malaysia after oil palm and rubber. In 2013, an approximately 674,332 hectares were planted with paddy including those that are planted twice a year (Department of Agriculutral, 2013). Nevertheless, rice tungro disease is greatly hampered the rice production in Southeast Asia including Malaysia. This disease is one of the most vicious and damaging disease causing serious risks to the increase of rice production (Mohd Daud *et al*.,2013).

This viral disease can affect thousands of hectares due to the absence of symptoms at an early growth stage. As a result, swift disease expansion in the outbreak area becomes one of the challenges in tungro management. The disease detection time and applying effective actions is a major challenge for the control of the disease (Yao *et al*., 2009).This disease can be calculated to cause production loss as high as 100%. Many farmers called this disease as a cancer disease because of the severe damage it causes and the difficulty of controlling it (Mohd Daud *et al*., 2013) . This disease is caused by two composite viruses which is rice tungro bacilliform virus (RTBV) a double-stranded DNA-containing in the Caulimoviridae family, Tungrovirus genus. Whereas, rice tungro spherical virus (RTSV) is a single-stranded RNA virus in the Sequiviridae family, Waikavirus genus (Nath *et al*., 2000).These two

viruses combined together in the host plant appears as tungro disease symptoms.

In South and Southeast Asian countries including Malaysia, Green leafhopper (*Nephotettix virescens)* are common vector that combat and disperses the virus agents of tungro disease in rice field. Two main symptoms occur when there is a sever outbreak of these viruses in the rice plantation. Firstly when rice is infected with RTSV it will causes mild or blurred symptoms. Most of researcher called RTSV as latent virus and causal virus of tungro for transmission of rice tungro bacilliform virus by green leafhopper (Lane & Louis, 1991) .On the other hand RTBV infections causes yellowing and reddening of the leaves with stunted growth.

Apart from that, RTBV can clearly show the tungro symptoms while RTSV have the capacity to enhance the symptoms. In addition, the green leafhopper has ability to acquire and spread RTSV but incapable to acquire RTBV for spreading the disease. Nevertheless, green leafhoppers that have fed previously on RTSV-infected plants are capable of acquiring RTBV from plants infected with RTBV (Nath *et al*., 2000). Due to the extreme importance of securing the production of rice, it is necessary to develop a simple and effective tool for tungro disease detection. Most of times, the symptoms of the disease are often detected too late to implement further actions to overcome the problems (Mohd Daud *et al*., 2013)

Generally, the farmers detect tungro diseases by visual observations. However, it is quite difficulty and not reliable to identify the symptoms through visual; due to the difficulty to differentiate it with non-pathogenic disorder such as nutritional deficiencies, excess water after drought or insect injury which cause similar symptoms (Nath *et al*., 2000) . In addition to that, most farmers are taking simple approach by using pesticides to control and monitor vector. However, this approach is not effective and it also affects the health of the operators (Mohd Daud *et al*., 2013). In this situation, an imperative method needs to be developed because the two types of viral infections are sometimes present independently in some plants which make it difficult to prevent during serious outbreaks.

Therefore, the detection of tungro disease required a fast, simple and sensitive method compare to convention methods that have been developed for tungro disease detection. Serological technique such enzyme linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) are excessively been used for the detection of tungro virus due to their capable to interact between antigen and antibody (Uda *et al*., 2013). Despite this exposure, the use of ELISA and SPR for tungro viruses are generally expensive as well as their operation need extension services and required some expertise with sophisticated equipment. A part from that, both methods give a major drawback which are labor-intensive and at least it takes 2 days for the results to be known.

Polymerase chain reaction (PCR) is another diagnostic tool used for tungro disease detection. It is very sensitive to target molecule detection and it can be able to detect low level of specific viral DNA and RNA present in variety of samples with highly sensitivity detection over ELISA. However, the total time frame of the analysis still takes several hours and requires trained personal to conduct the assays (Dasgupta *et al*., 1996). Nowadays, the use of the biosensor applications become a trend to facilitate the identification of microbial pathogens and the ability to perform simple detection, rapid and sensitive in the sample matrix (Alocilja & Radke, 2003) . These technologies has great advantages with unique capabilities for real time and on-site analysis in complex mixture without manipulate for treatment or required large number of sample for analysis (Velasco-Garcia & Mottram, 2003).

Due to the rapid development in research, there are many types of biosensor design based on signal transduction such as optical, electrochemical, piezoelectric, calorimetric and magnetic method which have been applied in various areas. Within these great advances in the design of sensor architectures, the integration of biological sensing (capturing) molecules can be in the form of antibodies, enzymes, nucleic acid and cells. At present, biosensors have expansive application in medicine, agriculture, environmental monitoring and the bioprocessing areas. Recent progress in these areas has already led to the introduction of new-generation biosensors into the competitive diagnostics market place (Saleem, 2013).

Presently, immunosensor technologies (Immuno-biosensor) have been developed to replace traditional analytical techniques which rely on the antibody-antigen binding reaction. The technique for immunosensors is based on the combination of specific antigens and antibodies in a solution on the surface support coupled to a signal transducer. Currently, the development of this device is for high degree sensitivity, specificity, reusability, speed ease and on-site analysis. Additionally, the recent advance in nanotechnology gives extraordinary sensing capabilities toward high selective for target analyte molecules.

1.2 Problem Statement

Rice is regarded as the most important crop in the food sub-sector in Malaysia. The government regards food security as an integral national policy objective for overall development and has stressed that food security is synonymous with rice security. Thus, the Malaysian government willingly support any program focused on rice because it is the staple food of the vast majority of the population (Swamy & Kumar, 2013; Tyagi & Mohanty, 2000). In major rice-growing countries, the outbreaks of rice disease remain the major threat to sustainable rice production. As one of the most destructive in paddy plantation, tungro disease has been recognized as nutritional disorder and widely disturbed not only in Malaysia but also affected in South and South East Asia (Uda *et al*., 2014a).

In the case of tungro disease, the symptoms are often detected lately, which makes it difficult to take any further action. A part from that, this disease is considered as a nutritional disorder of rice since 1950.Up to date, the control of Tungro disease is still a challenge, although many studies have been conducted to eradicate and prevent its outbreak in rice fields. As a result, a monitoring system is essential to provide a faster detection and to prevent a serious outbreak.

Conventional method remains the most reliable technique to be used as a tool to detect tungro disease such ELISA, SPR and PCR (Dasgupta *et al*., 1996;Uda *et al*., 2013). However, the major drawbacks of such techniques are labour-intensive which requires trained personnel and take 2-3 days for the results to be confirmation. This long testing time is inconvenience for industrial applications and particularly in the agricultural sector while screening methods generally require minimal technical expertise.

Currently, detection based on biosensor technology promises a fastest result over conventional method. Thus, immunosensor based detection of tungro disease in rice plant can be used for analytical tools and useful as diagnostic for early detection of physiological changes of the paddy. Thus, it becomes one of the potential benefits to sustain rice production.

The development of immunosensor is optimized for operating under specific condition for a special problem and most work is focused on sensitivity, speed, efficiency and simplicity of the assay procedure. Additionally, the ability of direct signal generation in immunosensor has the potential for real-time monitoring of analytes which is suitable tool for continuous monitoring in infected area (Tothill, 2009).

In this thesis, a screen-printed carbon electrode (SPCE) was used in the development of an immunosensor and designed with a three electrodes system (working, reference and counter). The SPCE have been broadly used because they are economical (easy to fabricate in bulk and disposable), easy to handle, have high sensitivity and a miniaturized portable system. Thus it is potential to be used for the detection of tungro disease in paddy fields.

1.3 Research Objectives

The aim of this study is to detect tungro disease using immunosensor platform by screen printed carbon electrode (SPCE) as diagnostic tool with high sensitivity for rice tungro disease virus detection in rice plant.

The specific objectives of this research are:

1.To isolate and produce specific antibody for two types of rice disease viruses which are rice tungro bacilliform virus (RTBV) and rice tungro spherical virus (RTSV) as bio-recognition element on the sensor surface.

2.To detect RTBV and RTSV using screen printed carbon electrode for highly sensitive detection of tungro disease.

1.4 Scope of Study

The study was conducted with the scope specified below:

a) In this study, propagation of viruses (RTBV and RTSV) through two artificially infected host varieties, which are MR81 for RTBV and Y1286 for RTSV. Due to continuous availability and suitability for laboratory experiments, these varieties were selected as model system and can be implemented to other rice varieties commonly using by farmers. Further, both varieties being used due to its susceptibility to produce more viruses. Rice plantation was limited to the laboratory scale to obtain precise results and to avoid spreading to the field. Viruses were purified and confirmed using Transmission Electron Microscopy (TEM) and UV-spectrophotometer before immunizing New Zealand White Rabbit, they are capable to produce specific polyclonal antibodies in laboratory scale. Rabbit was chosen as the host for immunization, due to its capability to recognize diverse epitopes, rabbit can also develops antibodies for small epitopes efficiently and tend to produce high-affinity antibodies. The purified antibody was optimized to determine the concentration for titer using ELISA, which revealed the right antibody titer for immunosensor development.

b) Immunosensor platform was developed using screen-printed carbon electrode (SPCE) and characterized the interaction between antigen–antibody to develop the standard curve for sensing purpose. Apart from that, limit of detection (LOD) of the sensor was determined to efficiently identify the unknown sample from paddy plantation. However, this research focused in lab scale without involving any field test in paddy plantation due to the above mentioned reasons.

1.5 Significant Contribution

The main contribution of this research is to develop a highly sensitive method as a diagnostic tool for tungro disease infection. The developed sensing system in this study can be the model to sense diseases in rice other crops. Another significant contribution is to be used for industrial applications and particularly in agricultural sector to sustain rice production and prevent economic impact in Malaysia and around the world. The sensor platform shown here is suitable for antibody based screening, applicable for downstream applications.

1.6 Thesis Organization

This thesis constituted of 5 chapters with reference and appendices attached. Each of the chapters will be briefly discussed and they are interrelated to each other.

Chapters 1, (Introduction), give a general overview of tungro disease outbreak. Therefore, an alternative solution is proposed concerning the specific problem which has been addressed in the objectives and scope of the study.

Chapter 2, Literature review, describes about history of tungro disease and the characteristics of infections. It also include the general concept of biosensor which specific to development of immunosensor, concept of electrochemical and the mechanism of reaction were also reviewed. Summary of the literature review is mentioned in this chapter.

Chapter 3, Methodology, gives the details process from inoculation procedure to host plant until the last part of the development of immunosensors using screen printed carbon electrode (SPCE). Details of purification of viruses, immunization into white rabbit New Zealand, purification of antibody and analysis of sensor analysis was described in this chapter.

Chapter 4, Results and Discussions, presents the experimental results and these results were discussed in light of previous findings.

Chapter 5, Conclusion and Recommendation, provides a comprehensive summary and conclusion of the study and also highlights recommendations for future studies.

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