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DEVELOPMENT OF AN ELECTROCHEMICAL IMMUNOSENSOR STRIP FOR EARLY DETECTION OF RICE BACTERIAL LEAF BLIGHT (BLB) DISEASE AND ITS APPLICATION ON A PORTABLE DEVICE

(Pembangunan Strip Imunosensor Elektrokimia Untuk Pengesanan Awal Penyakit Hawar Daun Bakteria Padi dan Aplikasinya Pada Peranti Mudah Alih)

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Abstract

We described here an electrochemical immunosensor strip based on a screen-printed carbon electrode (SPCE) for early detection of rice bacterial leaf blight (BLB) disease. The causal agent for this destructive disease has been identified as *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In order to circumvent the disease outbreak, an early detection system is required. Polyclonal antibody against *Xoo* was employed and immobilized on the SPCE strips modified with polypyrrole (PPy) and functionalized multi-walled carbon nanotube (fMWCNT) network. The anti-*Xoo* antibody is conjugated with horseradish peroxidase (HRP) as an enzyme label and used as the detection agent in the sensor development. Electrochemical detection was carried out via the chronoamperometry technique at a set potential of -200 mV. A fixed anti-*Xoo* antibody concentration at 0.03 mg/mL on the working electrode of the strip surface produced a standard linear curve for *Xoo* detection (R^2 = 0.9746). Two extraction methods for rice leaves (scissors-cutting and grinding) were compared for real samples application analysis. The scissors-cutting method had less matrix interference effect and gave a higher recovery rate than the grinding method. The optimal immunosensor configuration was then compared with the PCR technique for *Xoo* detection in inoculated leaves in a controlled environment. A good correlation of 92.7% was achieved between the two methods. The immunosensor strips were then tested on an Android-based portable biosensor device for on-site detection of BLB in hotspot areas at Bagan Terap, Selangor Northwest and Sg. Burong, Tanjung Karang. On-field detection has indicated that the immunosensor strips can detect BLB disease as early as 15 days after transplant (DAT) before symptoms appear.

Keywords: bacterial leaf blight, early detection, immunosensor, rice disease, *Xanthomonas oryzae* pv.*oryzae*

Abstrak

Satu strip imunosensor elektrokimia berasaskan elektrod karbon bercetak skrin (SPCE) untuk pengesanan awal penyakit hawar daun bakteria (BLB) pada pokok padi dilaporkan dalam kajian ini. Agen penyakit padi ini telah dikenalpasti disebabkan oleh bakteria *Xanthomonas oryzae* pv. *oryzea* (*Xoo*). Bagi mengekang penyebaran penyakit padi ini, satu sistem pengesanan awal penyakit padi adalah diperlukan. Antibodi poliklonal terhadap *Xoo* digunakan dalam kajian ini dan dipegunkan ke atas permukaan strip yang telah diubah suai dengan jaringan polipirol (PPy) dan nanotiub karbon berbilang dinding berfungsi (fMWCNT). Antibodi anti-*Xoo* dikonjugasikan dengan *horseradish peroxidase* (HRP) sebagai label enzim; dan digunakan sebagai agen pengesanan dalam pembangunan sensor. Pengesanan elektrokimia dilakukan dengan menggunakan teknik kronoamperometri pada set potensi -200 mV. Pada kepekatan antibodi 0.03 mg/mLyang dipegunkan pada permukaan elektrod bekerja strip, satu graf piawai linear untuk pengesanan *Xoo* berjaya dibangunkan (R^2 = 0.9746). Bagi aplikasi sampel sebenar, dua kaedah pengekstrakan daun padi (menggunting daun dan pengisaran daun) telah dibandingkan. Kaedah menggunting daun didapati memberikan kesan matriks yang lebih rendah dan kadar pulangan yang lebih tinggi berbanding kaedah pengisaran daun. Kaedah sensor ini kemudian dibandingkan dengan teknik PCR bagi analisis sampel daun teraruh dengan bakteria *Xoo* dalam persekitaran yang terkawal. Korelasi yang baik dengan nilai 92.7% telah dicapai bagi kedua-dua kaedah tersebut. Strip imunosensor ini kemudiannya diuji pada satu peranti biosensor mudah alih berasaskan Android untuk pengesanan BLB di lapangan di kawasan titik panas Bagan Terap, Barat Laut Selangor dan Sg. Burong, Tanjung Karang. Kajian lapangan menunjukkan bahawa strip pengesanan imunosensor ini berupaya mengesan penyakit BLB seawal hari ke-15 selepas transplan (HLT) sebelum kemunculan simptom penyakit.

Kata kunci: hawar daun bakteria, pengesanan awal, imunosensor, penyakit padi, *Xanthomonas oryzae* pv. *oryzae (Xoo)*

Introduction

Rice (*Oryza sativa* L.) is an important staple food in Malaysia and the Asia region. However, rice production has always been hampered by the recurrent attack of several rice diseases, causing massive yield and economic loss to the farmers and agricultural industry. In Malaysia itself, rice diseases in a susceptible variety's crop may cause yield reduction up to 70% [1] depending on the stage and duration of water deficit [2]. Bacterial leaf blight (BLB) disease is one of the major rice diseases in Malaysia. This destructive rice disease is caused by *Xanthomonas oryzae* pv.*oryzae (Xoo)*, a gram-negative bacterium. *Xoo* cells invade the rice plants through the wound opening on the leaves. At the initial stage, BLB symptoms appear as tannish-grey to white-yellowish lesions along the leaf's veins, and this alone causes yield losses up to 50% [3]. At advanced stages, however, BLB is difficult to distinguish from the bacterial leaf streak (BLS) disease, and although direct observation of the bacteria is preferred, the indication is not scientifically proven or confirmed.

Chemical control still remains the main method to reduce the infection of bacterial diseases incidence. However, in good plant disease management, the use of pesticides should be minimized. To prevent or control the spread of the disease, it is therefore crucial for farmers or extension agencies to detect the causal pathogens accurately at an early stage. Common practice for rice disease detection relies on the symptom's recognition by experienced plant pathologists or farmers. This technique, however is not scientifically validated. Other laboratory-based methods such as agar plate culture, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA), albeit specific, still require skilled and trained operators to operate them [4]. Furthermore, these techniques are generally not suitable for on-site analysis.

Under this circumstance, there is a considerable need to develop a biosensor system for early and accurate pathogen detection for rice disease management. With this regard, biosensor technology lends itself well due to its attractive features such as rapid detection sensitivity, reliability, inexpensive, simple performance, and most importantly, its ability to provide real-time and on-field analysis [5, 6]. Antibody-based biosensor or immunosensor application for agricultural plant pathogens detection is reported to hold great potential as it allows detection in various sample matrices such as water and seeds [7]. In this study, an antibody-based electrochemical biosensor strip is developed for the early detection of BLB disease. Electrochemical immunosensor has gained much attention in recent

decades owing to their versatility with wide sensing range and high sensitivity [8, 9].

The electrochemical immunosensor works on the principle that the specific binding interaction between antibody and antigen on an electrode strip is sensed and the signal generated is measured and amplified. Surface functionalization on an electrode surface is generally performed using conducting electroactive polymers (CEPs) and/or nanomaterials to provide antibody binding sites and enhance the signal generated. Here, we employ polypyrrole as the CEPs with functionalized multi-walled carbon nanotubes (fMWCNTs) to serve those purposes. MWCNTs-modified biosensor has been widely used due to their superior performance [10], sensitivity, and, most importantly, suitability for biomolecules exposure. When performing an immunochemical reaction, the binding interaction between the biomolecules and their targeted analytes can be performed either by direct or indirect assay format. Herein, a sandwich assay enzyme-linked immunosorbent assay (ELISA) format was adapted and employed in the sensor development.

Following the successful development and performance of the biosensor strips as indicated in the inoculated samples study, the strips were then tested on an Android-based portable electrochemical device for on-site BLB detection in rice fields located at Selangor Northwest. This device is integrated with the Internet-of-Things (IoT) application that allows the analysed data to be stored on a cloud server and accessed by supervisors at different locations instantaneously. The biosensor strips application with a handheld Android device offers flexibility and portability in the IoT-biosensor system [11]. Ultimately, integrating IoT with the biosensor system will greatly change the way plant disease management control, particularly for the early warning system for rice disease detection. Hence the objective of this study is first to develop an immuno-based strip for early detection of BLB disease in rice plants, and secondly, to demonstrate the strips' viability when attached to a custom-made portable biosensor device for on-site application. To the best of our knowledge, the latter has not been reported and ventured by any researchers in rice/plant disease management though several reports on plant disease biosensors have been published [3, 7, 12, 13, 14].

Materials and Methods

Production of polyclonal antibody against *Xoo*

Polyclonal antibody against *Xoo* was raised and developed in-house at Biotechnology and Nanotechnology Research Centre, MARDI Serdang using local isolated cultures from Paddy and Rice Research Center, MARDI Seberang Perai (P0.0, P1.0, and P7.3 strains). The procedure for polyclonal antibody production has been granted with MARDI Animal Ethics Committee Approval (20171103/R/MAEC27). Antibody purification was conducted using the Akta-Prime protein purifier system (GE Healthcare).

Chemicals and reagents

Ethanolamine, phosphate buffer saline (PBS) tablets, pyrrole, sodium bicarbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), and *3,3',5,5'*-tetramethylbenzidine (TMB) substrate solution were purchased from Sigma-Aldrich, USA. *N*-(3-Dimethylaminopropyl) - *N*'-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were from Sigma-Aldrich, Japan. Functionalized multi-walled carbon nanotubes (MWCNT-COOH) were supplied from Dropsens, Spain. Plastic-based screen-printed carbon electrodes (SPCEs) were from BioGenes Technologies Sdn. Bhd., Malaysia. EZ-Link™ Plus Activated Peroxidase Kit was from Thermo-Scientific, USA. Phosphate buffered saline (PBS) solution was prepared by dissolving one PBS tablet in 200 mL deionized (DI) water yielding 0.01 M phosphate buffer. All solutions were prepared using DI water from a Milli-Q Ultrapure water system with a resistivity of 18.2 MΩcm.

Surface modification for antibody immobilization

Electrodeposition of polypyrrole and functionalized-MWCNT on the SPCE strip surface was achieved by a one-step electropolymerization of 0.075 M pyrrole in the presence of 0.1 mg/mL MWCNT-COOH via chronoamperometry technique. The mixtures were electrodeposited for 900 s in PBS at a fixed potential of 1.0 V. The strip was then rinsed with DI water and air-dried under nitrogen (N_2) flow. The carboxylic acid group of MWCNT-COOH was activated in a 10 µL

mixture of 0.4 M EDC and 0.1M NHS (1:1) for 15 mins. The excess was washed out using PBS, rinsed with distilled water, and N_2 dried. Next, 5 µL of anti-*Xoo* antibody was dropped on the electrode surface for 1 hour and washed with PBS. The remaining active sites on the SPCE surface were deactivated for 30 mins using 0.1% ethanolamine. *Xoo* cells or samples were placed on the SPCE's working electrode (WE) and incubated for 1 hour. The unbound antigen was washed away with PBS, and 5 µL of purified anti-*Xoo* antibody conjugated with HRP was dropped onto the working electrode and incubated for 30 mins, washed again with PBS. All modification steps were performed at room temperature.

Electrochemical set-up and measurements

Sensor characterization and analysis for the SPCEs were performed on an Autolab PGSTAT 20 potentiostat (Eco Chemie, Netherlands). Unmodified and modified SPCEs were characterized and compared using the cyclic voltammetry (CV) technique in a redox solution of 1 mM FCA in PBS 0.01 M, pH 7.4 (scan rates of 500 mV/s, potential window -0.1 to +0.6 V). Electrochemical measurements for the modified SPCEs with bounded antibody and *Xoo* cells were carried out by placing 50 µL of TMB solution onto SPCEs covering all three-electrode and measured using chronoamperometry at the optimized potential for 300 s.

The developed SPCEs are attached to a handheld Android-based biosensor device for on-site analysis. This portable device with internet-of-things (IoT) integration has been fabricated and developed with BioGenes Technologies Sdn. Bhd., Malaysia. Besides 4G connection with the built-in mobile application, it also has Global Positioning System (GPS) function to pinpoint the location of sampling. This portable electrochemical device consists of two connected hardware units enclosed in a plastic casing. The first unit is a touch-screen Android-based device with developed electrochemical software. The second unit is an electrochemical transducer that converts the signal from chemical reactions into readable and analysable data. The data collected are saved in a central cloud server and can be accessed through the website **https://provenpac.solutions/MARDI/padi** by authorized users. The detection of BLB disease in the rice fields was conducted with a simple extraction method.

Surface morphological study

The microscopic analysis was performed using field emission scanning electron microscopy (FESEM, JOEL JSM-7600F). Samples for FESEM were mounted on the stub, followed by coating with gold. The samples were visualized at 20 kV accelerating voltage with 20,000-60,000x magnification.

Polymerase chain reaction (PCR)

PCR reaction was carried out in a Bio-Rad thermocycler following the conditions: denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, and final elongation at 72°C for 1 min. The primer used in this study is described in Table 1 below.

Table 1. Primer for BLB disease detection using PCR method

Primer Name	Sequences (5' - 3')
JLXocF	CAAGACAGACATTGCTGGCA
JLXocR	GGTCTGGAATTTGTACTCCG

Results and Discussion

Sensor surface modification and characterization

A sandwich enzyme-linked immunosorbent assay (ELISA) format was adapted and employed in the sensor development. Polyclonal antibody against *Xoo* is first immobilized on the modified sensor strip surface, capturing the antigen (i.e.,*Xoo* bacteria) (Fig. 1). Then, a secondary antibody with horseradish peroxidase (HRP) enzyme label, which is associated with the other site of the antigen, is added. Electrochemical detection

was carried out using *3, 3', 5, 5'*-tetramethylbenzidine dihydrochloride (TMB)/H_2O_2 as the enzyme mediator/substrate system and conducted using chronoamperometry at an optimized set potential of -200mV. The enzyme's reaction and its substrate will produce a signal current whereby the intensity of the signal generated is directly proportional to the amount of analyte present in the sample.

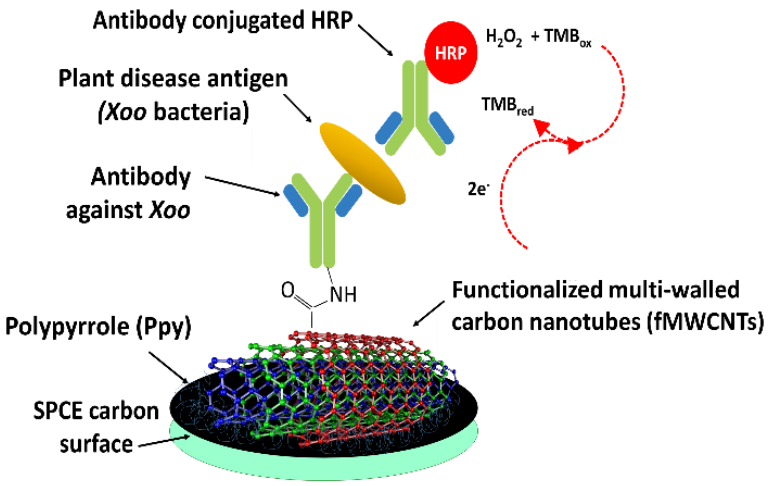

Figure 1. Schematic diagram of electrochemical immunosensor platform employing a sandwich immunoassay format for *Xoo* detection for bacterial leaf blight (BLB) disease

As can be seen from Figure 1, polypyrrole (PPy) and functionalized-multiwall carbon nanotube (MWCNT-COOH) were introduced to the sensor surface. By deliberately attaching or treating certain chemicals or nanomaterials on an electrode surface, the surface would take on the chemical properties of the attached compounds. As carbon material on the SPCE surface is inert, such modification will increase the sensor performance by enhancing the electrochemical properties on the electrode's surface [15]. The challenge of these modified electrodes, though is that they have to be durable and able to retain the activity of the biological receptors so that they do not denature easily and impair the chemical reaction activity. PPy was used in sensor platform modification due to its intrinsic chemical and electrical properties [16]. This type of conducting electroactive polymeris formed under mildly oxidative conditions from aqueous media. The mild conditions used for polymerizations are thus ideal for the immobilization of most of the biological elements in biosensor construction. PPy was incorporated with MWCNT-COOH to improve the sensitivity and selectivity of the biosensor besides providing a high surface area for antibody attachment owing to the carboxylic groups. Several unique properties of MWCNT, such as excellent mechanical strength, superior electrical conductivity, and electrochemically stable in aqueous and nonaqueous solutions, have received much attention in selecting the nanomaterial in sensor surface modification [17]. The EDC-NHS (1:1) was used to cross-link the COOH groups in the fMWCNT with the amine group of antibodies. Amine and carboxyl coupling are commonly achieved via carbodiimide chemistry that utilizes EDC with NHS esters; such has been reported to produce robust amide bond formation [18].

The performance of the modified electrode was characterized by cyclic voltammetry (CV). Figure 2 shows cyclic voltammograms of a bare (unmodified) and modified SPCE in the presence of 1mM ferrocenecarboxylic acid (FCA) in 0.01M PBS pH 7.4. After surface modification with electrodeposited PPy/MWCNT-COOH, peak current was found to increase due to the presence of PPy significantly. The modified electrode's oxidation and reduction peak currents increased two-fold more than the unmodified

electrode (Table 2). This indicates that active surface area has been successfully generated by incorporating the PPy/MWCNT-COOH. Such provides large binding sites and allows more antibody immobilization on the modified electrode surface. PPy has excellent conductivity, increasing the oxidation and reduction current [16] and improving electrochemical performance. On the other hand, MWCNTs incorporated with PPy have effective surface areas that act as supporting matrix for high density antibody attachment and provide a higher selectivity and sensitivity to the analyte of interest [19].

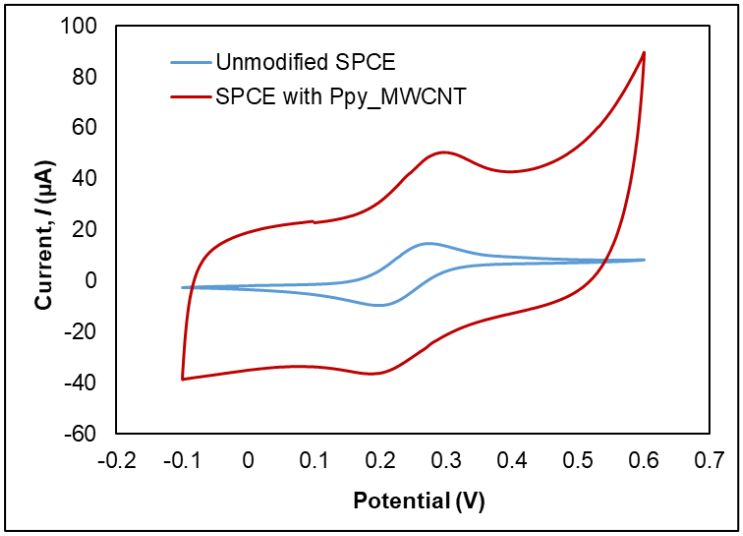

Figure 2. Cyclic voltammograms for an unmodified SPCE (blue line) and modified SPCE with electrodeposited PPy/MWCNT-COOH (red line)

Table 2. Electrochemical properties of unmodified and modified SPCE

	Peak Separation, ΔE(mV)	**Maximum i_{pa} (µA)**	**Maximum i_{pc} (µA)**	**Current Ratio (i_{pa}/i_{pc})**
Unmodified SPCE	63	19.0	-13.3	|0.98|
Modified SPCE	107	50.6	-38.5	|1.03|

Visual characterization of carbon surface prior to and after PPy/MWCNT electrodeposition was analyzed by FESEM. A scanning electron microscopic (SEM) image of the unmodified SPCE surface at 50,000x magnification showed a rough surface structure with the presence of a non-homogenous shape of carbon aggregate (Figure 3a). Following surface modification, typical cauliflower-like structures associated with successful PPy formation are observed (Figure 3b) [20]. Meanwhile, *Xoo* bacteria has rod-shaped cells with flagellum, where each cell has a width in the range of 300-400 nm.

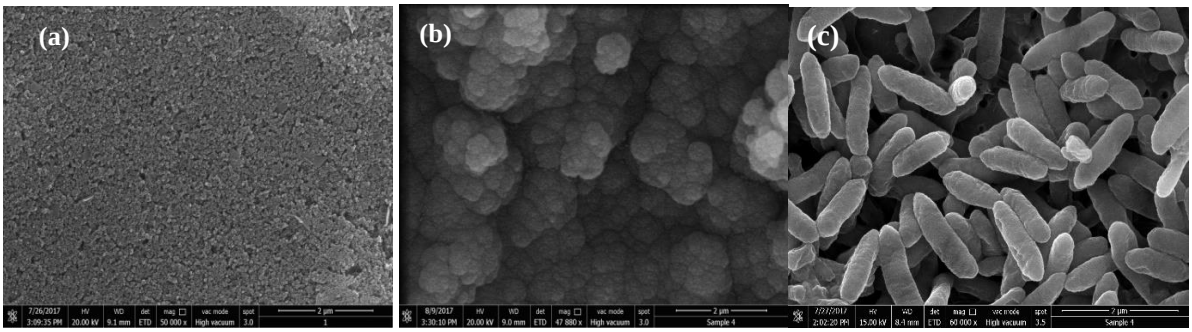

Figure 3. FESEM image of (a) unmodified working electrode (WE) on SPCE, (b) electrodeposited PPy on WE (Magnification of 50 000x); and (c) *Xoo* bacteria cells (Magnification 60,000x)

Development of electrochemical immunosensor

In order to obtain the best detection performance for biosensor development, the set potential was first optimized. Electrooxidative intermediate, produced by the enzyme-substrate interaction, is either oxidized or reduced by the potential applied to the biosensor. Set potential optimization was performed by scanning the potential from -0.6 V to +0.6 V for electrode coated with different concentrations of *Xoo* (i.e. 10^0, 10^2, 10^4, 10^6, 10^8 and 10^9 CFU/mL). The signal current to background current (S/B) ratio was measured. From Figure 4, we can see that the electrochemical current signal was optimum at -0.2 V with a significant S/B current ratio. Hence, -0.2 V was selected as set potential for further study.

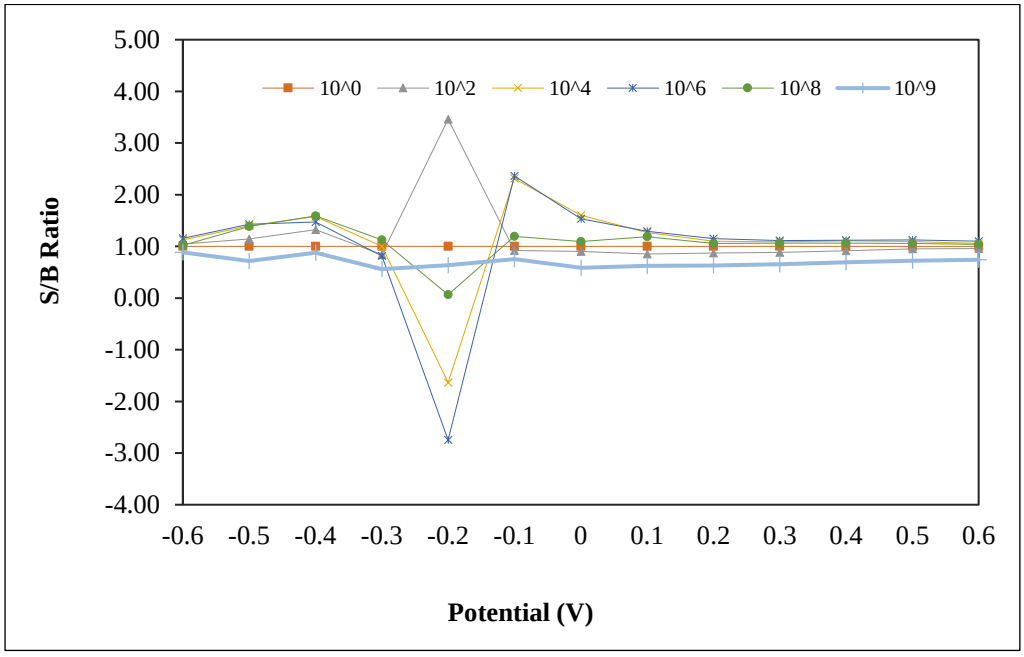

Figure 4. Set potential optimization by scanning the potential window from -0.6 V to +0.6 V

A standard graph was developed at optimized parameters using a mixed culture of *Xoo* (P0.0, P1.0, and P7.3) in carbonate-bicarbonate buffer, pH9.6. The current signal for each concentration of *Xoo* (0 - 10^9 CFU/mL) was recorded via chronoamperometry (CA) analysis at a set potential of -200mV for 300s.

Carbonate-bicarbonate buffer pH9.6 was used as blank control, accounted for 0 CFU/mL standard. An optimal *Xoo* antibody concentration of 0.3 mg/mL is immobilized on the SPCE as previously optimized [21]. The calibration plot of current generated against five concentrations of *Xoo* shows a linear current signal with a correlation coefficient of 0.926 for a linear regression graph (Fig. 5a). The immunosensor developed shows sensitivity with a limit of detection (LOD) at 10^2 CFU/mL. Several parameters have been optimized in order to reduce the non-specific binding in the sensor development, as reported before [22]. These include the dilution for *Xoo* cell cultures in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and the utilization of 0.1% ethanolamine as a blocking buffer as opposed to 0.5% BSA [23].

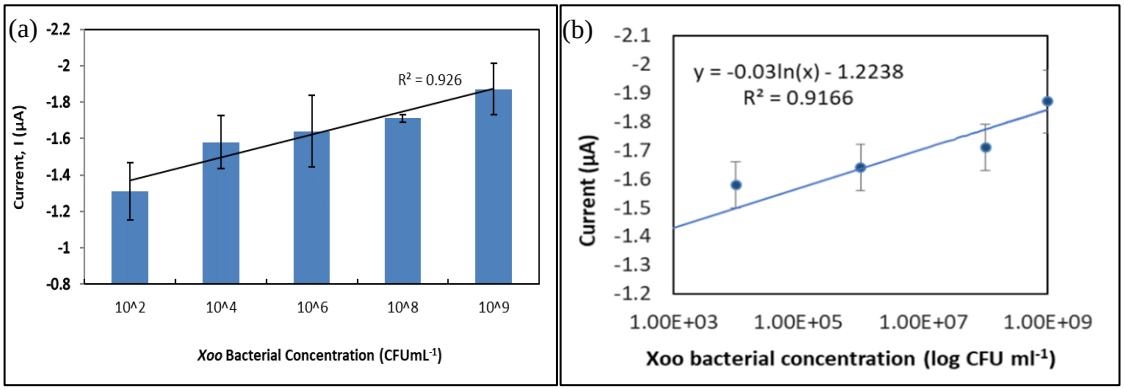

Figure 5. Calibration plot (a) linear regression graph and (b) logarithmic graph of *Xanthomonas oryzae* pv. *oryzae*(*Xoo*) using *Xoo* mixed culture of P0.0, P1.0, and P7.3

The matrix effect study was then performed to investigate the leaves effect in the electrochemical background current. *Xoo* with a concentration between 10^0 to 10^9 CFU/mL were studied in three different matrices, i.e pure culture, ground rice leaves, and cut (with scissors) rice leaves. Standard graph developed in the rice leaves cut using scissors showed excellent current increment response in accordance with the increasing concentration of *Xoo* up to 10^8 CFU/mL. Meanwhile, the standard plot in the grinded rice leaves matrix recorded the lowest current values compared to the other two matrices (Figure 6). Standard curve developed in cut rice leaves also showed good recovery besides exhibiting lower background current at 10^0 CFU/mL (i.e. blank control). Hence, cutting rice leaves with the scissors technique is selected for sample preparation and standard calibration plot development for the next biosensor studies with the real sample application.

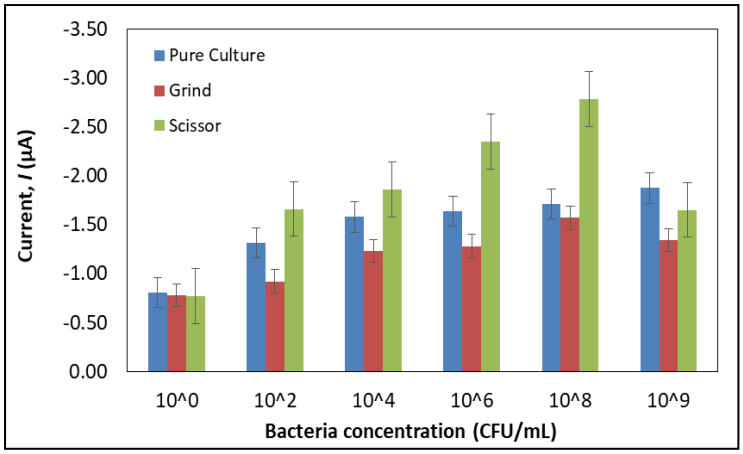

Figure 6. Matrix effect study and comparison of standard plot in different matrix

Performance of BLB disease immunosensor strips in a controlled environment

The performance of the developed electrochemical immunosensor was investigated by testing the strips on artificially infected rice leaves (MR 263 variety) with *Xoo* bacteria. Infection samples were prepared by inoculating *Xoo* in healthy rice leaves in a controlled environment. A total seven bacterial inoculation treatments (*n*=3) with two different inoculation techniques i.e.(i) brushing technique and (ii) scissors-cutting technique at a concentration of *Xoo* 0 CFU/mL (control study with sterile distilled water), 10^2, $10^{4,}$ and 10^8 CFU/mL were carried out. As presented in Table 3, only results from a second replicate (R2) are presented here. The results were determined from the standard calibration curve against the log concentration of *Xoo* cells (CFU/mL). T1R2 and T4R2 are the control study (blank) for brushing and scissors-cutting techniques. While T2R2 and T3R2 were inoculated with 10^4 and 10^8 CFU/mL of *Xoo* respectively by brushing techniques, and T5R2, T6R2, and T7R2 with 10^2, $10^{4,}$ and 10^8 CFU/mL respectively by scissors-cutting techniques.

The developed immunosensor was validated by PCR as a standard method. Table 3 shows the comparison between detection by electrochemical immunosensor strips and PCR for BLB disease detection in 41 inoculated samples. The immunosensor was able to detect the BLB disease as early as the third day of infection with disease agent for T5R2 (inoculation of 10^2 CFU/mL by scissors-cutting techniques) samples. For T3R2 and T7R2 samples, the immunosensor was able to detect the pathogens after 14 days. The results correlate with the PCR method for all the inoculated samples with a 92.7% correlation. This proves that the electrochemical immunosensor system developed has the potential as a bioanalysis tool for the early detection of bacterial leaf blight (BLB) disease and can be used in rice field areas.

Table 3. Detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in artificially inoculated rice leaves with two different inoculation techniques; (a) brushing (T1R2 – T3R2) and (b) scissors-cutting (T4R2 – T7R2)

Sample	*DAI (Day)	Sensor	PCR
T1R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**

	7	**-ve**	**-ve**
	14	**-ve**	**-ve**
T2R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**
	7	**-ve**	**-ve**
	14	**-ve**	**-ve**
T3R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**
	7	**-ve**	**-ve**
	14	**+ve**	**+ve**
T4R2	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**
	7	**-ve**	**-ve**
	14	**-ve**	**-ve**
T5R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**+ve**	**-ve**
	5	**-ve**	**-ve**
	7	**+ve**	**-ve**
	14	**-ve**	**+ve**
T6R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**
	7	**-ve**	**-ve**
	14	**-ve**	**-ve**
T7R2	0	**-ve**	**-ve**
	1	**-ve**	**-ve**
	3	**-ve**	**-ve**
	5	**-ve**	**-ve**
	7	**-ve**	**-ve**
	14	**+ve**	**+ve**

**DAI=days after inoculation*

Application of immunosensor strips on an IoT-biosensor portable device

A portable biosensor device with internet-of-things integration (IoT-biosensor) was employed for on-site detection of BLB disease using simple sample preparation (Figure 7). The modified strips with antibodies against *Xoo* are attached to the portable reader for sample analysis. Two hotspots for BLB disease are selected for this study, namely rice fields in Sg. Burong, Tanjung Karang and Bagan Terap, Selangor Northwest. The objectives of this study are to investigate the viability of the strips with the handheld device and to simulate a real sample application scenario in the field for future reference. The developed IoT-biosensor device prototype could provide fast data acquisition besides holds potential as a smart biosensor device that can reduce the dependency on bulky instrumentation for detection purposes.

Figure 7. Simple sample preparation on rice field for BLB detection using the fabricated IoT-biosensor handheld device

Table 4 shows the result of on-site analysis on 11 plots of BLB hotspots in Sg. Burong, Tanjung Karang. Samples were collected and tested on the field days after transplant (DAT) of DAT-15 and DAT-25. The results were obtained within 200 s by using the IoT-biosensor device. Results are displayed on the screen and can be stored on a cloud server. The analyzed data and further configurations can be accessed through a dedicated web page https://provenpac.solutions/MARDI/padi. All results were validated by the ELISA method, which used antibody-based detection similar to the immunosensor system developed. The immunosensor strips integrated with the IoT-biosensor device can detect BLB disease at early stage (DAT-15) before the symptoms appear. However, the ELISA method could not detect *Xoo* cells in all DAT-15 samples. All rice plants in the plots were confirmed to have BLB disease at DAT-25, as indicated by both ELISA and IoT-biosensor.

Table 4. Comparison of BLB detection analysis in Sg. Burong, Tanjung Karang hotspot

*DAT	Detection Method	A4	A5	A6	A7	A8	A9	A10	A11	A12	A14	A15
DAT-15	ELISA	-ve										
	IoT-BIOSENSOR	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
DAT-25	ELISA	+ve										
	IoT-BIOSENSOR	+ve										

**DAT=days after transplant*

A second on-field analysis was also conducted in two rice plots in Bagan Terap, Selangor Northwest, late before harvest (i.e. HLT 90-100). As validated by ELISA, Table 5 showed a 100% correlation between IoT-Biosensor with ELISA technique for Plot 1 and 80 % correlation for Plot 2. IoT-Biosensor reader was able to detect the *Xoo* presence even at a very low concentration (sample 2E) when the ELISA technique was unable to detect *Xoo*. The results prove that the IoT-biosensor developed has successfully detected the presence of BLB disease agent below ELISA's detection limit. As reported by Awaludin et al. [14], ELISA methods have limitations where their detection limits are merely in the range of 10^4-10^5 CFU/mL where as our immunosensor strip can detect as low as possible 10^2 CFU/mL.

Table 5. Comparison of BLB detection analysis in Bagan Terap, Selangor Northwest hotspot

BLB Hotspot	IoT-Biosensor	ELISA
1A	+ve	+ve
1B	+ve	+ve
1C	+ve	+ve
1D	+ve	+ve
1E	+ve	+ve
2A	+ve	+ve
2B	+ve	+ve
2C	+ve	+ve
2D	+ve	+ve
2E	+ve	-ve

Compared to other findings on BLB detection using an immunological approach, a Western Blot method using polyclonal antibody against *Xoo* has reported detecting *Xoo* cells at 3.5 x 10^3 CFU/mL [24]. Immuno-based assay using monoclonal antibodies developed by Wu et al. [25] have LOD of 1×10^4 CFU/mL for ELISA and 1×10^5 CFU/mL for immunochromatographic. The immunosensor strips described and developed here are superior to those afore mentioned techniques as they have better and lower LOD of 10^2 CFU/mL. Although a fluorescence-based immunoassay for BLB detection employing the same antibody source as ours has a lower LOD of 22 CFU/mL [14], this method still requires a fluorescence spectroscopy to measure the wavelength thus, portability is a major hindrance.

Conclusion

Here, we have demonstrated the development of an electrochemical immunosensor strip for the early detection of *Xoo* bacteria, the causal agent for bacterial leaf blight disease (BLB) in rice. Early detection mechanism in an efficient plant disease management can reduce the damage and losses due to pathogenic infections. A sandwich assay enzyme-linked immunosorbent assay (ELISA) format was adapted and employed in the sensor development. Surface modification with electrodeposited polypyrrole and functionalized multi-walled carbon nanotube (PPy-fMWCNT) network has greatly improved the sensor strip's performance, as indicated by electrochemical characterizations. A standard calibration plot in pure mix culture of *Xoo* (P0.0, P1.0, and P7.3) has been developed with R^2=0.9166. Scissors cutting for rice leaves was chosen for sample preparation and used in the development of a standard calibration plot for a real sample application. The efficiency of the developed electrochemical strips were tested on inoculated rice leaves samples, and show good sensitivity and high correlation with the PCR method. The electrochemical immunosensor strips were then integrated with an Android-based IoT-biosensor device suited for on-site detection purposes. The system was successfully applied

and tested on rice fields in BLB hotspot areas in Selangor. By using the immunostrips, early detection of BLB has been identified on DAT-15 as indicated by later infection stage using both sensor and ELISA techniques. This Android-based portable handheld reader with the developed immunostrips for BLB disease will eventually be an innovative on-site rice disease detection tool prior to the appearance of any visual symptoms so that corrective measures can be taken to prevent the spread of disease.

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