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Naked eye evaluation and quantitative detection of *Xanthomonas albilineans* in sugarcane xylem sap

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ABSTRACT

Sugarcane leaf scald caused by a bacterium, *Xanthomonas albilineans*, is a major disease of sugarcane worldwide. While erratic symptoms make phenotypic detection challenging, molecular methods require expensive instrument and manpower, and longer sample-to-answer times. Herein, we report a novel method for detection of *X. albilineans* DNA in sugarcane xylem sap. The method involved i) boiling lysis-based DNA extraction from sugarcane sap ii) magnetic purification of target sequences directly from the lysate using magnetic bead-bound capture probes iii) DNA sandwich hybridization platform for HRP/TMB/H₂O₂ reaction-based naked-eye and electrochemical detection of target. The method is sensitive (limit of detection 100fM), reproducible (relative standard deviation <7%) with linear dynamic range 100 fM-1nM (R² 0.99). The method was tested on a range of sugarcane cultivars of known resistance ratings, susceptible, intermediate resistant, and resistant, for leaf scald disease from an inoculated field trial. Detection levels were in agreement with the resistance rating of cultivars tested. qPCR results also strongly correlated with our assay (r=0.91 to 0.99, p<0.01) and cultivar resistance rating. We believe our assay could be useful for rapid screening as well as sensitive quantification of target pathogen DNA in infected sugarcane plants.

KEYWORDS: Leaf scald disease, *Xanthomonas albilineans* detection, naked eye evaluation of leaf scald disease, electrochemical detection of leaf scald disease, sugarcane xylem sap

ABBREVIATIONS:

GCEs: glassy carbon electrodes, CTV: Citrus tristeza virus, NTC: No target control SPGEs: Screen printed gold electrodes, HRP: Horseradish peroxidase, TMB: 3,3',5,5'-tetramethylbenzidine, SAM: Self assembled monolayer, RSD: Relative standard deviation, WPC: Wrong probe control, WTC: Wrong target control, NPC: No probe control, NHC: no-HRP control

1. INTRODUCTION

Plant pathogens are among the major threats to sugarcane productivity and biodiversity. Lost production and measures to control pests and diseases cost hundreds of millions of dollars every year (McLeod *et al.* 1999). Leaf scald is a major sugarcane disease caused by a xylem-inhabiting bacterium *Xanthomonas albilineans* (Ashby) Dowson (Garces *et al.* 2013). It has been reported in more than 60 countries, however, Australia, USA, Philippines, Vietnam and Thailand are among the most affected countries (Rott and Davies 2000). Leaf scald was first recorded in Australia in 1911 and its occurrence has since been reported in all sugarcane growing regions (Davis *et al.* 1997; Rott and Davies 1995). Leaf scald can cause severe yield loss or total destruction of crops if susceptible varieties are planted (Hoy 1994). Comstock *et al.* 2007 estimated a 19.5% to 32% yield loss in Florida, and Rott 1995 reported 12% to 21% yield loss in Guadeloupe, France. Besides reductions in yield and harvestable stalk number, leaf scald can also reduce the fiber quality and sucrose contents and affects the juice quality (Rott *et al.* 1997). Elimination of high yielding varieties in cultivar selection programs is an indirect but significant loss incurred by leaf scald. It has been reported that in Australia around 20% of potentially high yielding varieties are rejected due to leaf scald susceptibility (Birch *et al.* 2011). National plant biosecurity status report has identified leaf scald as a high priority pest threat (The National Plant Biosecurity Status Report, 2018 and 2019).

Narrow chlorotic (white) stripes or patches of chlorotic tissue on sugarcane leaves is one of the characteristic leaf scald symptoms. *X. albilineans* produces a highly potent pathotoxin, called albicidin, which is mainly responsible for these foliar symptoms (Birch and Patil 1985). Albicidin is a DNA gyrase inhibitor and blocks replication of chloroplast DNA, which ultimately leads to the inhibition of chloroplast differentiation and appearance of chlorotic symptoms on infected leaves (Cociancich *et al.* 2015). Albicidin also plays a key role in systemic invasion and possibly unpredictable transition from latency to active disease.

Although albicidin deficient mutant *X. albilineans* strains are able to efficiently colonize sugarcane, transgenic sugarcane varieties expressing albicidin detoxification gene *albD* show resistance to leaf scald (Birch *et al.* 2000). Thus, albicidin is considered necessary for development of leaf scald.

Plants infected with *X. albilineans* may show two forms of symptoms; chronic and acute, and two distinct phases; latent and eclipse (Rott and Davies 2000; Birch *et al.* 1985). Each of these pathogenic manifestations pose particular challenges in terms of disease diagnosis and control. The acute form which often appears subsequent to a rainy period followed by a prolonged dry weather (Rott and Davies 1995) leads to abrupt wilting of plants resulting in death, leaving little room for preventive interventions (Rott and Davies 2000). During the latency phase plants may remain asymptomatic for months or years before environmental conditions are favorable for the disease to trigger an outbreak (Ricaud and Ryan 1989). On the other hand, during the eclipse phase a plant can appear diseased or healthy depending on when it is inspected, as characteristic white lines on leaves continue to appear and disappear throughout the eclipse phase (Rott and Davies 1995). Many sugarcane cultivars can tolerate the pathogen without exhibiting symptoms or the symptoms may escape detection as the disease expression may be too insignificant to be recognized (Rott and Davies 1995; Rott *et al.* 1997; Ricaud and Ryan 1989). Disease diagnosis as well as assessment of sugarcane test cultivars for leaf scald resistance is generally based on observation of the phenotypic symptoms. Thus, latency and erratic nature of symptom expression not only make disease diagnosis challenging, but susceptible cultivars may also be assigned false ‘resistant’ ratings. Difficulty in accurately identifying infected plants has contributed to the worldwide spread of leaf scald via apparently “healthy” planting materials (Rott 1995). As alternatives to symptoms-based diagnosis, various other approaches like isolation on selective media (Davis *et al.* 1994), ELISA (Comstock and Irely 1992), end-point polymerase chain reaction (end-point PCR) (Pan

et al. 1999), and qPCR (Garces *et al.* 2013), have been employed for leaf scald diagnosis. However, these methods are not without shortcomings. Isolation on selective media though very efficient, especially for detecting *X. albilineans* in symptomless plants, is cumbersome and time-consuming (Wang *et al.* 1999). Immunological and molecular methods are robust and sensitive but require a centralized laboratory set up. High initial investment is required to set up laboratory infrastructure while reliance on skilled manpower significantly increases the costs of operation. In most of the cases, the testing site is located far from the disease affected areas, therefore additional measures and costs for processing, storage, and transport of samples from the field to labs are also involved. And lastly, there is a significant time lapse between sample collection and communication of the results back to farmers.

In recent years, electrochemical biosensors have emerged as potential contenders for the development of rapid, cost-efficient, and amplification free DNA/RNA detection platforms (Shiddiky *et al.* 2007, 2010; Boriachek *et al.* 2018). Although tremendous progress has been made towards the development of electrochemical methods for the detection of DNA sequences associated with human diseases and pathogens (Drummond *et al.* 2003; Ferapontova 2018), their application for plant pathogens detection has been relatively unexplored (Ferapontova 2018; Khater *et al.* 2017; Fang and Ramasamy 2015; Nezhad 2014). So far only a handful of electrochemical DNA sensors for plant pathogens detection have been reported. However, most of these genosensors have certain limitations. For example, Wongkaew *et al.* reported a voltametric method for the detection of sugarcane white leaf (SCWL) disease. The method involved direct hybridization of target organism genomic DNA with probes immobilized on chitosan modified glassy carbon electrodes (GCEs) (Wongkaew and Poosittisak 2000). However, high level of non-specific DNA binding to the electrode surface was observed in such platforms (Boriachek *et al.* 2018). Similarly, a highly sensitive method for the detection of soilborne fungus *Trichoderma* involved complicated sensor fabrications

(Siddiquee *et al.* 2014) while on the other hand a simple platform developed for the detection of *citrus tristeza virus* (CTV) could only achieve a limited sensitivity (0.1 μ M) (Khater *et al.* 2019). Signal amplification strategies based on redox reporters or chemical ligations sometimes lead to a loss of dynamic range (Qavi *et al.* 2010), which may be a particularly crucial consideration in pathogen detection where a high degree of inter-sample variability in levels of target analyte/s is observed. Some of the other electrochemical plant pathogen detection methods involve amplification of target DNA prior to electrochemical detection (Lau *et al.* 2017). Lastly, almost all of the electrochemical methods reported so far rely on commercial kits-based or other complicated chemical DNA extraction procedures. These challenges are the major impediments to the development of platforms that can carry out on-farm pathogen testing and are simple enough to be conducted by semi-trained personnel like farmers and extension workers.

In this study we sought to address several of these challenges. Specific aims of the study were to develop a leaf scald diagnostic method that: (i) is compatible with simple one-step DNA isolation method, (ii) can provide naked eye/visual evaluation capability, and (iii) can provide amplification free quantitative detection of *X. albilineans* DNA down to sensitivity levels suitable for routine diagnostics. Here we present a sandwich DNA hybridization assay for colorimetric (naked eye) evaluation as well as electrochemical quantification of *X. albilineans* specific target DNA sequences in sugarcane sap.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

All the reagents and chemicals used in this study were of analytical grade. Nuclease Free Distilled Water [Invitrogen™ Cat #. 10977015] was used for preparing all aqueous solutions.

Screen printed gold electrodes (SPGEs), DropSens DRP-220AT were purchased from Metrohm DropSens (Spain). Mercaptohexanol (MCH), Tris (2- carboxyethyl) phosphine hydrochloride (TCEP), Tris-HCl, EDTA, 3,3',5,5'-Tetramethylbenzidine (TMB), and Phosphate buffered saline (PBS) tablets (0.01M diphosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4 at 25°C) were purchased from Sigma-Aldrich (Sydney, NSW, Australia). Horseradish peroxidase (HRP)-conjugated Streptavidin was purchased from Life Technologies Australia (Cat # SA10001). All the synthetic probes and primers were purchased from Integrated DNA Technologies, Singapore. All electrochemical measurements were performed using a CH1040C potentiostat (CH Instruments, USA).

2.2 Sample Collection and DNA Isolation. Sugarcane stalks were collected from SRA Woodford research station's (26.929° E, 152.777° S, Woodford, QLD, Australia) disease screening trial, and immediately transported to the laboratory for extraction of xylem sap. Approximately 12 months old plants were inoculated with *X. albilineans* after six months of planting in the field. Stalk samples were stored in clean plastic bags at 4 °C until further analysis. Before extraction of DNA, stalks were cleaned thoroughly with Milli-Q® ultrapure deionized water and 80% ethanol. Vascular sap was extracted from internode sections of stalks aseptically. Using sterile scalpel blades, outer bark of stalks was shaved. A new blade was used to cut the peeled internal tissue into small pieces (roughly 3-5 cm long, 1 cm thick) and the cut pieces of internal tissue were transferred to clean nuclease free 1.5 ml microcentrifuge tubes. Using disposable polypropylene pestles, sugarcane sap was extracted by applying pressure on the cut internal tissue pieces within the microcentrifuge tubes. To avoid cross contamination between samples, only one sample was processed at a time and fresh and sterile scalpel blades and pestles were used for each sample. Bacterial DNA was isolated from sugarcane xylem sap following the method reported earlier (Garces *et al.* 2013). Briefly, 200µL of collected sap

samples were transferred to a clean microcentrifuge tube and were centrifuged at 9,000x g for 5 min to precipitate bacterial cells. Pellet was resuspended in 100 μ L of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl and 0.2% Tween-20; pH ~8.3) by vortexing vigorously followed by boiling at 95°C for 10 min. DNA from cultured *X. albilineans* was isolated using DNeasy Plant Mini Kit (Qiagen Cat # 69104). Isolated DNA samples were stored at -20°C until further use.

2.3 Capture and purification of target. Probes were designed targeting a 39bp region (synthetic XALB, Table S1) within the albicidin pathotoxin biosynthesis gene cluster XALB1 (GenBank accession # AJ586576) (Royer *et al.* 2014). The target region was selected considering the pivotal role albicidin plays in leaf scald pathogenesis. The target region (synthetic target) corresponded to positions 1740266 to 1740304 in *X. albilineans* GPE PC73 complete genome (GenBank accession # FP565176.1) (Pieretti *et al.* 2009). Sequences of the probes and synthetic target sequence are shown in Table S1. Known concentrations of synthetic target sequence were prepared by diluting stock solution (100 μ M) in nuclease free H₂O. DNA purified from cultured bacteria was diluted to a concentration of 1 ng μ L⁻¹, while DNA from xylem sap samples was used without any further dilution. Each concentration was run in triplicate throughout (from target capture to detection).

The target sequences were separated and magnetically purified following our previously described protocol with slight modifications (Koo *et al.* 2016; Islam *et al.* 2018). Unless otherwise stated, all incubations in this section were carried out for 30 min at room temperature and with shaking at 300 rpm. Briefly, 10 μ L sample (or nuclease free H₂O₂ for no target control, NTC) was mixed with 15 μ L of 10 μ M biotinylated capture probe 1 (CP1) and 10 μ L of 5X saline-sodium citrate (SSC) buffer (pH 4.0), heated at 95°C for 5 min followed by cooling on ice and incubation. Required volume (10 μ L/sample) of Dynabeads™ MyOne™ Streptavidin

C1 beads (Invitrogen™, Cat# 65001) were washed three times with 1X binding and washing (B&W) buffer (10 mM Tris-HCL, pH 7.5; 1 mM EDTA; 2 M NaCl) and resuspended in 35 μ L 2X B&W buffer. Washed beads were mixed with CP1-target mixture and incubated. The target-attached beads were magnetically separated, washed (two times with 1X B&W, once with 1X SSC buffer) and resuspended in 10 μ L 5XSSC buffer. The captured target samples were stored at -20°C until further processing.

2.4 Sensor Fabrication. Unless otherwise stated, electrodes were washed between each step using 10 mM PBS and dried by gentle air flow. The electrodes were protected from direct exposure to light throughout the experiments. The screen printed gold electrodes (SPGEs) were pre-treated electrochemically as described earlier (Zhang *et al.* 2007). The effective area of working electrode was estimated by measuring the peak current obtained as a function of scan rate under cyclic voltammetric conditions for the one-electron reduction of $[\text{Fe}(\text{CN})_6]^{3-}$ [2.0 mM in PBS (0.5 M KCl)] (Shiddiky *et al.* 2009) and was determined by using the Randles - Sevcik equation:

$$i_p = (2.69 \times 10^5) n^{3/2} A D^{1/2} C v^{1/2} \dots \dots \dots \dots \quad (1)$$

where, i_p is the peak current (Amp), n is the number of electrons transferred ($\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$, $n = 1$), A is the effective area of working electrode (cm^2), D is the diffusion coefficient of $[\text{Fe}(\text{CN})_6]^{3-}$ (taken to be $7.60 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$), v is the scan rate (V s^{-1}), C is the concentration (mol cm^{-3}).

Thiolated capture probes (CP2) were reduced by incubating 1 μ L of 1 mM TCEP with 99 μ L of 0.2 μ M CP2. Equal volumes I-buffer [10mM Tris-HCl + 1mM EDTA + 0.1M NaCl + 10mM

TCEP (pH 7.4)] and reduced CP2 were mixed and 5 μL of mixture was disposed on to the SPGE working electrode and incubated for 1 h. The working electrode was passivated with 5 μL MCH (1 mM) for 20 min.

Density of CP2 on fabricated sensor was measured as described earlier (Shiddiky *et al.* 2010; Wong and Gooding 2003). Briefly, chronocoulometric (CC) measurements were taken in 50 μL of E-Buffer (10 mM Tris-HCl; pH 7.0) and 50 μM ruthenium hexamine $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (RuHex) using following parameters; initial potential 0.2V, final potential -0.5V, number of steps 1, pulse width 0.25 sec, and sample interval 0.002 sec. Charge (Q) versus $t^{1/2}$ for both CC runs was plotted and capacitive charge (Q_{dl}) and total charge (Q_{total}) were obtained from the intercept at $t = 0$, from the E-Buffer and RuHex plots respectively (Figure S1). The charge corresponding to RuHex electrostatically bound to surface-confined ssDNA (Q_{ss}) was calculated as described earlier (Masud *et al.* 2017).

$$Q_{\text{ss}} = Q_{\text{Total}} - Q_{\text{dl}} \quad \dots \quad \dots \quad \dots \quad \dots \quad (2)$$

The probe density (Γ_{ss}) was calculated by using the following equation:

$$\Gamma_{\text{ss}} = \left(\frac{Q_{\text{ss}} N_A}{nFA} \right) \left(\frac{z}{m} \right) \quad \dots \quad \dots \quad \dots \quad \dots \quad (3)$$

where n is the number of electrons ($n = 1$), A is the area of the working electrode, m is the number of nucleotides in the DNA, z is the charge of the redox molecules, F is Faraday constant, and N_A is Avogadro's number.

2.5 Colorimetric and Electrochemical Detection. Target solutions (10 μL) were added onto the sensor surface for 1 h followed by the incubation with 10 μL biotinylated DP (of 10 μM) for 30 min and 4 μL of streptavidin conjugated HRP (0.1 ng μL^{-1} in PBST) for another 20 min. The electrode was then incubated with 50 μL TMB single solution (Invitrogen™ Cat #. 00-

2023) for 10 min in dark. Naked-eye evaluation was done by observing blue color development. Reaction was stopped by adding 4 μL stop solution (0.2 M H_2SO_4) to generate yellow colored complex. The amount of the enzymatically generated yellow colored complex as an indicative of surface-bound target was measured by chronoamperometry (CA) at a fixed potential of +100 mV. All the measurements were performed at room temperature.

2.6 Detection of leaf scald bacteria in samples collected from field trial. In order to further demonstrate the suitability of our method for field application, we analyzed a series of sugarcane xylem sap samples. Samples were collected from nine cultivars of sugarcane from SRA leaf scald screening trial. The trial was inoculated by manual decapitation of cane tops above the growing point, between the 3rd and 4th dewlap, followed by application of infected juice within a few minutes, by brushing the cut tissue with a paintbrush dipped in the inoculum. Sugarcane stalks were collected from cultivars with three resistance categories to *X. albilineans* infection. The name of the cultivars used in this study along with their resistance categories are provided in figure 6. For each cultivar, stalks from two different plants were collected initially (18 stalks in total). However, only 13 of the stalks were included in final analysis. Rest of the 5 stalks were excluded mainly because of difficulties in obtaining sufficient volume of sap for further analyses. Stalks of sugarcane cultivars cut into approximately six 12 cm pieces away from the nodes in order to extract xylem saps. Xylem sap was collected from three different segments of a single stalk as described in section 2.3, and sap from each segment was processed independently. In total, sap samples extracted from 13 different plants corresponding to 9 different cultivars mentioned in Figure 6 were included in final analysis.

2.7 Validation with qPCR. A qPCR assay was designed with primers targeting 123 bp region from positions 1740257 to 1740379 in *X. albilineans* GPE PC73 complete genome (GenBank

accession # FP565176.1). The qPCR primers encompassed the whole synthetic target region (Table S1). qPCR validation was carried out at CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD™) in a 20µL reaction using SensiFAST Sybr No-ROX Kit [Cat No. BIO-98005] and forward and reverse primers at a final concentration of 400 nM each (TableS1). qPCR reaction conditions were as follows: initial denaturation at 95°C for 2 min, 40 cycles of; 95°C for 5 seconds, 50°C for 30 seconds and 72°C for 20 seconds followed by melt curve analysis (65°C to 95°C, with an increment of 0.5°C, for 5 seconds). Serial dilutions of known *X. albilineans* genomic DNA quantity (10ng, 1ng, 100pg, 10pg, 1pg) were used to generate standard curve for the absolute quantification of *X. albilineans* in sugarcane sap samples. For the validation and repeatability measurement, *Proc Corr* in SAS version 9.4 (SAS Institute, Cary, NC) was used to calculate the correlations between electrochemical detection and qPCR measurements.

3. RESULTS AND DISCUSSION

3.1 Assay Design. The assay is schematically outlined in Figure 1. Most of the DNA biosensing platforms reported so far rely on costly, time consuming, and tedious DNA extraction procedures. We adopted a simple boiling lysis-based method for bacterial DNA isolation directly from sugarcane xylem sap. Target DNA sequences (39bp region within the XALB1 gene cluster of *X. albilineans* genome) were specifically captured directly from this crude lysate using complementary biotinylated probes (CP1) attached on the surface of streptavidin coated magnetic beads. Subsequently, probe bound target sequences were magnetically purified followed by heat release and magnetic purification. Self-assembled monolayer (SAM) of thiolated-CP2 on gold electrodes was prepared. CP2 are complementary to the target region adjacent to CP1 complementary region. Surface density of the thiolated capture probes was in the range of 1.2×10^{12} to $< \sim 5.0 \times 10^{12}$ molecules cm^{-2} to ensure efficient capture and sensitive

detection of target (section 3.2). The resultant target-CP2 hybrid on gold electrode surface was reacted with biotinylated DP (CP1) to generate a sandwich CP2-DP:target assembly followed by binding of streptavidin-HRP to DNA assembly. HRP catalyzed-TMB oxidation reaction yields a blue colored product which on the addition of concentrated acid is converted to a yellow colored diimine compound. The intensity of the blue or yellow colored products provides an indirect measure of target concentration and was used for naked eye visualization (blue color) or electrochemical quantification via fixed potential chronoamperometry of electroactive yellow colored diimine compound (Figure 1).

3.2 Determination of Probe Density. Performance of DNA-probe based sensors (e.g., selectivity, sensitivity, and stability) is highly dependent on the characteristics of the immobilized DNA probes, such as conformation, orientation, and surface density. The assembly of DNA probes at sensor surface is affected by various parameters such as incubation time as well as concentration and ionic strength of immobilization buffer (I-buffer) as described earlier (Zhang *et al.* 2007, Shiddiky *et al.* 2010). MCH used in these experiments not only serves as spacer by finely modulating surface density of the DNA probes but also acts as a blocker that effectively blocks non-specific gold-DNA interactions on the electrode surface and helps DNA probes ‘stand up’ on the electrode surface, favoring the efficient hybridization of incoming target sequences. DNA surface density was quantitatively measured by characterizing the redox process of RuHex using CC readout. The electroactive label, $[\text{Ru}(\text{NH}_3)_6]^{3+}$ (RuHex), is stoichiometrically bound to the anionic phosphate backbone of DNA strands via electrostatic interaction. RuHex complexes serve as signaling molecules whose cumulative redox charge is a direct function of the amount of DNA strands proximal to the electrode surface (Supplementary Figure 1). The probe densities obtained were in the range of 1.4×10^{12} to 5.3×10^{12} molecules cm^{-2} which is within the range previously recommended 1.2

$\times 10^{12}$ to $< \sim 5 \times 10^{12}$ molecules cm^{-2} (Zhang *et al.* 2007). For supplementary Figure 1, the surface density of thiolated capture probes was calculated as 5.3×10^{12} .

3.3 Assay performance. In order to demonstrate the viability of assay we compared the current response obtained in the presence of two designated synthetic target concentrations (1 nM and 100 fM) with that of no-target control (NTC). NTC corresponds to a control reaction where nuclease free water instead of synthetic target was mixed with CP2 functionalized beads keeping all other assay components and subsequent steps consistent. As shown in Figure 2A, more than 10 times higher amperometric signal was obtained for 1 nM target DNA concentration compared to the NTC (mean current density $2.5 \mu\text{Acm}^{-2}$ vs. $0.23 \mu\text{Acm}^{-2}$, $n = 3$). Similarly, low target concentration (100 fM) also showed significantly higher current response ($0.71 \mu\text{Acm}^{-2}$) compared to NTC (>3 times higher). Our assay design is based on “sequential correlation” whereby target concentration in sample is directly correlated to the number of DNA molecules hybridized to the SAM of CP1 on SPGEs, which in turn is proportional to the concentration of biotinylated DPs confined to the target-CP1 hybrid. This way, streptavidin-HRP concentration immobilized onto this sandwich DNA assembly and ultimately biocatalytic oxidation of TMB is directly proportional to the target concentration in sample. Thus, current response obtained from the electrocatalysis of the yellow colored diimine compound provides direct measure of target concentration. Each concentration as well as NTC were analyzed in triplicate and intra-assay variability was evaluated by determining the percentage relative standard deviation (RSD) between the current responses obtained for each replicate. As indicated by error bars in Figure 2A, our assay showed high repeatability as RSD values of <5% were obtained. Representative chronoamperograms obtained from the analysis of known target concentrations and NTC are shown in Figure 2B.

High specificity of the signal is a prerequisite for electrochemical DNA assays. Several components of the assay may be able to contribute to background signal such as: interaction of probes with non-complementary sequences (a major concern when starting sample is not a highly purified DNA and is rather a crude lysate which is essentially a heterogenous pool of various biomolecules like proteins and RNA as well as sugarcane genomic DNA or DNA from any other microorganisms), non-specific adsorption of target and/or non-target DNA sequences on to the bead surface, and nonspecific interaction of HRP with the gold electrodes. We systematically determined the possible effect of each assay component on signal. As shown in Figure 3 and S2, current response obtained for all the controls was comparable or less than NTC thus indicating minimal interference from any of the assay components and highly specific target detection. In wrong probe control (WPC), non-complementary biotinylated and thiolated probes were used (sequences not shown), while in wrong target control (WTC), a synthetic DNA fragment corresponding to hsa-miR-891 was used keeping all other components of the assay constant. Amperometric response obtained for both the controls (mean current densities $0.13 \mu\text{Acm}^{-2}$ for WPC and $0.20 \mu\text{Acm}^{-2}$ for WTC, $n = 3$) was similar or less than NTC indicating probes used in our assay are capable of capturing *X. albilineans* sequences with high specificity. No probe control (NPC) comprised of “mock purified” high target concentration (1nM) where magnetic beads not functionalized with biotinylated CP1 were mixed with target DNA. All the subsequent assay steps remained unchanged. In no-HRP control (NHC), all the assay steps were carried out similar to synthetic target reaction using high concentration of target (1nM), except at the stage of streptavidin-HRP immobilization where 0.1% PBST was introduced into the system rather than streptavidin-HRP. Mean current densities obtained for NPC and NHC were 0.18 and $0.042 \mu\text{Acm}^{-2}$, respectively. All the control experiments were carried out in triplicate and showed high reproducibility (RSD <5%). Taken together these results suggest high specificity of probes (WPC and WTC), low non-specific

adsorption of DNA on beads or gold electrodes (NTC and NPC), and very little non-specific interaction of HRP with the sensor surface (compare NHC with other controls). Corresponding chronoamperograms for control experiments is given in Figure S2.

Broad and linear dynamic range is an essential performance matrix for pathogen detection assays as high variability in target concentration is observed. Therefore, to assess the sensitivity and linearity of the assay, we analyzed five different serially diluted concentrations of synthetic target DNA sequence (100 fM to 1 nM). As discussed previously, electrochemical signal obtained in our assay is directly proportional to the analyte concentration. As shown in Figure 4A an increment in amperometric current response proportional to the increasing concentration of target DNA was observed. Our assay was able to reproducibly detect synthetic target DNA over a broad dynamic range between 100fM and 1nM. The linear regression equation was estimated to be $y = 0.46 \text{ Log}(\text{target concentration}) + 6.64$, with a correlation coefficient of $R^2 = 0.99$ indicating the good linearity of the assay (inset figure 4A). Limit of detection (LOD) of our assay, defined as the concentration of the analyte that can be reliably distinguished from NTC, was found to be 100fM ($S/N = >3$). All the reactions were carried out in triplicate and RSD was calculated. RSD values for all the samples were found to be $< 7\%$ showing minimum intra-assay variability and excellent reproducibility. Our assay showed 100 times better sensitivity compared to one of the earlier reported sugarcane pathogen detection platform where the authors reported a detection limit in the range of 10pM (Wongkaew and Pooittisak 2000). On the other hand a recently reported gold nanoparticles based plant virus detection platform could only achieve a lower detection limit of 100nM. Moreover, a logarithmic relationship was only observed within a very narrow range of concentrations (0.1 – 10 μ M) (Khater et al. 2019). However, the platform reported by Siddiquee et al. achieved attomolar level sensitivity and detection over a very broad dynamic range ($1.0 \times 10^{-18} - 1.82 \times 10^{-4} \text{ mol L}^{-1}$). Although this platform was several orders of magnitude more sensitive to our

assay, it involved complicated sensor fabrication and relied on tedious phenol-chloroform DNA extraction process (Siddiquee *et al.* 2014). In comparison, our assay provided a rapid naked-eye readout streamlined with a simple field deployable DNA isolation method and without extensive sensor fabrication steps.

3.4 Detection of leaf scald in samples collected from field trial. In order to further demonstrate the suitability of our method to real world applications, we analyzed a series of sugarcane sap samples. Samples were collected from SRA Woodford leaf scald screening trial in an operator blind fashion. Samples were collected from varieties with various degrees of *X. albilineans* susceptibility (highly resistant to highly susceptible). Names of the varieties tested in this study along with their resistance ratings are included in Figure 6. The susceptibility/resistance status of the collected samples were only known to SRA staff. Griffith team involved in conducting the electrochemical and molecular analysis were not aware of the sample susceptibility/resistance status during the method development stage. The target DNA was detected both colorimetrically (naked eye) and electrochemically. As shown in figure 5, development of blue color was observed only in samples with known concentration of synthetic target DNA or sugarcane sap samples while NTC did not show any color development. The intensity of the color also provided a rapid qualitative result of bacterial concentration in the sample. Bright blue color in sample 133 (1) (cultivar Q133) indicated high bacterial concentration while sample 208 (2) (cultivar Q208) showed very low intensity suggesting low bacterial concentration in the sample.

Target DNA was quantified electrochemically by carrying out chronoamperometric (CA) measurements as described earlier. As shown in figure 6A, the sensor successfully detected varying concentrations of target sequence in the samples collected from field trial.

The target DNA detected in most of the samples was within the LOD of our assay indicating its applicability to real world samples. In most of the cases detected *X. albilineans* DNA were in agreement with the resistance rating of cultivars. For example, highly resistant variety such as Q208^A (sample 208) consistently provided low response. However, some discrepancies between the expected bacterial concentrations and electrochemical response were also observed. For example, Q44 which was collected from susceptible cultivar provided reduced current response. These discrepancies can be attributed to the age and the location of the stalks or where the samples were taken from. (Garces *et al.* 2013) reported variation of bacterial concentrations among types of tissues and plant age. Nonetheless, all infected sugarcane samples collected were positive to the bacteria in our study. Figure 6B shows the amperograms for selected field trial samples.

3.5 Validation of assay with qPCR. A qPCR assay was designed to validate the outcomes of our biosensor platform. qPCR primers target a 114bp segment within the XALB1 gene cluster (corresponding to positions 1740266 to 1740379 in *X. albilineans* GPE PC73 complete genome GenBank accession # FP565176.1) that encompassed the region targeted in electrochemical assay. Standard plot for qPCR based absolute quantification was obtained by plotting C_q values against log of corresponding known *X. albilineans* starting quantity. Absolute quantification of starting DNA copy number in field trial samples was calculated by comparing with standard plot. The qPCR results depicted an excellent concordance with the electrochemical quantification demonstrating that the newly developed biosensing assay is suitable for detecting *X. albilineans* in sugarcane sap samples (Figure 7). The qPCR values were strongly correlated ($r=0.91$, $p<0.001$) with biosensor assay (Table 1). The strong correlation ($r=0.99$, $p<0.0001$) of C_q value of biosensor and qPCR samples implicated repeatability of the test.

In this proof-of-concept study we have demonstrated sensitive (up to femtomolar level) detection of sugarcane pathogen *X. albilineans* in xylem sap samples. The assay offers some significant advantages. Firstly, we have demonstrated that our assay is compatible with boiling lysis-based DNA extraction method. When compared with kit-based or organic extraction-based DNA isolation methods, boiling lysis method is not only low-cost, it is potentially field deployable as it requires relatively simple instruments (centrifuge, heat block). Also, crude lysate is relatively more stable sample source and resistant to degradation and contamination compared to cut stalks. Thus, instead of shipping sugarcane stalks, the bacterial DNA can be extracted using a simple on-farm setup and sent to remote labs for testing. In case of colorimetric detection, the process takes less than an hour and gives a first pass yes/no answer. Electrochemical assay utilizes disposable screen-printed electrodes which cost as low as ~USD \$3 each making overall per sample cost of the assay less than \$10. And lastly, unlike most of the electrochemical assays reported previously, our method achieves sensitive target detection without complicated sensor fabrication steps.

This generic technology is flexible in the sense that it can potentially be applicable to almost all types of pathogens using specific probes. Our assay could be applicable for the diagnosis and management of diseases in other agricultural and horticultural crops, and potentially in other environments (e.g. in soil). Moreover, using multi-well screen printed electrodes, the platform can be further extended for high throughput and/or multiplex detection of various pathogens simultaneously. The assay could also form the basis of a fully integrated next-generation handheld device, associated with a purpose-built smartphone application. Such a device can be particularly useful in developing geographic information system (GIS) for surveillance as well as prevalence and risk mapping of various diseases. The proposed integrated device can also be used for developing rapid screening and warning systems for exotic diseases to be implemented at entry points like airports and seaports.

4. Conclusion

We have developed a sensitive method for the detection of leaf scald disease of sugarcane. This method offers a dual detection where naked eye color change evaluation provides qualitative detection of *X. albilineans* while quantification up to femtomolar levels of target DNA was achieved by electrochemistry. Our assay showed high specificity, good linear response ($R^2 = 0.99$) for detection of target concentrations 100fM – 1nM, and excellent repeatability (RSD <7%, n =3). The proposed assay can potentially be used for detection of a range of sugarcane and other agricultural pathogens and can also form the basis of next generation portable integrated devices for on-farm applications.

ASSOCIATED CONTENT

Supporting Information

Table S1 (oligonucleotide sequences), Figure S1 (chronocoulometric response for self-assembled monolayer of thiolated capture probes on screen printed gold electrodes), and Figure S2 (Representative chronoamperograms for assay specificity experiments) (PDF).

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Notes

The authors declare no competing financial interest.

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Figure Legends

Figure 1. Schematic representation of the assay for naked eye evaluation and electrochemical detection of *X. albilineans* DNA in sugarcane sap samples.

Figure 2. A) Current densities (μAcm^{-2}) obtained for the analysis of known synthetic target concentrations (1nM and 100 fM) compared with NTC (1nM). Error bars represent the relative standard deviation of three independent experiments (RSD <5%, $n = 3$) B) Corresponding representative amperograms for synthetic target detection.

Figure 3. Current densities (μAcm^{-2}) obtained for the control experiments compared with target (1nM). Error bars represent the relative standard deviation of three independent experiments (RSD <5%, $n = 3$) NTC = no target control; NPC = no probe control, WTC = wrong target control, WPC = wrong probe control, NHC = no-HRP control.

Figure 4. Sensitivity and linearity of the biosensor: (A) The biosensor can reliably detect target DNA concentrations as low as 100 fM. Amperograms show distinguishable difference in current responses obtained for 100fM and NTC. (B) The assay showed a linear response over a broad range of concentrations. Inset shows linear regression analysis of the data. Error bars represent the relative standard deviation of three independent experiments (RSD <7%, $n = 3$)

Figure 5. Concentration dependent increase in the intensity of TMB color (blue) development (synthetic target DNA 100 fM to 1nM). Representative photos for naked eye detection of *X. albilineans* DNA in sugarcane xylem sap: 133 (dark blue), 44 (moderate intensity), and 208 (light blue) indicate high, intermediate, and low bacterial concentrations in sap samples, respectively.

Figure 6. (A) Current density bar graphs for all the analyzed samples from SRA Woodford leaf scald screening trials (xylem sap extracted bacterial DNA) Error bars represent the relative standard deviation of three independent experiments. Data labels represent resistance rating: S = Susceptible, In = Intermediate, R = Resistant. Note: For discussion on discrepancies between the expected bacterial concentrations (based on resistance rating) and current response, see section 3.4. (B) Amperograms for a selected set of samples.

Figure 7. Validation of the biosensor data using qPCR. (A) starting quantity (copy number) for a selected set of samples as determined by qPCR based absolute target quantification (B) Current density (μAcm^{-2}) obtained for the corresponding sugarcane xylem sap samples. Error bars represent the RSD of three independent experiments.

Table 1. Pearson correlation coefficients to compare relationship current density (μAcm^{-2}) from the samples collected from field trial and qPCR validation samples.

| Parameters | log starting quantity (qPCR sample) ¹ | Current density (μAcm^{-2}) qPCR sample |
|---|--|--|
| Current density (μAcm^{-2}) field trial sample | 0.91** | 0.99*** |
| N | 7 | 7 |
| logstarting quantity (qPCR sample) | | 0.89** |
| N | | 7 |

¹ Significant (P-value) ** ≤ 0.001 , *** ≤ 0.0001

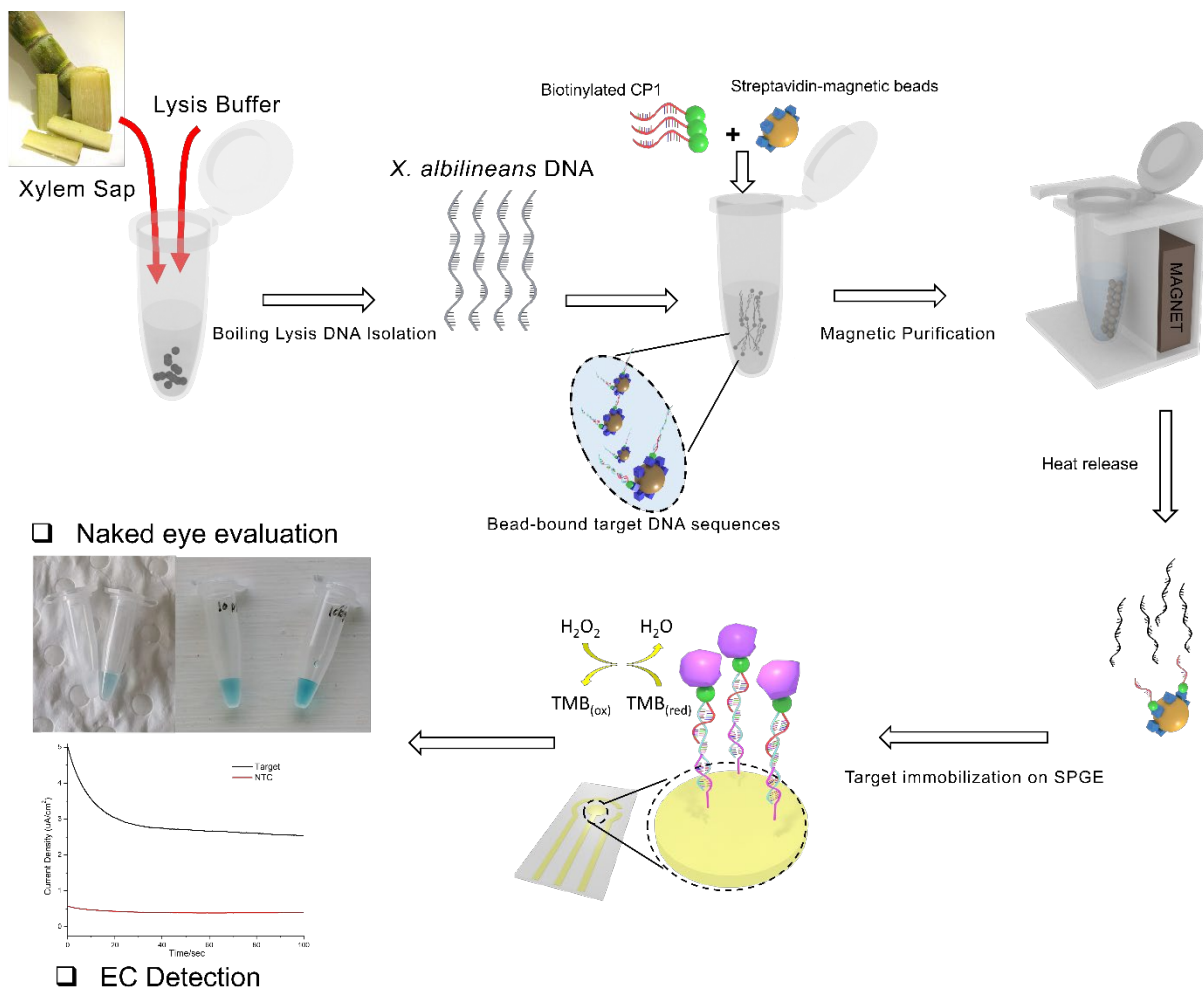


Figure 1

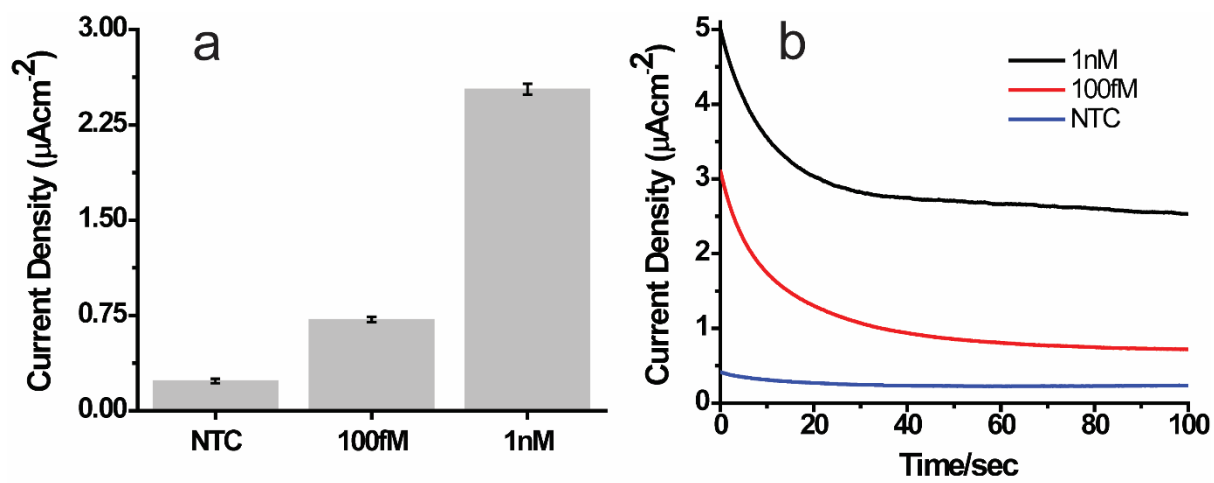


Figure 2

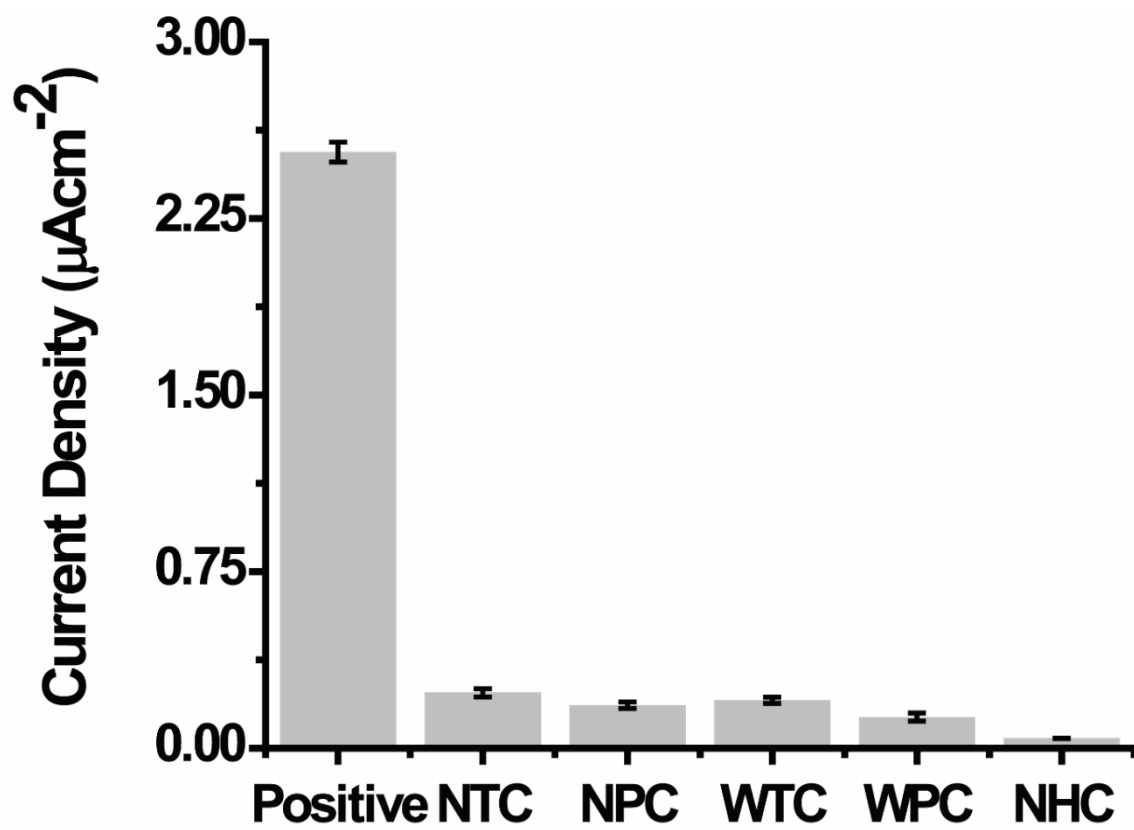


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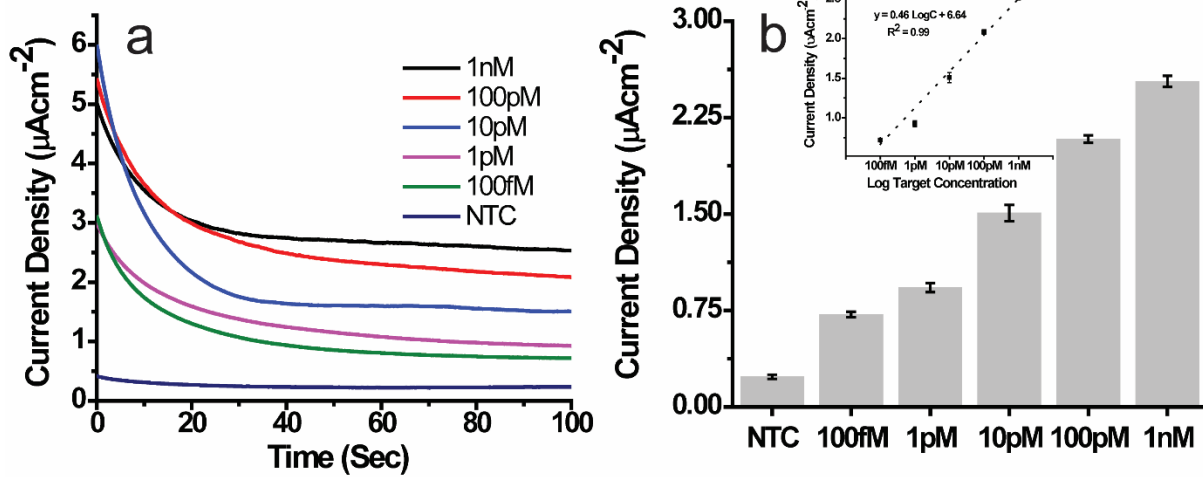


Figure 4

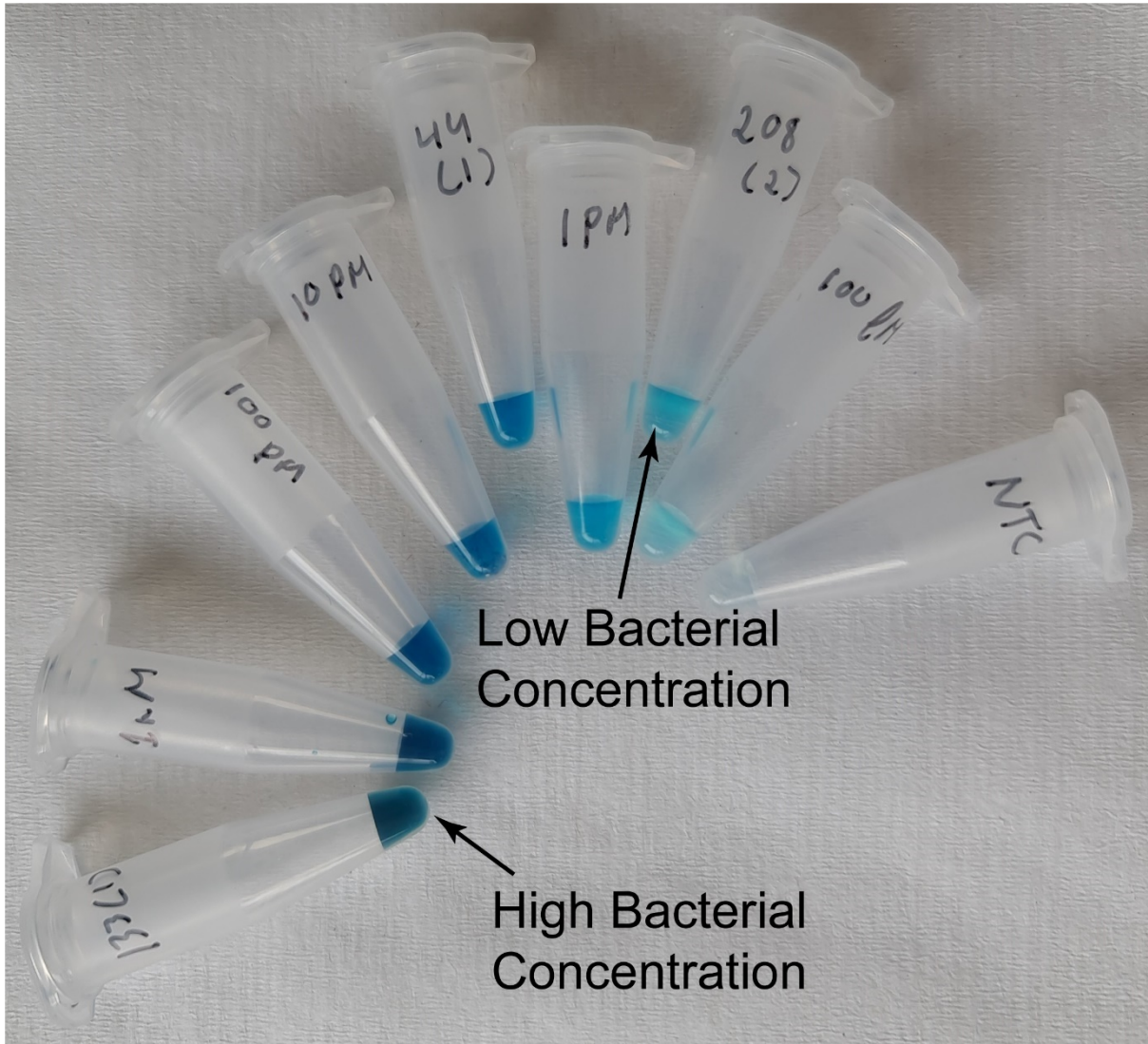


Figure 5

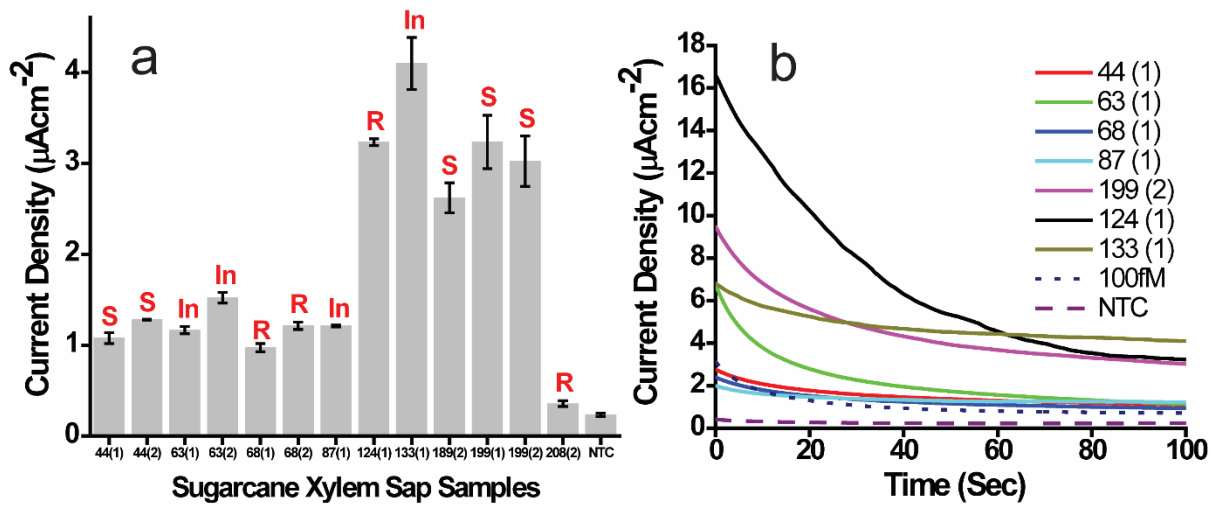


Figure 6

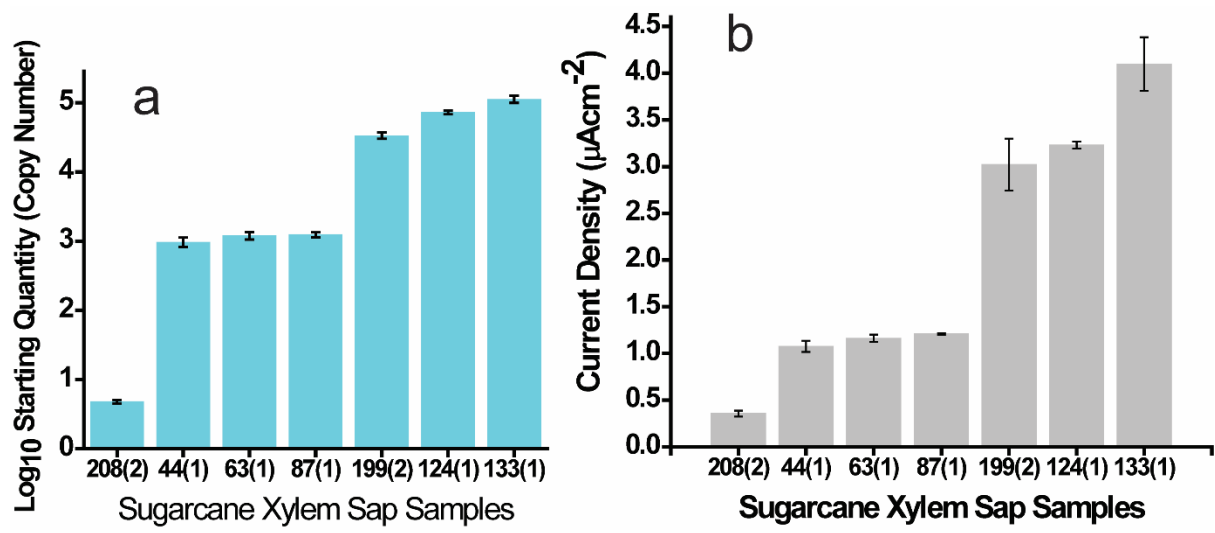


Figure 7