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Evaluation of a lateral flow device for in-field detection of Banana Xanthomonas Wilt and its application in tracking the systemicity of *Xanthomonas campestris* pv. *musacearum*

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Early detection of Banana Xanthomonas Wilt (BXW) in the field and immediate destruction of infected plants or plant tissue are key control methods to prevent the introduction and spread of BXW. This requires rapid, cost-effective and an on- site diagnostic tool to detect the bacterium, *Xanthomonas campestris* pv *musacearum* (*Xcm*). Polymerase chain reaction (PCR) detection technique for BXW is efficient but requires expensive equipment and knowledgeable expertise; this limits PCR application to the laboratory. This study therefore was carried out to evaluate the enzyme-linked immunosorbent assay (ELISA) tool configured as a lateral flow device (LFD) for detection of *Xcm*. Studies on the systemicity of *Xcm* in banana were carried out using the BXW-LFD in a field trial of 300 banana plants of Pisang Awak inoculated with the *Xcm* at Kiifu Forest, Mukono District, Uganda. Pseudo-stem samples from symptomatic and asymptomatic suckers were collected and tested with the LFD and the results compared with conventional PCR using the GspDm BXW primers. The LFD was able to detect *Xcm* 3 days post inoculation (dpi), 2 cm above and below inoculation site, 15 to 35 days in the pseudo-stem, 35 to 42 days to reach the corm and 81 days in the lateral roots. The rate of *Xcm* movement in banana was found to be sigmoid in nature, leveling off as the bacteria moved down the pseudo-stem towards the corm. Conventional PCR was only 24% more sensitive than the LFD. The use of the BXW LFD can therefore boost BXW control measures through improved surveillance and quarantine services to arrest the introduction and spread of the disease within and between national borders.

Key words: Banana Xanthomonas Wilt lateral flow device (BXW LFD), Banana Xanthomonas wilt, *Xanthomonas campestris* pv musacearum, complete systemicity, incomplete systemicity, lateral flow device.

INTRODUCTION

Banana *Xanthomonas* wilt (BXW) caused by *Xanthomonas campestris* pv. *musacearum* (*Xcm*) is a

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serious threat to the livelihood of millions in East and Central Africa, who depend on banana (*Musa* spp.) for food and income (Tushemereirwe et al., 2003). The disease causes up to 100% yield loss, attributed mainly to rapid death of the mother plant and premature fruit ripening and subsequent rotting of the bunch that renders the fruit completely inedible (Tripathi et al., 2009a). Additional symptoms of BXW include the progressive yellowing and wilting of leaves, shriveling and blackening of the male buds. None of the grown cultivars are resistant to BXW, although East African highland cultivars (AAA) are significantly less affected compared to Sukali Ndizi (AAB) and Pisang Awak (ABB) (Ssekiwoko et al., 2006a; Karamura et al., 2010). The causal bacterium (*Xcm*) was first detected on ensete (*Ensete ventricosum*), a close relative of banana that is native to the highlands of Ethiopia (Yirgou and Bradbury, 1968). Later it was reported in Central Uganda in 2001, and subsequently in other banana-growing areas of the Great Lakes region (Tushemereirwe et al., 2003; Ndungo et al., 2006; Reeder et al., 2007; Carter et al., 2010). Disease mainly spreads within fields primarily by insects such as bees, wasps, and cockroaches that frequently visit the male inflorescence while foraging for nectar. In the process, their limbs and appendages come in contact with bacterial ooze that is exuding from fresh scars left by the dehisced floral bracts (Tinzaara et al., 2006).

Field studies reveal that once inside the inflorescence, *Xcm* spreads systemically along the true stem downwards to the corm and subsequently into the entire root system (Ssekiwoko et al., 2006b; 2010). The infection process via the inflorescence to corm takes about 28 days, although the bacteria can survive in lower parts of the mat for over two years without causing visible symptoms (Ocimati et al., 2013a). Such prolonged durations of latency present a major concern, especially in the light of recent disease resurgence in growing areas wherein it had been contained (Tinzaara et al., 2013). Research shows that latent infections in host tissues can result in long distance spread through movement of infected planting materials (Mwangi, 2007; Lewis Ivey et al., 2010). Notably, inoculum concentrations below 10^4 colony-forming units per milliliter (cfu mL^{-1}) have great potential of causing latent infections (Ochola et al., 2014). Accurate detection of BXW is critical, since prompt deployment of cultural control options is strongly associated with faster subsidence of symptoms and reduced spread across fields in smallholder banana systems.

Majority of smallholder farmers in East and Central Africa rely on visual symptom expression for disease diagnosis. Unfortunately, studies reveal that by the time symptoms are expressed, *Xcm* is already fully established itself in the plant. Besides, symptoms are non-specific and quite easy to confuse with infections by Fusarium wilt (*Fusarium oxysporum* fsp. *cubense*), Moko disease (*Ralstonia solanacearum*) and Blood disease (*Pseudomonas solanacearum*). This consequently results in incorrect recommendations and deployment of inappropriate disease management options. Culture-based methods are not appropriate for a large number of samples in a short period of time, because it is time consuming. In addition, culture-based techniques are prone to give false negative results when used to assess latently infected plants due to the low bacterial load (Mwangi, 2007; Tripathi et al., 2007). Recent developed molecular approaches, that is, (Polymerase chain reaction) PCR need only slight amount of DNA for detection, however they are often costly, time consuming, and require tissue or DNA samples to be transported to the laboratory (Aritua et al., 2008; Lewis Ivey et al., 2010; Adikini et al., 2011; Adriko et al., 2011).

Multi-stakeholder efforts to address these limitations occasioned in the development of a novel, easy-to-use lateral-flow-device (LFD) for quick and accurate onsite detection of *Xcm* (Hodgetts et al., 2014). The immuno-chromatographic *Xcm*-LFD incorporates a polyclonal antibody (PAb) that detects a specific epitope on a glycoprotein antigen secreted during the active growth of *Xcm* inside the banana plant. In a positive reaction, *Xcm* binds with the PAb and conjugate-coloured particles to give a coloured complex seen as a line. The conjugate-coloured particles also migrate and bind with a second fixed control, hence the two observed coloured bands. Screenhouse experiments have proven that the *Xcm*-LFD takes guesswork out of disease identification and reduces the need for unnecessary laboratory testing of banana specimens (Hodgetts et al., 2014). As a basis of this study, the *Xcm*-LFD was evaluated to assess its application in field tracking *Xcm* spread among suckers emerging from infected mother plants and to generate knowledge on its capacity to detect latent infections in banana.

MATERIALS AND METHODS

Study site

The study was conducted at Kifu Forest Reserve (00°28'N and 32°44'E, 1250 m.a.s.l.) the only location in Uganda designated for controlled BXW epidemiological studies. Generally, the thick forest provides perfect seclusion from neighbouring farmers' fields, which minimizes any long-distance vector transmission (Ochola et al., 2014). The climate is warm-humid with an average temperature of 25°C and precipitation of 1560 mm per annum distributed in two seasons (March-June and August-November). The site is located in a crystalline basement characterized by metamorphosed granites and quaternary alluvial and lacustrine deposits. Kifu soils are mainly ferralsols with gleysols in the swamps (Okorio, 2000).

Experimental design and treatments

Tissue culture-derived plants of Pisang Awak (ABB-genome) were established at a spacing of 3 × 3 m in a newly opened field where banana has never been planted. This ensured that the subsequent infection symptoms are not the outcome of *F. oxysporum* fsp.

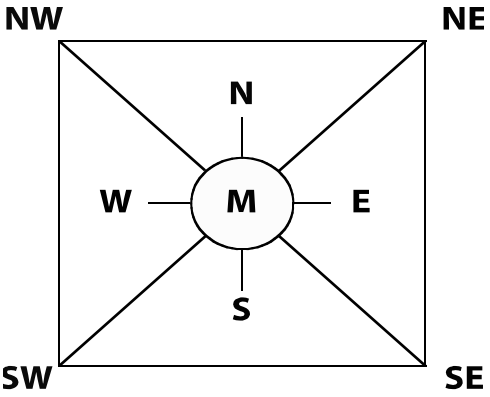

Figure 1. Orientation of treatments (NE, SE, SW and NW) with respect to the inoculated mother plant (M).

cubense. The field experiment consisted of four treatments and uninoculated control laid out in a randomized block design (RBD) with three replications. The four treatments were defined by the north-east (NE), south-east (SE), south-west (SW) and north-west (NW) orientation of the inoculated leaf petiole on the mother plant (Figure 1). The treatments and control (20 plants each) were randomly assigned per replication (that is, each replication = 100 plants). The chronological pattern of sucker emergence was closely monitored and each sucker geographically mapped in respect to its position on the mother plant.

Bacterial cultures and inoculum preparation

Bacteria were obtained from fresh bacterial ooze cultured and grown at 24°C for 72 h, on a semi- selective growth media, cellobiose cephalexin agar (CCA) (Mwebaze et al., 2006) containing yeast extract (1 gL^{-1}), glucose (1 gL^{-1}), peptone (1 gL^{-1}), NH_4Cl (1 gL^{-1}), $MgSO_4.7H_2O$ (1 gL^{-1}), K_2HPO_4 (3 gL^{-1}), agar (14 gL^{-1}), beef extract, (1 gL^{-1}), cellobiose, (10 gL^{-1}), cephalexin (40 mgL^{-1}), 5-fluorouracil (10 mgL^{-1}) and cycloheximide (120 mgL^{-1}). Pure *Xcm* colonies were harvested into sterile water and with optical density at 600 nm adjusted with sterile water to 0.5 *c.* 1×10^8 colony-Forming-units (cfu) mL^{-1} using a spectrophotometer (Biomate-3, Thermo Electron Corporation, USA).

Inoculation of banana plants

The point of inoculation on the petiole of the youngest leaf was cleaned with 95% ethanol and 1 mL of the bacterial cell suspension injected into the plant with an insulin syringe (Micro-Fine Plus, 0.33 x 12.7 mm, Beckton Dickinson, USA) (Ochola et al., 2014). Information on *Xcm* colonization of distal parts of banana including the suckers was collected for a period of two months.

Tracking the movement of *Xcm* in banana

Tracking the movement of *Xcm* began 2 days post inoculation (dpi). This was done by first measuring 2 cm from the point of inoculation downwards direction and cutting deep into the leaf stalk/pseudostem using a sterile knife. The plant tissue is then placed into the extraction buffer bottle that comes with the LFD kit and shaken for a minute. A drop of the tissue-extraction buffer mixture is placed onto the LFD and results read 5 min after. Blue twin bands indicated that the sample was positive for *Xcm* and one single band means the sample was negative for *Xcm*. Tracking *Xcm* was done every two days.

Detection of *Xcm* in suckers

Corm and pseudostem tissues were collected aseptically and tested from each sucker using a knife sterilized in sodium hypochlorite (NaOCl) to avoid cross contamination. Symptomatic and asymptomatic suckers whose samples tested positive were labeled, put into sampling bags, and taken to the laboratory for PCR confirmation using GspDmF/R primers (Adriko et al., 2011). These same suckers were also visually diagnosed (Visual Diagnosis, plants were determined to be unhealthy or healthy based on the symptoms seen) and any symptoms were noted down for purposes of comparison with LFD and PCR test results.

Relative sensitivity and specificity of LFD and PCR

Sensitivity, in this experiment was the probability that a test result will be positive when the disease is present (true positive) while specificity was the probability that a test result will be negative when the disease is not present (true negative). Sensitivity was calculated as true positive/ (true positive + false negative) while specificity was calculated as true negative/ (true negative + false positive).

Genomic DNA extraction

Plant genomic DNA was extracted using the CTAB method (Murray and Thompson, 1990). 300 mg of banana pseudo-stem tissue was frozen with liquid nitrogen and ground to a fine paste using mortar and pestle. The fine powder was then placed in 2 ml eppendorfs and 500 µl of CTAB buffer (2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide), 1 M Tris pH 8.0, 0.5 M EDTA pH 8.0 (Ethylenediaminetetra Acetic acid Di-sodium salt) and 5 M NaCl, at pH 5) added to the mixture. The CTAB/plant extract mixture was then incubated in a water bath at 65°C for 30 min. 1.5 µl RNase at 10 mg/ml was added to each tube and incubated at room temperature for 10 min. The eppendorfs were then placed in the centrifuge to spin at 12000 rpm for 5 min to bring down cell debris. The supernatant transferred to clean eppendorfs and to each tube 250 µl of Chloroform: Iso Amyl Alcohol (24:1) added and the solution mixed by inverting. After mixing, the tubes were spun at 13000 rpm for 1 min. The upper aqueous phase that contains the DNA was then transferred to a clean tube and to each tube 5 µl of 7.5 M Ammonium Acetate was added followed by 500 µl of ice-cold absolute ethanol. The tubes were inverted several times slowly to precipitate the DNA for 5 min and then centrifuged at 13000 rpm for 1 min to pellet the DNA. The extraction yielded was 50 ng/µl of DNA. The DNA was then resuspended in sterile DNase free water of 100 µl and stored at 4°C.

PCR and cycling conditions

To test for the presence of *Xcm* in the plant samples, conventional PCR was run against the plant DNA samples using primers GspDm-F/R (Adriko et al., 2011). PCR reactions were composed as follows: each 25 µl PCR reaction contained 1 µl of DNA template, 12.5 µl of 2 × PCR Master Mix (GoTaq), 1 µl of each forward and reverse primer from a stock of 10 µM and 9.5 µl of nuclease-free water. Water was used as a non-template control (in the place of DNA) for each run. The PCR reactions were performed in a

GeneAmp PCR system 9700, Applied Biosystems. PCR amplicons of 5 µl were then separated by agarose gel electrophoresis in 1% agarose gels in 1x TBE (Tris/borate/EDTA) at 130 V for 60 min. O'gene ruler 1kb plus DNA ladder was used to evaluate the sizes of the PCR amplicons under ethidium bromide. 250 bp PCR amplicons were expected for any positive result.

RESULTS

Symptom expression after artificial inoculation

Wounding is essential for entry of *Xcm* into banana whether by vector-mediation via the inflorescence or through contaminated tools. The inoculated sites at the leaf stalk showed signs of cell death, typical of hypersensitive reactions to the bacteria. About 28% inoculated mother plants displayed typical BXW symptoms on their leaves. The incubation period ranged between 14 and 21 days post inoculation (dpi). Although *Xcm* is also introduced in intensively managed systems by contaminated tools, data reveals that artificial wounding followed by pathogen introduction may not necessarily result in disease.

Tracking the movement of *Xcm* in banana

The LFD allowed for screening of a reasonable number of plant samples in the field without need for movement of infected banana tissues for laboratory testing. Results of testing were achieved in 5 minutes. LFD was deployed for the determination of the speed of movement of *Xcm* from the inoculated leaf petiole downwards to the corm. The device was sensitive to detect *Xcm* within 2 cm proximity of the site of infection after 3 days post inoculation (dpi). The bacteria was noticed to move at 0.4 cm daily for the initial 12.5 days post inoculation (dpi), thereafter the rate of movement tended to rapidly increase at 20.5 dpi from 3.2 to 7.8 cm at 32 dpi before starting to level off. Since the distance between inoculated petiole and corm among plants was fairly uniform (280-320 cm), *Xcm* was predicted to rich the corm from between 35 and 42 dpi. The pattern of *Xcm* movement downwards the infected mother plant was sigmoid in nature.

Xcm colonization of lateral shoots

BXW symptom expression on lateral shoots was observed from 81 days onwards. Moreover, in some instances all suckers on the mat remained asymptomatic even after the demise of the mother. In general, the pattern of spread from the infected mother to lateral suckers was however random irrespective of the orientation of wounding site for *Xcm* to quickly access nutrients.

Comparison of the visual, LFD and PCR diagnosis of *Xcm*

On-site testing of 16 randomly selected mats confirmed a random pattern of BXW transmission from infected mother to suckers. Testing of the asymptomatic and symptomatic suckers proved that LFD and PCR were more sensitive than using visual diagnosis (VD).

LFD and PCR testing relative to VD

Out of 83 pseudo-stem samples from the suckers, 30 were positive and 53 negative by LFD while 21 were positive and 62 negative by PCR. Of the 30 that were positive by LFD, only 28 were diagnosed as positive by VD. Of the 53 that were negative by LFD, 51 were diagnosed as negative VD. Of the 21 that were positive by PCR only 16 had diagnosed as positive by VD. Of the 62 that were diagnosed as negative by PCR, only 48 were negative by VD (Table 1).

Relative sensitivity and specificity of LFD and PCR

Of the 30 that were positive by LFD, 17 were positive by PCR while of the 53 negative by LFD, 49 were found to be negative by PCR. Of the 21 positive by PCR, 16 were positive by LFD and of the 62 negative by PCR, 48 were negative by LFD. PCR detection of *Xcm* by the primers GspDmF/R was 24% more sensitive than the LFD. However the LFD was 13% more specific than the PCR (Table 2).

DISCUSSION

Xcm infection and tracking the movement of Xcm

A scenario in which artificial wounding and introduction of *Xcm* into the leaf petiole does not result in symptom expression and disease, suggests that infection via wounding may actually be passive or accidental. In this study, localized cell death in response to wounding and pathogen invasion of the leaf petiole was typical of hypersensitive (Hpr) defense mechanism of the plant. Specifically, restricted movement or entrapment of bacteria within 2-cm diameter of the site of inoculation is consistent with secretion of reactive oxygen species (ROS) that play a major role in signal transduction pathway that elicits programmed cell death. Studies show that when induced responses occur very early, Hpr is of great benefit to the plant, and reduces the subsequent pathogen attack (Heath, 2000; Gechev et al., 2006). Moreover, the transient nature of Hpr-mediated cell death portrayed by a later rapidity in *Xcm* movement suggests that the bacterium was able to totally decompose the

Table 1. Sensitivity and specificity of LFD and PCR relative to VD.

LFD vs VD and PCR vs VD	LFD	VD	95%CI	PCR	VD	95% CI
Total = 83						
+	30	28		21	16	
-	53	51		62	48	
Sensitivity	TP/(TP+FN)*100 28/(28+2))*100 = 93.3%		77.89 - 98.99%	TP/(TP+FN)*100 16/(16+5)*100 = 76.19%		52.83 - 91.69%
Specificity	TN/(TN+FP)*100 51/(51+2)*100 = 96.23%		87 - 99.43%	TN/(TN+FP)*100 48/(48+14)*100 = 77.42%		65.02 - 87.06%

LFD, Lateral flow device; VD, visual diagnosis; PCR- polymerase chain reaction; CI, confidence interval; TP, true positive; FP, false positive; TN, true negative; FN, false negative. + means pseudo stem samples that tested positive while – means pseudo stem samples that tested negative. Note that sensitivity = true positive/ (true positive + false positive), specificity = true negative/ (true negative + false negative).

Table 2. Comparison of sensitivity and specificity relative to PCR and LFD.

LFD vs PCR and PCR vs LFD	LFD	PCR	95%CI	PCR	LFD	95% CI
Total = 83						
+	30	17		21	17	
-	53	49		62	49	
Sensitivity	TP/(TP+FN)*100 17/(17+13)*100 = 56.67%		37.44 - 74.52%	TP/(TP+FN)*100 17/(17+4)*100 = 80.95%		58.08 - 94.44%
Specificity	TN/(TN+FP)*100 49/(49+4)*100 = 92.45%		81.77% - 97.86%	TN/(TN+FP)*100 49/(49+13)*100 = 79.03%		66.81 - 88.33%

LFD, Lateral flow device; VD, visual diagnosis; PCR- polymerase chain reaction; CI, confidence interval; TP, true positive; FP, false positive; TN, true negative; FN, false negative. + means pseudo stem samples that tested positive while – means pseudo stem samples that tested negative. Note that sensitivity = true positive/ (true positive + false positive), specificity = true negative/ (true negative + false negative).

oxidative activity of associated ROS.

The infection pathway in inflorescence-infected plants involves the systemic movement of *Xcm* along the true stem, into the leaf sheaths then downwards to the corm and subsequently into entire root system (Ssekiwoko et al., 2006; Ocimati et al., 2013a). However, despite being equally systemic, leaf petiole-internal spread was actually bi-directional within proximity of the area of infection. Internally, banana petioles are pierced by large air canals that are separated by narrow longitudinal partitions and lateral plates of stellate parenchyma that contain very few vascular bundles (Ennos et al., 2000). This most likely allows for the pathogen to rapidly spread and multiply in adjacent parenchyma tissues and intercellular spaces. Our findings show that the *Xcm* bacterium takes about 39 days to travel from the point of inoculation to reach the corm, and an extra 42 days for expression of typical symptoms in lateral shoots. These results contravene the claim that symptom development in attached lateral shoots may take up to 24 months (Ocimati et al., 2013a). The predicted increased systemicity under tool-mediated infections is likely to have severe implications on successful disease control particularly in intensively managed smallholder systems with relatively high probability for deployment of contaminated cutting tools.

Random nature of infection of lateral roots

Astoundingly some lateral shoots emerging from infected mother plants remained disease free - a phenomenon now referred to as incomplete systemicity (Ocimati et al., 2015; Sivirihauma, 2013). Insights into the causes of incomplete systemicity apparently revealed that the bacterium was capable of surviving for long periods in lower parts of the mat without causing visible symptoms (Ocimati et al., 2013a). Latent infection of plants by pathogens has been long recognized (Gäumann, 1951). Most latent pathogens tend to persist and later produce symptoms of disease when prompted by environmental or nutritional conditions or changes in phenology of the host (Agrios, 2005). Although this study has not corroborated the occurrence of latent infections, it has reliably confirmed the random nature of *Xcm* spread to lateral shoots emerging from previously infected mother. A number of reasons could account for this observation:

(1) Presence of other microbes and endophytes in the corm hence competition for nutrients, and (2) existing differences in the type of plant cells found in corm making it difficult for *Xcm* to move. However, according to Ssekiwoko et al. (2006), *Xcm* does not actually spread easily once in the inner cylinder and cortex of the corm. Ocimati et al. (2013b), also went on to prove that there was actually low transmission efficiency of *Xcm* from the corm or corm roots.

LFD application in the use of single diseased stem removal (SDSR)

Consequently, proposed a less labor intensive novel control is in practice known as the single diseased stem removal (SDSR), involving the removal of only the visibly diseased plants on the mat in order to reduce the inoculum level and lower disease incidence to an acceptable level. In context of latency and random infection pattern of lateral shoots, reliance on disease symptom expression to manage the XW is not sufficient (Ocimati et al., 2013a). Field application of SDSR requires early detection of BXW symptoms and tracking the systemic movement of the bacteria towards the corm. This study has demonstrated the LFD to be an essential field diagnostic kit that can be used to guide the implementation of SDSR. Since it takes about 14 to 21 days for symptom expression, and 35 to 42 days for *Xcm* to get to the corm, it is recommended that farmers deploy the LFD the moment foliar symptoms are expressed to first ascertain how far the bacterium has moved down the pseudostem towards the corm. Thereafter, SDSR must be implemented provided the bacteria have not reached within 45 cm from the corm.

However, this study has also revealed that the efficacy of SDSR in preventing further colonization of the mat is likely to be undermined by the random pattern of *Xcm* expression in lateral shoots. Since latently infected plants appear asymptomatic before diagnosed by traditional symptoms, culture-based techniques and the lateral flow device (LFD).

LFD sensitivity and specificity

Xcm was detected 3 dpi and in respect to the point of inoculation (2 cm from the point of inoculation) by the LFD, it is safe to assume that the LFD is sensitive enough to detect even smaller concentrations of *Xcm* bacterial inoculum in any suspected plant tissue. This is especially in regards to the fact that transmission of the *Xcm* within fields is commonly by contaminated tools and insect vectors that are suspected of carrying bacteria inoculum of more than a concentration of 10^8 cfu/ml or as low as 10^4 cfu/ml respectively. This level of LFD sensitivity was proven useful where *Xcm* was detected in asymptomatic plants. This is important for early detection of BXW in the field and immediate destruction of the affected plant, as a key control method of BXW.

Conventional PCR detection by GspDmF/R primers (Adriko et al., 2011) was found to be more sensitive than the LFD detection. This should be expected as studies have shown PCR to be at least 10 times more sensitive than antibody based methods of detection (Omrani et al., 2009) and is usually the confirmatory test. However due to its high sensitivity PCR is also able to pick up more false positives and negatives than antibody based methods (Alvarez and Kaneshiro, 1999; Kaneshiro, 2003). In some instances antibody based methods or immunodiagnostic assays can be used a confirmatory tests next to PCR (Alvarez, 2004). The LFD was only 24% more sensitive than the PCR and is therefore still reliable and precise enough to be used in the field. PCR detection of *Xcm* can then be used as a confirmatory test of the LFD.

Management of BXW using the cultural methods such as destruction and infected plant materials and use of clean plant materials has only slowed down the spread of the disease (Karamura et al., 2010; Tinzaara et al., 2009). The LFD will be useful in the integrated approach that not only involves cultural control methods but also surveillance of disease outbreak and routine screening at borders. The LFD provides rapid detection of the *Xcm* pathogen without the need for the laboratory environment. Quick results of the detection are achieved in less than 10 min.

Conflict of Interests

The authors have not declared any conflict of interests.

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