Agro-Science Journal of Tropical Agriculture, Food, Environment and Extension
Volume 14 Number 3 September, 2015 pp. 27-34

ISSN 1119-7455

EVALUATION OF DIFFERENT DETECTION ASSAYS FOR USE IN *Leifsonia xyli* subsp. *xyli* ASSESSMENT PROGRAMS

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ABSTRACT

Ratoon stunting disease (RSD) caused by Leifsonia xyli* subsp. *xyli is a major constraint to sugarcane production worldwide. Importation of infected germplasm exposes Nigeria to risk of introduction of the pathogen since it has no unique external and internal symptoms. Therefore, different detection techniques were investigated. L. xyli* subsp. *xyli inoculated and uninoculated sugarcane varieties comprising susceptible (CP72-1210), moderately resistant (CP80-1743), most commonly grown (Co957, Co997, SP71-6180, Co62175, chewing cane, B47419, Co6806) and locally bred (NCS001, NCS002, NCS003, NCS005, NCS006, NCS007, NCS008, ILS001 and ILS002) were evaluated in the screenhouse for both plant crop and ratoon crop. RSD detection assays of PCR on leaf and stalk saps, evaporative-blot enzyme immunoassay (EB-EIA) on sap from stalk and tissue-blot enzyme immunoassay (TB-EIA) on stalk were carried out using inoculated and uninoculated varieties. L. xyli* subsp. *xyli was not detected in any of the uninoculated varieties. In inoculated varieties, L. xyli* subsp. *xyli was not detected by PCR in leaf extracts but was detected by EB-EIA, TB-EIA and PCR in stalk extracts from all the varieties in both plant crop and ratoon crop. Evaporative-blot enzyme immunoassay is recommended for quarantine detection of RSD bacterium in stalk because it is simple, less costly, sap used for detection can be stored for a long time and result read rapidly with a microplate reader compared to counting visually with stereo microscope in TB-EIA. PCR is much more expensive and accurate; it can be used to confirm results that are either questionable or negative from these serological techniques.

Key words: Ratoon stunting disease, *Leifsonia xyli* subsp. *xyli* detection, Sugarcane varieties, Nigeria

INTRODUCTION

Leifsonia xyli subsp. *xyli* (*Lxx*) is one of the most economically damaging diseases of sugarcane in the world (Davis *et al.,* 1980; Davis and Bailey, 2000; Evtushenko *et al.,* 2000; Dest´efano *et al*., 2003; Gao *et al.,* 2008). It only affects sugarcane resulting in 5 to 50% yield reduction (Pan *et al.,* 1998; Croft, 2002; Taylor *et al.*, 2003; Westpal and Mirkov, 2003; Comstock and Lentini, 2005; Comstock, 2008), depending on variety and growing conditions (Comstock, 2002; Monteiro-Vitorello*et al.*, 2004; Young *et al.*, 2006; Sutcliffe and Hutchings, 2007). It is a fastidious organism, Gram positive, non-motile, xylem–limited bacterium which causes a systemic infection of the vascular bundle called Ratoon Stunting Disease (RSD) (Davis *et al.,* 1980; Evtushenko *et al.,* 2000) or Ratoon Stunting (ISPP, 2012). It is found in many sugarcane growing countries but absent in Nigeria (Kazeem *et al*., 2015).

Symptoms of RSD is not always apparent in the field because it has no unique external symptoms except a non-specific stunting of affected cane which can also be attributed to other diverse factors such as poor cultural practices, inadequate moisture, nutrient deficiency (Gillaspie and Teakle, 1989; Hoy *et al.*, 1999; Comstock and Lentini, 2005). Internal symptoms, which do not develop adequately in all cultivars, include red-orange discoloration of the vascular bundles at the basal nodes of the matured stalks and faint pink discoloration in the immature nodes near the apical meristem when the stalk is sliced open longitudinally (Pan *et al.,* 1998; Viswanathan, 2001; Croft, 2002; Comstock and Lentini, 2005; Gao *et al.,* 2008).

DOI: http://dx.doi.org/10.4314/as.v14i3.6

The standard control practice is to establish a new crop with pathogen-free plant cuttings through the use of hot water treatment (50^{o}C for 2 hours), micropropagation using apical meristem and effective sanitation practice during planting and harvesting to prevent plant-to-plant transmission from harvesting equipment (Hoy *et al.,* 1999; Iglesia, 2003; Comstock and Miller, 2004; Comstock and Lentini, 2005; Young *et al.,* 2006; Grisham *et al.,* 2007). This requires monitoring through diagnostic technique to ensure that quarantine treatments and sanitation procedures are effective in preventing infection of the sugarcane plants in the field. This is important in safe movement and transfer of sugarcane germplasm where low level of *L. xyli subsp. xyli* can escape detection (Hoy *et al.,* 1999; Comstock, 2002).

Various diagnostic tools such as microscopic, immunological and deoxyribonucleic acid (DNA) based procedures have been developed to detect its presence. These tools are influenced by the sensitivity of the diagnostic protocol, bacterial titre, plant tissue, time of sample collection, inhibitors, instrument and personal skills (Hoy *et al*., 1999; Gao *et al*., 2008). Diagnosis typically has focused on the stalk because bacterial titre is highest there. In the stalk, maximum titre is reached first in the basal internode and then in internodes progressively towards the apex of the stalk (Harrison and Davis, 1988). Each method has distinct advantages and disadvantages as far as cost, accuracy, and ease of processing large samples but for quarantine purpose it should be simple, quick and sensitive enough to detect low population of the pathogen.

Thus, this study compared tissue blot, evaporative blot and PCR of sugarcane stalk along with PCR of leaf sheaths to evaluate different detection assays for use in RSD assessment programs in Nigeria.

MATERIALS AND METHODS

RSD Detection Assay

Sugarcane varieties used in the assay

Imported commercial sugarcane varieties grown in the sugarcane estates, one soft (chewing) cane and nine locally bred commercial sugarcane commonly grown in Nigeria as well as one RSD susceptible and one moderately resistant varieties (Table 1) were used for the study.

Inoculation of sugarcane varieties

Inoculum was prepared by removing a 1cm diameter longitudinal core tissue using a cork borer from *Leifsonia xyli subsp. xyli* (*Lxx*) infected sugarcane stalks provided by Dr. Sharon MacFarlane, South African Sugarcane Research Institute. *Lxx*-infected xylem sap was extracted into 2ml microcentrifuge tubes by spinning the tissue in a microcentrifuge at 6000 rpm for 5 mins.

Stalks of sugarcane varieties listed in Table 1 were first washed in tap water, cut into two-nodes and each varieties packed in a "fishing type net" to allow contact with water. Thermotheraphy using hot water to disinfect the sugarcane varieties was done by placing the canes in a circulatory hot water bath containing 10 litres of water at 50^{o}C for 2 h. The stalks were cool down and air-dried by spreading in flat tray on a table overnight (17 h) prior to inoculation and planting.

The hot water treated two-node cuttings from each variety were inoculated with *Lxx* by immersing the stalks at both cut ends for 5 mins in undiluted *Lxx* infected xylem sap, air-dried before they were planted. Another set of hot water treated cuttings from each variety were used for planting as control without inoculation with *Lxx* infected sap.

Planting of Sugarcane varieties

Leifsonia xyli subsp. xyli inoculated and non-*Lxx* inoculated two-node cuttings from each varieties shown in Table 1 were pre-planted separately in seed trays for a month in order to select those that germinated. The pre-germinated cuttings, *Lxx* inoculated and non-*Lxx* inoculated, from each variety were then planted in pre-washed 16 litre perforated plastic bucket filled with 14kg steam sterilized mixture of top soil, sand and ground granite (7:2:1) (NAQS, 2014). The buckets were laid out in a Randomized Complete Block Design with three (3) replicates in three screenhouses of Nigeria Agricultural Quarantine Service (NAQS), Ibadan with each screenhouse used as a block. NPK fertilizer (15:15:15) at rate of 100 kg/ha was added a month after transplanting.

Plants were grown in the screenhouse for 12 months for inoculated first plant crop and nine (9) months for the re-growth of the plant crop after harvest (first ratoon crop). These plants from the plant crop and first ratoon crop in the first and second year of planting respectively were used to screen for detection of *Lxx*.

Detection of Leifsonia xyli subsp. xyli in planted sugarcane crop

Ratoon Stunting Disease detection assay was conducted on *Lxx* infected sugarcane crop planted in the screenhouse by comparing three RSD detection techniques: PCR on both stalk sap and leaves; Evaporative blot enzyme immunoassay (EB-EIA) on sap and Tissue blot enzyme immunoassay (TB-EIA) on stalk.

Polymerase chain reaction (PCR)

DNA extraction procedures of Llop *et al*. (1999) and Mahuku (2004) on the sugarcane leaves were conducted with some modifications. The DNA was also extracted using the procedure of Llop *et al*. (1999) and Gao *et al*. (2008) on saps of

Table 1: Sugarcane varieties planted for detection of RSD of sugarcane

Sugarcane varieties	Parents	Sources	Comment
B47419	Unknown	Kadawa, Auyo, Numan	Imported commercial variety
"Hausa" or "Black" or "Beke" cultivar	Unknown	grown all over Nigeria	Chewing cane
Co62175	Unknown	Bacita	Imported commercial variety
Co6806	Unknown	Kadawa, Auyo, Numan	Imported commercial variety
Co957	Unknown	Bacita, Edozhigi, Numan	Imported commercial variety
Co997	Unknown	Auyo, Edozhigi, Numan	Imported commercial variety
CP72-1210	CP65-357 x CP56-63	Badeggi	Susceptible variety
CP80-1743	CP72-2083 x CP68-1067	Badeggi	Moderately Resistant variety
ILS001	Polycross	University of Ilorin	Local commercial Breed
ILS002	Polycross	University of Ilorin	Local commercial Breed
NCS001	LS-8 x Polycross	NCRI,Badeggi	Local commercial Breed
NCS002	LS-8 x Polycross	NCRI,Badeggi	Local commercial Breed
NCS003	OG-06 x Co biparent	NCRI,Badeggi	Local commercial Breed
NCS005	Composite open varieties	NCRI,Badeggi	Local commercial Breed
NCS006	B47419 Mutant	NCRI,Badeggi	Local commercial Breed
NCS007	BJ6502 Mutant	NCRI,Badeggi	Local commercial Breed
NCS008	KD-11 x Polycross	NCRI,Badeggi	Local commercial Breed
SP71-6180	Unknown	Bacita, Kadawa, Numan	Imported commercial variety

nine months sugarcane stalk from where leaves were taken for comparison. Gao *et al*. (2008) PCR technique was used on the leaves and stalk of the first ratoon crops (after the harvest of the plant crop) when the *Lxx* titre was expected to be high.

Three oldest green leaf sheaths were taken from RSD infected nine month-old sugarcane stalk (from previously PCR determined *Lxx* positive sugarcane stalk saps) of the first ratoon sugarcane varieties. The sheath was chopped and immersed overnight with occasional shaking in phosphate buffer saline (PBS) (50 mM of each of Na_2HPO_4, KH_2PO_4 and KCl pH 7.2; 0.5M NaCl). The supernatant was centrifuged at 13,000 rpm for 30 mins and the pellet suspended in PBS. A 500 µl of the suspension was centrifuged at 13,000 rpm for 12 mins and the pellet suspended in 500 µl extraction buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS and 2% PVP) vortexed and left for 1 hour at room temperature (28ºC) with continuous shaking. This was centrifuged at 6000 rpm for 5 mins and 450 µl of the supernatant was taken. Then 450 µl of isopropanol was added, mixed gently and left for 1 hour at room temperature (28ºC). The mixture was then centrifuged at 13,400 rpm for 15 mins; the supernatant was discarded and the pellet was dried over an airflow before the pellets was re-suspended in 100 µl sterile distilled water to be used as DNA template.

A 1-cm diameter from the stalk was cut with an alcohol-flamed sterilized cork borer into a 2ml sterile microcentrifuge tube. Xylem sap was extracted into a centrifuge tube at 6000rpm for 10 mins and stored (-20ºC) until used for DNA extraction (Davis and Dean, 1984; Pan *et al.,* 1998; Taylor *et al.,*2003; Croft *et al*., 2012). DNA was extracted using the method of Gao *et al.* (2008) by centrifuging 1 ml sap at 3,000 rpm for 5 min to separate impurities and large sized microorganisms. The supernatant (350 µl) was centrifuged at 12,000 rpm for 10 min and the supernatant discarded while the pellet lyzed in freshly prepared 50µl buffer A (0.1m NaOH and 2% Tween-20) by vortexing at full speed, centrifuged at 1000rpm for few seconds heated in a water bath at 95ºC for 10 min and finally cooled on ice for 5 mins. This was then neutralized by adding 50 µl of buffer B (0.1mM Tris-HCl[pH 8.0] and 2mM EDTA) into the suspension before vortexing and centrifuge at 1000 rpm for few seconds to collect the supension to the bottom. Positive controls using the same procedure above were obtained from infected sugarcane stalks (provided by Dr. Sharon MacFarlane, South African Sugarcane Research Institute) and RSD DNA samples (provided by Dr. Pan extracted from known infected sugarcane sap) and negative control DNA obtained from sap of hot water treated sugarcane respectively.

PCR reaction was conducted by preparing 25µl reaction mixture consisting of 1µl DNA template (either from sugarcane leaves or sap), 12.5µl Econo Taq Plus Green 2x Master mix (0.1 unit/µl Econo Taq DNA polymerase, Reaction buffer (pH 9.0) 3mM MgCl, 400µM each of dNTPs), 1µl each of Cxx1(5′- CCG AAG TGA GCA GAT TGA CC-3′) and Cxx2(5′- ACC CTG TGT TGT TTT CAA CG-3) primers and sterile distilled water (SDW) was used to make up the volume. PCR amplification modified from Gao *et al*., 2008 was done in Mygene series Peltier MG96G thermal cycler at a program of 1 cycle of 94ºC for 2 min; 40 cycles of 94ºC for 15 sec; 54ºC for 15sec and 72ºC for 1 min with a final extension of a cycle at 72ºC for 10 min. The PCR products were analysed in VWR international electrophoresis unit in 1% agarose gel with ethidium bromide (0.5µg/ml) and visualized under VWR international UV (302nm) transilluminator. A 439 bp bands amplified from the internal transcribed spacer region between the 16S and 23S rDNA of *L. xyli subsp. xyli* was used to determine its presence in the samples.

Evaporative blot enzyme immunoassay (EB-EIA)

Leifsonia xyli subsp. *xyli* population in the sap was determined using evaporative blot enzyme immunoassay (EB-EIA) described by Croft (2002) with modification by centrifuging 300µl sap at 3000 rpm for 30 mins. Supernatant was removed and the pellet was re-suspended in 300µl of $CaCO_3$ buffer (1.59g Na_2CO_3, 2.93g $NaHCO_3$ and 10g diethyldithiocarbamate (DIECA) in 1litre distilled water at pH adjusted to 9.6). A subsample of 100µl was added to the ELISA (microtitre) plate and placed in a drying oven at 37ºC for 1 hour or 4ºC overnight. After incubation, the plate was washed with three (3) changes (3 mins each) of PBS-T (50mM of each of Na_2HPO_4, KH_2PO_4 and KCl pH 7.4; 0.5M NaCl and 0.5% Tween-20). After washing, the plate was tap-dried on a paper towel to drain the wells. A 100 µl anti-*Leifsonia xyli* subsp. *xyli* rabbit antibody diluted 1:10,000 with conjugate buffer (0.05 g of Albumin, 0.5 g of Polyvinyl Pyrrolidone (PVP) and 12.5 ml of 10 X PBS made up to 250 ml with distilled water) was dispensed into each well of the ELISA plate and then incubated 37ºC for 1 hour. The plate was washed with PBS-T as above.

Alkaline phosphatase (ALP) conjugated anti-rabbit goat antibody (Sigma) was diluted (1:5000) in conjugate buffer and mixed thoroughly. This (100µl) was dispensed into each well of the ELISA plate and then incubated at 37ºC for 1 hour. The plate was washed as above with PBS-T. After that, 100µl *p*-nitro phenyl phosphate (PNPP) substrate solution [prepared by dissolving 0.5mg of PNPP/ml of substrate buffer (10% diethanolamine in distilled water, at pH 9.8)] was added to each well. The plate was incubated in the dark for 30mins and 1 hour at room temperature (28ºC) and overnight (approximately 16 hours) at 4ºC to allow for colour development. Absorbance of the colour reaction in the ELISA plate was read on a Biorad microplate reader at 405nm. Samples with an absorbance reading of >0.05 were considered positive using the EB-EIA technique (Croft *et al*., 2012).

Tissue blot enzyme immunoassay (TB-EIA)

TB-EIA technique was performed using the methods of Harrison and Davies (1988) and Croft *et al*. (2012), with slight modifications with 1cm tissue core taking from basipetal end of the internode. The core was then place on a 0.22µm nitrocellulose membrane placed on absorbent material in 5ml Eppendorf tubes. This was then centrifuged at 3000rpm for 15 mins to extract the sap and filter the bacterium in the sap on the membrane. The membrane was dried at 80ºC for 1 hour or overnight (approximately 16 hours) at 4ºC in a refrigerator before performing indirect enzyme-linked immunosorbent assay (ELISA) technique on it.

Blotted membrane was immersed for 1hour at room temperatue (28ºC) in *Lxx* rabbit polyclonal antibody IgG (provided by Rafaela Degaspari, CanaVialis S/A, Brazil) diluted (1:10,000) in ELISA buffer (50mM of each of Na_2HPO_4, KH_2PO_4 and KCl and 0.5M NaCl pH 7.2 containing 2% polyvinylpyrrolidone, 0.5% bovine albumin serum and 0.02% sodium azide). It was washed with 3 rinses of 5 min interval in ELISA washing solution (50mM of each of Na_2HPO_4, KH_2PO_4 and KCl and 0.5M NaCl pH 7.2 containing 0.2% tween and 0.02% sodium azide). It was then immersed for 1 hour at room temperature (28ºC) in goat antirabbit antibody conjugated with alkaline phosphatase (SIGMA) diluted (1:1000) with ELISA buffer. After 3 rinses with ELISA washing solution at 5 min interval, membrane was immersed in dissolved BCIP/NBT alkaline phosphatase substrate (SIGMA) (1 tablet:10ml sterile distilled water) and incubated in the dark for 30 mins for colour change (stain) detection in vascular bundles. The reaction was stopped by dipping the membrane in distilled water. After drying the membrane, stained colonized vascular bundles observed using stereo-microscope was regarded as positive detection.

RESULTS

There was no amplification using Cxx1 and Cxx2 primers (439bp) of DNA extracted from infected sugarcane leaves to confirm presence of *Lxx* and test for possibility of detection of the bacterium in the leaves. This is indicated in Lanes 3, 5, 7, 8, 9, 12, 14, 19, 20, 23, 25, 27, 28, 31, 33, 34 and 35 from leaves obtained *Lxx* inoculated and Lanes 4, 6, 10, 13, 15, 18, 21, 24, 26, 29 and 32 from leaves of uninoculated sugarcane varieties on Figure 1. Amplifications of DNA extracted from *Lxx* infected sap from the stalk of same sugarcane varieties from where the leaves were obtained detected the bacterium in figure1 on lanes 2, 11, 16, 17, 22 and 30.

Colonized vascular bundles (CVB) results of tissues from stalk of the sugarcane varieties showed the dot stain within the vascular bundles indicating the presence of *L. xyli subsp. xyli* (*Lxx*) in the samples (Figure 2) while un-colonized vascular bundles had no stain indicating absence of *Lxx* (figure 2). All eighteen (18) inoculated and 18 uninoculated sugarcane varieties grown in the screenhouse and used for diagnosis tested positive and negative for *Lxx* respectively (Table 2).

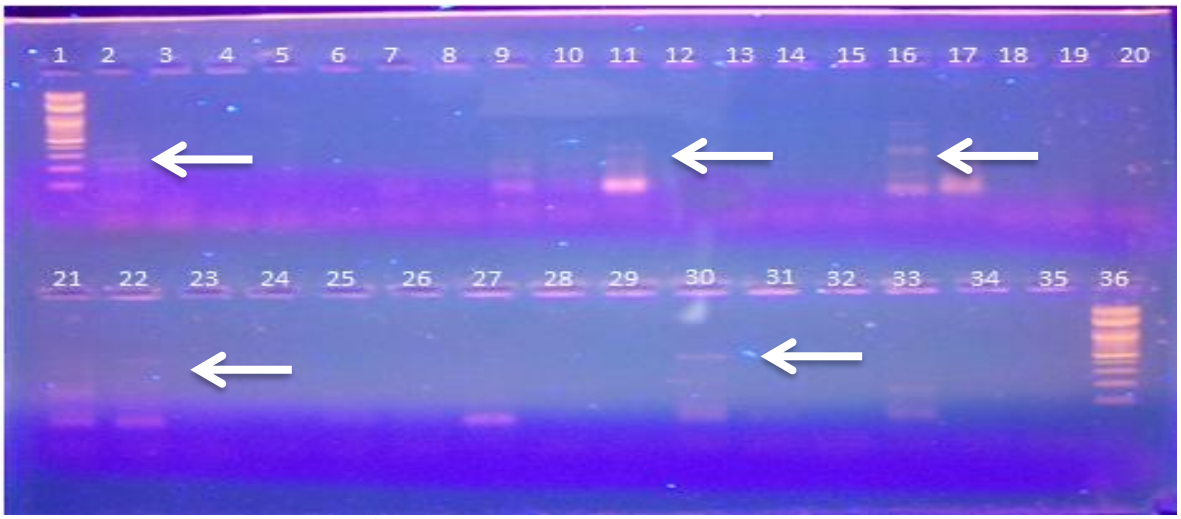

Figure 1: Ethidium bromide stained amplification with Cxx1 and Cxx2 primers (439bp) of DNA extracted from *Lxx* inoculated sugarcane leaves of NCS005, B47419, NCS003, NCS008, NCS007, NCS006, NCS001, CP80-1743, Co6806, Co997, Chewing cane, CP72-1210, ILS001 Co957, Co62175, NCS002, ILS002 and SP71-6180 (Lanes 3, 5, 7, 8, 9, 12, 14, 19, 20, 23, 25, 27, 28, 31, 33, 34 and 35 respectively); from healthy sugarcane leaves of NCS005, B47419, NCS007, NCS006, NCS001, CP80-1743, Co997, Chewing cane, CP72-1210, Co957 and Co62175 (Lanes 4, 6, 10, 13, 15, 18, 21, 24, 26, 29 and 32 respectively) and from sugarcane sap (Arrow) of Chewing cane (Lane 2), ILS002 (Lane 11), ILS001 (Lane 16), SP71-6180 (Lane 17), NCS001 (Lane 22), CP72-1210 (Lane 30), Ladder (Lane 1,36).

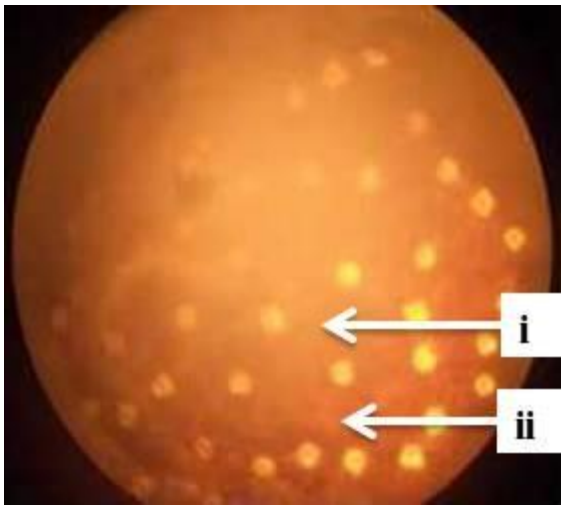

Figure 2: Vascular bundles of sugarcane variety with (i) no dot stain (ii) dot stain

Absorbance values obtained from evaporative binding-enzyme immunoassay (EB-EIA) detection technique on sugarcane sap of the different varieties of sugarcane used in the study varies with varieties as shown in Table 2. Absorbance reading of 0.05 was used as critical value to determine the presence or absence of *L. xyli subsp. xyli*. All the uninoculated samples had values lower than the critical values needed for the detection of *L. xyli subsp. xyli* except CP80-1743 and NCS003 which had absorbance of 0.05 from the plant and ratoon crops indicating the absence of RSD bacterium. Absorbance readings from the 18 inoculated varieties ranges from 0.96-3.21 and were higher than the critical value of 0.05 indicating the presence of *Lxx* in the samples. Susceptible control (CP72-1210) had the highest absorbance followed by inoculated varieties of CP80-1743, ILS001, chewing cane, Co62175 and NCS005. The lowest absorbance occurred in inoculated varieties of ILS002, SP71-6180, NCS001 and NCS007.

This result agrees with the two other techniques (PCR on sugarcane sap and CVB on stalk) in all cases with all inoculated and uninoculated samples from the sugarcane varieties producing positive and negative responses respectively for detection of RSD bacterium (Table 2).

DISCUSSION

Result of the different detection assays used in this study on *Lxx* infected sugarcane saps and stalks from the plant crop and first ratoon crops showed that it is possible to detect RSD bacterium using Tissue-blot enzyme immunoassay (TB-EIA) on stalk, Evaporative-binding enzyme immunoassay (EB-EIA) and Polymerase chain reaction (PCR) on sugarcane saps as previously done by several researchers (Harrison and Davis, 1988; Croft *et al.*, 1994; Hoy *et al.*, 1999; Pan *et al.*, 2001; Croft, 2002; McFarlane, 2002; Shen *et al.*, 2006; Gao *et al*., 2008). There was 100% detection of RSD bacterium from the stalk extract in all *Lxx* inoculated sugarcane varieties while none of the uninoculated varieties gave indications of the presence of the bacterium for all the detection techniques used. The accuracy of these methods were confirmed with no false positive or false negative results observed in both plant and ratoon crops. This result supports previous comparisons of the two techniques which showed that TB-EIA and EB-EIA could be used to detect infected *Lxx* sugarcane varieties even though TB-EIA was more

Table 2: Result of PCR on leaf with the Tissue-blot enzyme-linked immunosorbent assay (TB-EIA), Evaporative enzyme-linked immunosorbent assay (EB-EIA) and PCR for detection of *Leifsonia xyli subsp. xyli*

Varieties	Treatments	TB-EIA	EB-EIA	PCR stalk	PCR leaf
B47419	uninoculated	-	0.040^{-}	-	-
	inoculated	+	1.91^{+}	+	-
Chewing cane	uninoculated	-	0.035^{-}	-	-
	inoculated	+	2.50^{++}	+	-
Co62175	uninoculated	-	0.047^{-}	-	-
	inoculated	+	2.26^{++}	+	-
Co6806	uninoculated	-	0.044^{-}	-	-
	inoculated	+	2.07^{++}	+	-
Co957	uninoculated	-	0.045^{-}	-	-
	inoculated	+	1.80^{+}	+	-
Co997	uninoculated	-	0.042^{-}	-	-
	inoculated	+	2.11^{++}	+	-
CP72-1210[a]	uninoculated	-	0.049^{-}	-	-
	inoculated	+	3.21^{++}	+	-
CP80-1743[b]	uninoculated	-	0.050^{+}	-	-
	inoculated	+	2.67^{++}	+	-
ILS001	uninoculated	-	0.044^{-}	-	-
	inoculated	+	2.60^{++}	+	-
ILS002	uninoculated	-	0.044^{-}	-	-
	inoculated	+	0.96^{+}	+	-
NCS001	uninoculated	-	0.047^{-}	-	-
	inoculated	+	1.30^{+}	+	-
NCS002	uninoculated	-	0.039^{-}	-	-
	inoculated	+	1.69^{+}	+	-
NCS003	uninoculated	-	0.050^{+}	-	-
	inoculated	+	1.92^{+}	+	-
NCS005	uninoculated	-	0.042^{-}	-	-
	inoculated	+	2.26^{++}	+	-
NCS006	uninoculated	-	0.048^{-}	-	-
	inoculated	+	1.86^{+}	+	-
NCS007	uninoculated	-	0.043^{-}	-	-
	inoculated	+	1.55^{+}	+	-
NCS008	uninoculated	-	0.042^{-}	-	-
	inoculated	+	1.99^{+}	+	-
SP71-6180	uninoculated	-	0.049^{-}	-	-
	inoculated	+	1.24^{+}	+	-

[a]resistant and[b]susceptible checks; -, RSD negative (≤ 0.05 absorbance value of 1 hour ELISA reading at 405_{nm} Absorbance); +, RSD positive (represents ≥ 0.05-2 absorbance value of sugarcane sap); ++, highly RSD positive (represents ≥ 2 absorbance value of sugarcane sap) (Croft,2002).

accurate than EB-EIA (Hoy *et al*., 1999; Gao *et al*., 2008; Croft *et al*., 2012) but less sensitive than PCR (Fegan *et al*., 1998; Pan *et al*., 1998; Taylor *et al*., 2003). These methods utilized the stalk or stalk extracts for detection of *Lxx* in infected sugarcanes (Miller *et al*., 1996; Croft, 2002; Taylor *et al*., 2003; Westpal and Mirkov, 2003; Gao *et al*., 2008).

EB-EIA is recommended for quarantine detection of RSD bacterium in stalk because it is simple, less costly, sap used for detection can be stored for a long time and result read rapidly with a microplate reader compared to counting visually with the stereo microscope in TB-EIA. TB-EIA is more appropriate in the determination of resistant status of sugarcane varieties. Both methods (EB-EIA and TB-EIA) were performed on large samples within the same time frame and require virtually the same materials except nitrocellulose membrane used for TB-EIA which is more expensive than microtitre plate used for EB-EIA. Although, PCR is much more expensive and accurate, it can be used to confirm results that are either questionable or negative from these serological techniques.

This study also adapted the suggestion of Gillaspie (1987) on the need to explore the use of leaves as a non-destructive method for detection of RSD due to bulkiness of stalks during transportation and in quarantine situations for indexing planting materials or where it is important to conserve stalks for propagation. PCR was selected as a method of choice to evaluate RSD bacterium on leaf sheath because it can detect low titre of *Lxx* in infected sugarcane varieties compared to TB-EIA and EB-EIA as reported by Fegan *et al*. (1998) and Pan *et al*. (1998). This was based on report that diffusates from the leaf sheath, leaf mid-ribs and leaf blades contain *L. xyli* subsp. *xyli* in the ratio of 25:5:1 (Anonymous, 1988) with the highest concentration in the oldest green leaf sheath (Teakle *et al.*,1975; Croft *et al*., 2012) though 2-10 times lower when compared to that of the stalk (Gillaspie, 1987; Anonymous 1988). This is with a view to validating and comparing the non-destructive technique for the detection of *Lxx* in sugarcane leaf for quarantine screening of sugarcane germplasm with that from the stalk.

DNA was successfully extracted from infected leaf sheath from sugarcane first ratoon crop using the technique of Llop *et al*. (1999). Amplification of the DNA from the leaf using conventional PCR for identification of *L. xyli* subsp. *xyli* was not successful compared with TB-EIA, EB-EIA and conventional PCR on stalk and stalk extracts. Several factors which might have been responsible for the non-detection of the bacterium was identified by Gao *et al*. (2008) to include bacterial titre, inhibitors, plant tissue, instruments and personal skill.

Failure to detect the bacterium could be attributed to interference from PCR inhibitors which occur because green matured leaves from older ratoon plants (9 months) were used. The quality of extracted DNA was high and Polyvinlypyrrolidone 40 (PVP-40) was added to reduce effects of PCR inhibitors as recommended by Fegan *et al*. (1998) and Pan *et al*. (1998). Fegan *et al*. (1998) observed that PCR inhibitors will still affect the efficiency of PCR detection even with the addition of PVP-40.

Similar result using conventional PCR technique on sugarcane leaf was obtained by Grisham *et al*. (2007) with decline in the number of positive samples in both susceptible and resistant varieties from 100 to 0% detection of *Lxx* in infected sugarcane leaf from early growth to late growth stages. Grisham *et al*. (2007) also reported that real-time PCR (RT-PCR) was not affected by the stage of growth of the plant but depending on resistance status of infected sugarcane varieties was able to detect 75-100% of *L. xyli* subsp. *xyli* in the same leaf samples used in the conventional PCR technique. Also, investigation on the efficacy of leaf-tissue assays for RSD by researchers from Australian Government Sugar Research and Development Corporation (SRDC) showed an average consistency in detection of RSD and recommended further work to improve the technique to help test greater number of sugarcane pla (SRDC Annual Report, 2006).

A factor that was responsible for this result (reduced detection of *L. xyli* subsp. *xyli*) was attributed by Grisham *et al*. (2007) to build-up of PCR inhibitors as the plant matures which was not controlled by the addition of compound to limit the effect of PCR inhibitors. Two other studies (Gillaspie, 1987; Croft *et al.*, 2012) using different techniques had shown that RSD bacterium could be detected in leaves from young plants, though it contains fewer bacterium and less PCR inhibitors (Gillaspie, 1987; Westpal and Mirkov,2003; Grisham *et al*., 2007; Croft *et al.,* 2012). Westpal and Mirkov (2003) also raised the issue of resistant status of the sugarcane varieties as increased resistant would result in low titre of the pathogen in the stalk. This was also supported by Grisham *et al*. (2007) that there was reduced detection in resistant compared to susceptible varieties using RT-PCR.

From the observations made from this and previous studies (Gillaspie, 1987; Grisham *et al*., 2007; Croft *et al.,* 2012) age and position of the leaf on the stalk, age of sugarcane plants in both plant and ratoon crops, PCR inhibitors, titre of the bacterium and resistant status of the sugarcane varieties should be considered as a way of improving the technique of using non-destructive part of the cane for detection of RSD bacterium in future studies.

ACKNOWLEDGEMENT

We thank COMSTECH/IFS for the financial grant (Grant No. C/4971-1) to conduct the study. Dr. S. MacFarlane (SASRI, South Africa) and Dr. Y-B. Pan (USDA-ARS, USA) for RSD positive controls. Dr. O.J. Alabi (Texas A. and M. University, USA) and Dr. L. Kumar (IITA, Nigeria) for PCR assistance, Jibrin M.O. of Ahmadu Bello University, Nigeria for reading through the manuscript.

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