2014

Detection and quantification of Xanthomonas albilineans in sugarcane tissues with quantitative polymerase chain reaction and evaluation as methodology for monitoring resistance

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DETECTION AND QUANTIFICATION OF *XANTHOMONAS ALBILINEANS* IN SUGARCANE TISSUES WITH QUANTITATIVE POLYMERASE CHAIN REACTION AND EVALUATION AS METHODOLOGY FOR MONITORING RESISTANCE

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

by
Andres Felipe Gutierrez Viveros
B.S., Universidad del Valle, 2006
May 2014

ACKNOWLEDGMENTS

I would like to thank my Major Professor, Dr. Jeffrey W. Hoy for the guidance and encouragement through the completion of this project, and for helping me to develop scientific skills.

I also want to thank Dr. Niranjan Baisakh, Dr. Collins Kimbeng, Dr. Jong Ham and Dr. Lawrence Datnoff for serving on my research committee and for providing valuable advice in the design and revision of the experiment results.

I want to extend my acknowledgment to Carolyn Savario of the Sugarcane lab, as well as, Alejandro and Kara for their friendliness and constant support in the lab and field. I thank my lab mate and unconditional friend Carolina Avellaneda for all the support in the US.

Finally, without the economic support of the United States Department of Agriculture (USDA) and American Sugar Cane League, it never would have been possible to earn my degree.

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ABSTRACT

Leaf scald, caused by *Xanthomonas albilineans*, is a major sugarcane disease worldwide that is controlled primarily with host plant resistance. Since visual evaluation of disease resistance can be uncertain due to erratic symptom expression, a more reliable screening method is needed for resistance research. A quantitative polymerase chain reaction (qPCR) assay was developed previously with demonstrated potential for resistance screening. However, only four cultivars with extreme reaction against the disease (two highly susceptible and two highly resistant) were compared. Therefore, additional research was conducted to demonstrate assay specificity, compare bacterial populations in 31 clones with varying levels of susceptibility at different times after inoculation, and evaluate the correlation with the visual rating method currently used for resistance evaluation. SYBR Green and TaqMan qPCRs were tested against DNA from different bacteria and a fungus, and the assays showed high specificity amplifying only *X. albilineans* isolates. Inhibitors of amplification during PCR were not detected in DNA extracts from four cultivars. Comparison between the bacterial population quantified by qPCR and visual symptom severity ratings showed variable results with the highest correlation between the data at 8 weeks after inoculation ($\rho = 0.62$, $P < 0.0001$). In order to measure consistency, the correlation was determined among three different inoculations for data obtained with the same method at different times after inoculation. The qPCR assay was more consistent among the different inoculations ($r = 0.77$, $P < 0.0001$) compared with the visual rating system ($r = 0.53$, $P = 0.0026$) at 8 weeks after inoculation. Bacterial quantification was more consistent in field inoculations compared to greenhouse inoculation. The high specificity and consistency suggest that qPCR can provide an improved method to evaluate resistance to leaf scald in sugarcane.

CHAPTER 1: GENERAL INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is an important tropical grass crop that accounts for 70% of the raw sugar produced worldwide (Le Cunff, *et al*., 2008; Wei, *et al*., 2006). Sugarcane is a C4 photosynthetic plant which, combined with its perennial nature, has made it one of the most productive cultivated plants (Le Cunff, *et al*., 2008). It is able to partition carbon to sucrose in the stem, in contrast with other cultivated grasses that usually accumulate their reserve products within seeds (Le Cunff, *et al*., 2008). Recently, it has gained increased attention because it represents an important source of renewable biofuel via ethanol production and electricity generation (Le Cunff, *et al*., 2008; Wei, *et al*., 2006). In the United States, sugarcane is grown for sucrose in Florida, Louisiana, Texas, and Hawaii. Florida produces 48% of the total cane sugar (Baucum & Rice, 2009), while Louisiana produces nearly 43% (NASS, 2010).

Among the 120 diseases that have been described on sugarcane (Rott, *et al*., 2000), leaf scald, caused by *Xanthomonas albilineans* (Ashby) Dowson, is one of the major diseases and occurs in most sugarcane-producing countries of the world (Rott & Davis, 2000; Rott, *et al.* 1997; Wang, *et al*., 1999). The disease is characterized by chronic and acute symptoms varying in severity from a single, white, narrow, sharply defined longitudinal leaf stripe to death of shoots or entire plants (Ricaud & Ryan, 1989; Rott, *et al*., 1997; Wang, *et al*., 1999). Latent infection can occur, making visual diagnosis problematic (Ricaud & Ryan, 1989; Rott, *et al*., 1997). Leaf scald can be a serious disease due to high losses in tons of cane per hectare and reduction in the juice quality (Ricaud & Ryan, 1989; Rott & Davis, 2000). In addition, decreases in yield have been associated with the acute form of the disease, in which whole fields planted with a susceptible variety could be destroyed in few months (Ricaud & Ryan, 1989; Rott, 1993).

Xanthomonas albilineans is a xylem-inhabiting gamma-proteobacterium that belongs to the order Xanthomonadales (Janse, 2005). It is a Gram-negative, aerobic, rod 0.25 - 0.3 µm by 0.6 – 10 µm, occurring singly or in chains, with a single polar flagellum (Ricaud & Ryan, 1989). The colonies are buff yellow but non-mucoid with optimal growth at 25 °C, and the bacteria grow slowly and appear after 4 – 6 days as circular, moist and shiny, transparent honey-yellow colonies (Ricaud & Ryan, 1989). *X. albilineans* is an unusual bacterium because it apparently does not possess avirulence or pathogenicity genes that are typically found in phytopathogenic bacteria (Champoiseau, *et al*., 2006).

Xanthomonas albilineans can cause three different phases of infection and symptomatology on sugarcane: latent (no symptoms), chronic, and acute (Ricaud & Ryan, 1989; Rott & Davis, 2000; Saumtally & Dookun, 2004). Symptom expression and severity are associated with the level of cultivar resistance, environmental conditions, and pathogen aggressiveness.

The chronic phase is characterized by symptoms that vary in severity, including white longitudinal streaks along leaf veins termed “pencil lines”, general leaf chlorosis or bleaching, leaf necrosis progressing basipetally initially along pencil lines, abnormal development of side shoots exhibiting symptoms on stalks, reddish discoloration of vascular bundles at the node level, stunting, wilting, and death (Birch, 2001; Ricaud & Ryan, 1989; Rott & Davis, 2000; Saumtally & Dookun, 2004). Symptoms could be caused by bacterial xylem blockage and by the metabolic wastes produced (Birch, 2001), while bleaching, chlorosis and necrosis are associated with changes in the cells caused by a toxin, albicidin, produced by the pathogen. Albicidin is a phytotoxin that inhibits DNA replication and blocks plastid development (Hashimi, *et al*. 2008).

The acute phase occurs as a sudden wilting of plants resulting in death, with few or no previous symptom expression. Large areas of a field may be affected in this manner (Rott & Davis, 2000; Saumtally & Dookun, 2004). The acute phase was observed in a highly susceptible cultivar after a period of drought stress following rainy conditions (Ricaud & Ryan, 1989). The latent phase occurs and ends for reasons which are unknown (Rott & Davis, 2000). Latency is sometimes observed in young shoots that emerge from infected setts and in ratoon crops. Symptomatic young shoots also can recover during stalk development (Ricaud & Ryan, 1989). Detection of the disease is difficult when infection is latent, and this resulted in worldwide spread of leaf scald during sugarcane germplasm exchanges (Daugrois, *et al*., 2003).

Leaf scald was reported for the first time in Louisiana in 1993. A survey found that leaf scald was widely distributed in the Louisiana industry and had the potential to cause severe symptoms and yield losses under Louisiana environmental conditions (Hoy & Grisham, 1994). An indirect loss is caused by the elimination of promising clones in cultivar selection programs (Hoy & Grisham, 1994; Ricaud & Ryan, 1989). Up to 20% of sugarcane clones in the selection population are rejected annually due to leaf scald susceptibility in Australia, even though crosses between susceptible parents are avoided (Birch, 2001).

The disease is spread locally by the use of infected cuttings for planting and contaminated tools used at harvest (Ricaud & Ryan, 1989; Rott & Davis, 2000). Hurricane conditions have also been associated with disease spread (Ricaud & Ryan, 1989; Hoy & Grisham, 1994). Aerial transmission was reported in Guadeloupe (Klett & Rott, 1994), where the bacterium was exuded from the leaf hydathodes and then spread by aerial means. In addition, maize and several weeds have been reported to be naturally infected by the bacterium (Rott & Davis, 2000).

Breeding and selecting for host plant resistance has been the most important control measure for leaf scald. The use of hot water treatment and tissue culture techniques to produce healthy seed-cane for moderately susceptible cultivars, disinfection of cutting and harvest tools with bactericides, and quarantine measures during germplasm exchanges are additional methods used to prevent and control the disease (Ricaud & Ryan, 1989; Rott & Davis, 2000).

Screening trials to evaluate resistance are carried out in many countries where the disease is a problem, but assessment of cultivar reactions is difficult and time-consuming (Rott, *et al*., 1997). Assessments generally are based on observation and subjective rating of symptom severity after artificial inoculation (Rott, *et al*. 1997). However, the troublesome aspect of evaluating resistance to leaf scald is that some sugarcane cultivars can tolerate the pathogen without exhibiting symptoms, and symptom expression even in susceptible cultivars is affected by environmental conditions (Rott, *et al*., 1997). Erratic symptom expression results in the failure to accurately detect susceptibility and the need for repeated inoculations.

The worldwide distribution of leaf scald and the consequences in field production create a need for efficient pathogen detection methodologies. Current methodologies for leaf scald detection are based on the isolation and culture of the bacteria on XAS Wilbrinks semi-selective plating media (Davis, *et al*., 1994), or serological assays, such as enzyme-linked immunosorbent assay [ELISA] (Comstock & Irey, 1992), dot immunobinding assay [DIA] (Rott, *et al*., 1994), and tissue-blot enzyme immunoassay [TBIA] (Comstock & Irey, 1992). However, the sensitivity of the serological methodologies is low (threshold levels of detection around 10^5 CFU/ml), and detection of the bacterium is not always accurate, especially in plants that show no symptoms (Wang, *et al*., 1999). Culturing is very sensitive and detects a low concentration of bacteria, but the bacteria require up 7 days to form characteristic colonies (Rott, *et al*. 1995; Wang, *et al*.,

1999). Moreover, diagnosis based only on the isolation of bacterial colonies with characteristic morphology can be affected by contamination and needs corroboration with another more specific method. For these reasons, there is a need for a more sensitive and rapid *X. albilineans* detection method for quarantine, epidemiological, and resistance research.

Molecular techniques providing faster and more sensitive detection have been used for different plant pathogenic bacteria (Wang, *et al*. 1999). Some detection methods utilizing polymerase chain reaction (PCR) were developed previously for *X. albilineans* (Davis, *et al*., 1994; Pan, *et al.*, 1997; Wang, *et al*., 1999); however, methods based in conventional PCR have a serious limitation for epidemiological studies and resistance screening because they only give qualitative results of the infection (presence or absence of the bacteria in the tissue examined). The qualitative information does not reflect the differences in susceptibility observed in the field among different clones of sugarcane, especially between moderately and highly susceptible varieties.

Quantitative PCR (qPCR) can determine the amount of a target sequence or gene that is present in a sample. A qPCR assay was developed that uses a video camera to detect the accumulation of double-stranded DNA in the PCR using the increase of fluorescence of ethidium bromide that results from its binding duplex DNA (Higushi, *et al*., 1993). The kinetics of fluorescence accumulation during thermocycling is directly related to the starting number of DNA copies (Higushi, *et al*., 1993; Gao, *et al*., 2004). Since 1993, though its basic principle remains the same, the qPCR assay has been optimized (Gao, *et al*., 2004).

Real-time PCR is a modification of the conventional PCR in which amplification of the target based on the 5'-3' exonuclease activity of *Thermus aquaticus* (Taq) DNA polymerase is

measured by the fluorescence produced by a special dye (SYBR Green) or probe (TaqMan) (Gao, *et al*., 2004). This activity releases two different chemicals that bind to a special probe, and this separation produces fluorescence. The increase in the fluorescence is directly related to the increase in DNA amplification, and the degree of fluorescence accumulation is related with the starting number of the DNA copies. In other words, the fewer amplification cycles necessary to produce detectable fluorescence, the greater the number of DNA copies present in the sample.

Currently, two common methods of analyzing data from qPCR experiments are employed: absolute quantification and relative quantification (Gao, *et al*., 2004). Relative quantification describes the change in expression of the target gene relative to some relative group, such as an untreated control, using a housekeeping gene for the comparison of expression changes of the target gene (Gao, *et al.* 2004). Absolute quantification determines the input copy number of the transcript of interest based on the comparison of the fluorescence of the unknown concentration sample with a standard curve of samples with known concentration (Gao, *et al*., 2004). Absolute quantification has been used to detect and quantify plant pathogenic fungi, bacteria and viruses, as well as biocontrol agents of plant pathogens (Gao, *et al*., 2004).

Real-time, quantitative PCR (qPCR) is a highly sensitive, reproducible and accurate method that is being used for qualitative and quantitative analysis of nucleic acid molecules (Ginzinger, 2002; Higuchi, *et al*., 1993). Real-time PCR assays have been developed for the detection of pathogens causing other sugarcane diseases, including yellow leaf, caused by *Sugarcane yellow leaf virus* (Korimbocus, *et al*., 2002; Yun et al., 2010), and ratoon stunt, caused by *Leifsonia xyli* subsp. *xyli* (Grisham, *et al*., 2007). The potential for high sensitivity and specificity could make qPCR a superior method for reliable detection and quantification of *X. albilineans*.

Previously, it was reported that *X. albilineans* populations in the shoot apex were highly correlated with disease severity in field and greenhouse experiments (Rott, *et al*., 1997). The correlation between pathogen population and disease severity could be a useful feature for resistance studies. If the relationship between bacteria population dynamics inside the plant, symptom severity, and resistance level is confirmed, *X. albilineans* quantification with qPCR could provide a more reliable method for determining resistance levels in sugarcane genotypes in inoculation tests (Garces, 2011).

A qPCR for improved diagnosis and quantification of *X. albilineans* with demonstrated potential for resistance screening was recently developed (Garces, 2011). However, only four cultivars with extreme reaction against the disease (two highly susceptible and two highly resistant) were compared. For that reason, additional research is needed to determine the sensitivity and specificity of the qPCR method and compare bacterial populations in more clones with varying levels of susceptibility.

CHAPTER 2: SPECIFICITY AND PLANT EXTRACT INHIBITION OF QUANTITATIVE PCR FOR *XANTHOMONAS ALBILINEANS*

2.1 INTRODUCTION

Leaf scald, caused by *Xanthomonas albilineans* (Ashby) Dowson, is a major disease of sugarcane worldwide. It is a systemic, vascular bacterial disease that can cause severe cane yield reductions and reduce juice quality in susceptible cultivars, eliminate potential cultivars, and require special attention for germplasm exchange (Rott and Davis, 2000). Widespread distribution resulted from the exchange of symptomless, infected vegetative germplasm (seed-cane) in the absence of adequate detection techniques in quarantine programs (Daugrois, *et al*., 2003).

Multiple methods have been developed to detect and quantify *X. albilineans* (Xa), including selective media (Davis, *et al*., 1994), serological based (Alvarez, *et al*., 1994; Comstock & Irey, 1992; Rott, *et al*., 1994), and polymerase chain reaction (PCR) based techniques (Jaufeerally, *et al*., 2002; Pan, *et al*., 1999). However, all these techniques have different problems. Immunoassays have low sensitivity with threshold levels of detection around 10^5 CFU/ml (Wang, *et al*., 1999). Bacteria isolated on selective medium require 5-7 days to form characteristic colonies (Rott, *et al*., 1995; Wang, *et al*., 1999). In addition, the visual identification of bacterial colonies based on characteristic morphology is risky and needs corroboration with another more specific method. Finally, there is a lack of quantitative information in the conventional PCR assays. For these reasons, there is a need for a more sensitive and rapid Xa detection method for quarantine use, epidemiological studies, and resistance research (Wang, *et al*., 1999).

Leaf scald has been controlled primarily through breeding, selection, and cultivation of resistant cultivars (Rott and Davis, 2000). Traditionally, resistance evaluation has been based on rating symptom severity after inoculation with the pathogen; however, erratic symptom expression makes visual rating an inconsistent method to measure resistance to the disease. A previous report that *X. albilineans* populations in the shoot apex were highly correlated with disease severity (Rott, *et al*., 1997) opened the possibility of using bacterial quantification as an indirect method to measure resistance.

Quantitative PCR (qPCR) is a sensitive, reproducible and accurate method for quantitative analysis of nucleic acids (Higuchi, *et al*., 1993). Real-time, quantitative PCR assays have been developed for the detection of pathogens causing other sugarcane diseases, including yellow leaf, caused by *Sugarcane yellow leaf virus* (Goncalves, *et al*., 2002; Korimbocus, *et al*., 2002; Yun, *et al*., 2010), ratoon stunt, caused by *Leifsonia xyli* subsp. *xyli* (Grisham, *et al*., 2007), and brown and orange rust, caused by *Puccinia melanocephala* and *P. kuehnii*, respectively (Glynn, *et al*., 2010). The high sensitivity and specificity could make qPCR a superior method for reliable detection of Xa. If the relationship among bacterial population dynamics inside the plant, symptom severity, and resistance level is confirmed, Xa quantification with qPCR could provide a reliable method for determining resistance levels in sugarcane genotypes in inoculation tests.

A qPCR was developed for detection of Xa with demonstrated potential for resistance screening (Garces, 2011). TaqMan and SYBR Green PCR assays were developed utilizing primers from the bacterium-specific albicidin toxin gene cluster for the detection and quantification of Xa in sugarcane. The qPCR assays for Xa detection were faster and more sensitive than conventional PCR. However, only four cultivars with extreme reaction against the

disease, based on disease severity data determined in multiple field evaluations, were compared. Therefore, additional research was needed to demonstrate assay specificity, determine the bacterial extraction efficiency, and evaluate the possibility of inhibition due to the method used for DNA extraction, all factors that might affect the accurate quantification of Xa. The study objectives were to determine whether the qPCR amplifies DNA from different bacteria and fungi associated with sugarcane or related to the pathogen, if bacterial DNA is efficiently obtained from infected tissues, and whether DNA extracts from different host genotypes inhibit Xa amplification.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial isolation and DNA extraction

Xanthomonas albilineans was isolated from a longitudinal section of leaf with a characteristic bleached vein "pencil-line" symptom. Tissue was surface-sterilized with NaOCl (0.5%) for 30 s and rinsed with water. The leaf sections were dried, 6-mm-diameter discs were removed with a sterilized hole punch, and 20 discs were placed in an 1.5 ml microcentrifuge tube (Eppendorf, Hauppauge, NY) containing 1 ml of sterile, distilled water. The tubes were incubated overnight at 4°C. A loop of bacterial suspension was transferred to semi-selective XAS medium (Davis, *et al*., 1994) and incubated at 28°C. After 5-8 days, single colonies were selected and transferred to XAS medium without antibiotics, and single colonies were again selected. Pure cultures were incubated at 28°C, and after 48 h, 5 ml of sterile, distilled water were added to each plate. The bacterial suspension was diluted to obtain 3.5×10^8 CFU/ml based on spectrometric absorbance (0.18 optical density at 590 nm).

This suspension was used for DNA extraction using a similar methodology used for leaf diffusates. One ml of the suspension was transferred to 1.5 ml tubes and centrifuged at 9000 g

for 5 min, the supernatant was discarded, and the bacterial pellets were suspended in 1 ml of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl, 1% Tween 20, pH 8.3). Genomic DNA for qPCR was prepared by lysing the cells in the suspension at 95-100 °C for 15 min and immediately incubating the samples on ice for 10 min (Jacobs, *et al*. 2008). The bacterial DNA was diluted and used to generate a standard curve for the qPCR experiments.

2.2.2 qPCR conditions

Previously, Garces (2011) designed sets of primers for SYBR Green and TaqMan qPCR from the *X. albilineans* genome sequence using the program Beacon Designer (Premier Biosoft International, Palo Alto, CA). The gene cluster of albicidin bio-synthesis corresponding to the *alb*I gene was targeted for *X. albilineans* specific primers. A TaqMan Double-Quenched ProbeTM (5'FAM/ZEN/3'ABkFQTM) with two quenchers, ZEN and ABkFQ, and the FAM reporter was developed following the manufacturer instructions (IDT Integrated DNA Technologies, Skokie, IL).

Amplification conditions for SYBR Green qPCR

From each sample of DNA, 2 µl were mixed with 7.5 µl of SYBR Green master mix (2X), 0.1 µl of each forward and reverse primer (100 µM), and 5.3 µl of sterile, distilled water. The conditions of amplification were as follows: an initial step at 95°C for 10 min, followed by 40 cycles of DNA denaturation at 94°C for 10 s, and annealing-polymerization at 60°C for 30 s. The melting curve analysis consisted of 81 cycles with step-wise increases in set point temperature after cycle 2 by 0.5°C from 55 to 95°C for 30 s. A 10-fold dilution series of Xa DNA extracted from a 3.5 x 10^7 CFU/ml suspension was diluted five times to be used as

standards with concentrations from 10^7 to 10^3 CFU/ml to determine the qPCR amplification efficiency, cycle threshold value (CT), and concentration of unknown samples.

Amplification conditions for TaqMan qPCR

From each sample of DNA, 2 µl were mixed with 10 µl of TaqMan universal master mix (Roche, Basel, Switzerland), 1 µl each of forward and reverse primers (10 uM), and TaqMan double-quenched probe XaQ (2 µM), and 5 µl of sterile, distilled water. The conditions of amplification were as follows: an initial step at 50°C for 10 min., a second step of 95°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 15 s, and annealing-polymerization at 60°C for 1 min.

Positive control samples for qPCR experiments were DNA from Xa culture or DNA from diffusates from leaves collected from plants showing symptoms of leaf scald that previously tested positive for *X. albilineans*. Negative control samples were diffusates from known non-infected plants. A no template sample (NTS) consisting of purified water was always included. All controls were added to the reaction plate in triplicate wells for all experiments.

2.2.3 Specificity of *X. albilineans* detection study

Different bacteria and a fungus associated with sugarcane or bacteria related to Xa (Table 2.1) were tested for amplification by the qPCRs to determine assay specificity for detection and quantification of Xa populations. In addition, some unidentified bacteria that grew on the XAS semi-selective media were tested for amplification by the qPCRs.

For the evaluation of specificity, DNA was extracted from the different bacteria and fungus using the same method used for the DNA extraction of Xa (for *Xanthomonas oryzae*, the DNA was provided by the USDA). Positive controls were bacteria isolated and cultured on semi-

selective medium. The DNA extraction for all the samples were based in the boiling-lysis method previously described. Total DNA extracted was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Table 2.1. Microorganisms included in the specificity test of the TaqMan and SYBR Green qPCRs

Source of DNA	Taxonomic group	Relevant features
Cryptococcus albidus	Fungi (Basidiomycota)	Isolated from sugarcane leaf surface
Burkholderia gladioli	Bacteria (Beta Proteobacteria)	Plant pathogen in onions, gladiolus, iris and rice
Tanticharoenia sakaeratensis	Bacteria (Alpha Proteobacteria)	Osmotolerant acetic acid bacteria isolated from sugarcane
Asaia bogorensis	Bacteria (Alpha Proteobacteria)	Ultrafine cellulose producer isolated from sugarcane
Pantoea ananatis	Bacteria (Gamma Proteobacteria)	Common epiphyte, plant pathogen in mono and dicotyledonous isolated from sugarcane
Xanthomonas oryzae	Bacteria (Gamma Proteobacteria)	Plant pathogen, rice blight disease
Gluconacetobacter liquefaciens	Bacteria (Alpha Proteobacteria)	Isolated from sugarcane
Herbaspirillum rubrisubalbicans	Bacteria (Beta proteobacteria)	Causal agent of mottled stripe disease of sugarcane and sorghum
Leifsonia xyli* subsp. *xyli	Bacteria (Actinobacteria)	Causal agent of ratoon stunt of sugarcane

2.2.4 Evaluation of *X. albilineans* extraction efficiency from plant tissue

The method used for extracting Xa from plant tissue is diffusion of the bacteria from the tissue into water at 4°C overnight. The diffusate obtained is the starting point for bacterial DNA extraction and is then a critical step in the qPCR for Xa. Therefore, the effects of different factors, such as the tissue source and the cultivar extracts, on the efficiency of extraction of Xa needed to be evaluated to validate the use of qPCR for the evaluation of resistance.

The Xa concentration in leaf diffusates and the bacteria concentration remaining in the plant tissue were compared for two susceptible cultivars, HoCP 85-845 and HoCP 89-846,

varying in degree of resistance to leaf scald. Plants of the cultivars were inoculated with a suspension of Xa (10^8 CFU/ml) in the greenhouse by cutting the shoot above the apical meristem and applying bacteria to the cut surface. After 10 weeks, tissue of the second youngest not yet fully emerged leaf above the youngest fully emerged leaf known as the top visible dewlap (TVD) leaf and designated as the TVD-2 leaf was collected from inoculated and control (non-inoculated) plants. Bacteria and DNA were extracted as described previously. *Xanthomonas albilineans* concentrations were evaluated using TaqMan qPCR. However, the leaf discs used for bacterial diffusion were not discarded. The leaf discs were washed with tap water and macerated with a mortar and pestle in DNA extraction buffer (0.05 M KCl, 0.01 M Tris-HCl, 1% Tween 20, pH 8.3) to release remaining bacteria in the tissue. After that, DNA was extracted from both diffusate types using the same method as described previously and subjected to qPCR.

DNA concentrations in the two diffusate types were compared for both cultivars using a t-test performed using SAS software v. 9.3 (SAS Institute Inc., Cary, NC, USA).

2.2.5. Inhibition of qPCR amplification study

Pure DNA extracts containing no inhibitors to PCR is critical for precise determination of the bacterial population in the sugarcane tissues (Gao, *et al*., 2004). The reaction inhibition can be total or partial and can manifest itself as a complete reaction failure or as reduced sensitivity (Gao, *et al*., 2004). For the evaluation of the presence of inhibitors in the sugarcane tissues after the DNA extraction, four sugarcane cultivars were used, LCP 85-384, Ho 95-988, HoCP 85-845, and HoCP 89-846. According to previous visual symptom severity evaluations, LCP 85-384 and Ho 95-988 are resistant and HoCP 85-845 and HoCP 89-846 are susceptible to leaf scald. Plants not inoculated with *X. albilineans* were evaluated using SYBR Green qPCR, TaqMan qPCR, and culture on semi-selective XAS media to demonstrate the absence of Xa in the tissues. Leaf tissue

was collected as for the qPCR assay from the TVD-2 leaf. Samples consisting of a 10 cm section collected from the leaf base were placed on ice and kept at 4°C until processing.

Leaf diffusate was obtained by immersion of 20 discs of leaf tissue 6 mm in diameter in sterile distilled water overnight. Leaf diffusates were transferred to 1.5 ml tubes and centrifuged at 9000 g for 5 min, the supernatant was discarded, and the bacterial pellets were suspended in 100 µl of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl, 1% Tween 20, pH 8.3). Genomic DNA for qPCR was prepared by lysing the cells in the suspension at 95-100°C for 15 min and immediately incubating the samples on ice for 10 min (Jacobs, *et al*. 2008). The DNA extracts from each of the four cultivars were used to suspend three different DNA concentrations of Xa, 3.5×10^8, 3.5×10^6, and 3.5×10^4 CFU/ml. The different dilutions were compared to similar dilutions using distilled, deionized, sterile water as control.

The selected Xa concentrations reflect high (3.5×10^8, a bacteria concentration similar to that found in infected susceptible cultivars), medium (3.5×10^6) and low (3.5×10^4, a concentration near the detection threshold of the qPCR) concentrations of bacteria in the sample. The CT value (threshold cycle value) was used to compare the controls to the different DNA extract treatments. Differences higher than one CT value between the appropriate water control and the treatments would be attributed to possible inhibition (Gao, *et al*. 2004). The CT values also were compared in an ANOVA with three repetitions for each treatment. Analysis of Variance was performed using SAS software v. 9.3 (SAS Institute Inc., Cary, NC, USA).

2.3 RESULTS

2.3.1 Specificity of SYBR Green and TaqMan qPCR for *X. albilineans* detection

The specificity of the SYBR Green and TaqMan qPCRs was determined using sources of DNA from different bacteria and a fungus isolated from sugarcane tissues or other bacteria for amplification. Positive amplification occurred only with DNA samples of Xa from culture or isolated from symptomatic leaf tissue of sugarcane. All the other species evaluated showed negative amplification for both kinds of qPCR (Table 2.2).

Table 2.2. Specificity of the TaqMan and SYBR Green qPCRs for amplification of *X. albilineans*

Source of DNA	**DNA concentration (ng/µl)**[a]	**TaqMan qPCR**		**SYBR green qPCR**	
		CT value[b] (average)	CFU[c]/ml (average)	CT value (average)	CFU/ml (average)
Cryptococcus albidus	87.4	NA[d]	0	NA	0
Burkholderia gladioli	111.2	NA	0	NA	0
Tanticharoenia sakaeratensis	124.4	NA	0	NA	0
Asaia bogorensis	215.3	NA	0	NA	0
Pantoea ananatis	409.4	NA	0	NA	0
Xanthomonas oryzae	202.3	NA	0	NA	0
Gluconacetobacter liquefaciens	186.8	NA	0	NA	0
Herbaspirillum rubrisubalbicans	126.0	NA	0	NA	0
Leifsonia xyli* subsp. *Xyli	536.5	NA	0	NA	0
Xanthomonas albilineans[e]	60.2 - 154.8	20.69	2.88×10^9	26.91	6.92×10^6

[a] For qPCR, all the organisms evaluated, except *X. albilineans*, were tested at different DNA concentrations (25 ng/µl, 50 ng/µl and the concentration obtained after the DNA extraction).
[b] CT value = Ct or threshold cycle is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
[c] CFU = colony-forming unit is an estimate of viable bacterial or fungal numbers.
[d] NA = no amplification. These samples did not reach the threshold.
[e] For *X. albilineans*, five different samples from diffusates of leaves from plants with symptoms were evaluated.

2.3.2 Evaluation of efficiency of *X. albilineans* extraction from leaf tissue

The Xa concentration in the initial DNA extraction was always higher than the bacterial concentration detected remaining in the tissue after extraction for both susceptible cultivars (Table 2.3). In some cases, the remaining tissue of initial extraction positive samples was negative in the qPCR for the detection of Xa. The calculated proportion of bacteria remaining in

the tissue ranged between 0 and 0.32. Generally, the remaining tissue concentrations were low (Table 2.3), and they were significantly different (Table 2.4).

Table 2.3. Comparison of the *Xanthomonas albilineans* concentrations in the initial extraction and a second extraction of the remaining tissue

Cultivar	Sample	Initial concentration (CFU/ml)	Concentration in the remaining tissue (CFU/ml)	Proportion in the remaining tissue[a]
HoCP89-846	1	1.82×10^8	2.34×10^7	0.11
	2	1.16×10^8	1.66×10^7	0.13
	3	1.35×10^7	2.16×10^6	0.14
	4	1.95×10^6	6.56×10^5	0.25
	5	2.89×10^5	0	0
	6	1.77×10^6	8.23×10^5	0.32
	7	1.06×10^6	0	0
	8	8.63×10^6	3.52×10^6	0.29
	9	4.03×10^5	0	0
	10	1.76×10^6	0	0
	11	8.86×10^5	0	0
Mean		2.98×10^7	4.29×10^6	0.13
HoCP85-845	1	1.72×10^{10}	1.89×10^7	0.00
	2	2.41×10^6	0	0
	3	6.02×10^9	8.89×10^7	0.01
	4	9.07×10^8	5.25×10^6	0.01
	5	1.24×10^9	8.54×10^6	0.01
	6	6.03×10^9	1.29×10^7	0.00
	7	6.64×10^9	1.87×10^7	0.00
	8	1.73×10^6	0	0
	9	2.62×10^9	2.64×10^7	0.01
	10	5.10×10^8	3.12×10^6	0.01
	11	6.77×10^8	1.17×10^7	0.02
	12	8.26×10^9	1.71×10^7	0.00
	13	7.55×10^7	0	0
	14	1.26×10^7	3.82×10^6	0.23
	15	2.15×10^7	9.20×10^6	0.30
	16	4.35×10^6	3.64×10^5	0.08
	17	7.55×10^6	2.16×10^6	0.22
Mean		2.95×10^9	1.25×10^7	0.00

[a] The proportions are calculated by dividing the remaining tissue bacteria concentration by the initial bacteria concentration plus the remaining concentration in the same tissue.

Table 2.4. Paired t-test analysis and non-parametric t-test options comparing the initial *X. albilineans* DNA concentration and the concentration of bacteria DNA in the remaining tissue

Test	Statistic	*P*
Shapiro-Wilk (Normality)	0.54	< 0.0001
Paired *t*-test	2.48	0.0199
Paired sample sign[a]	14	< 0.0001
Wilcoxon signed rank[a]	203	< 0.0001

[a] Alternative non-parametric methods.

In the paired t-test analysis, the difference between the initial extract concentration and the concentration in the remaining tissue was different than zero (t= 0.54, P = 0.0199). However, the use of a paired t-test for these data is not advisable based on the result of the Shapiro-Wilk test. The Shapiro-Wilk rejects the null hypothesis of normality in the data (Shapiro-Wilk = 0.54, $P < 0.0001$), and normality is an important assumption for the use of t-test. For that reason, a non-parametric analysis for paired data was performed as the paired sample sign test (based in the sign of the difference) and the Wilcoxon signed rank test (based in the sign and the magnitude of the difference). With both tests, the results reject the null hypothesis that the difference is equal to zero ($P < 0.0001$), confirming that the bacteria concentrations of the initial extraction and the concentrations in the remaining tissue were different, and these concentrations were always higher in the initial DNA extraction.

2.3.3 Inhibition of *X. albilineans* amplification by plant DNA extracts in SYBR Green and TaqMan qPCRs

To determine whether inhibitors of DNA amplification are present when using the simple method for DNA extraction, extracts from four different cultivars without Xa infection (LCP 85-384, Ho 95-988, HoCP 85-845, and HoCP 89-846) were used to dilute Xa before qPCR. Extracts from all four cultivars were first tested with qPCR to demonstrate the absence of Xa DNA, and all were negative. The results are shown for one TaqMan qPCR plate for LCP85-384, Ho95-988

and HoCP85-845 (Table 2.5) and HoCP89-846 (Table 2.6). However, all the experiments were replicated three times, and the results were similar. The differences between the CT values of the treatments and the water dilution controls were never higher than 1, and there were no significant differences among the treatments.

For SYBR Green qPCR, the results were similar to the TaqMan qPCR in single qPCR plates for LCP 85-384, Ho 95-988, and HoCP 89-846 (Table 2.7) and another plate for HoCP 85-845 (Table 2.8). Differences among the CT values of the treatments and the respective controls were never higher than 1, and no differences were detected.

Table 2.5. Inhibition of TaqMan qPCR amplification by DNA extracts of cultivars LCP85-384, Ho95-988 and HoCP85-845 with three concentrations of *Xanthomonas albilineans*

DNA source and concentration	**Mean CT[a] value (SD[b])**	**Concentration in CFU/ml (SD[c])**	**CT difference (ΔCT value)[d]**
Concentration of DNA = 3.5 x 10^8 CFU/ml (ANOVA p-value = 0.5771[e])			
LCP85-384	22.69 (0.1692)	1.39 x 10^8 (1.44 x 10^7)	-0.24
Ho95-988	23.06 (0.4021)	1.13 x 10^8 (2.57 x 10^7)	0.13
HoCP85-845	22.82 (0.1833)	1.29 x 10^8 (1.37 x 10^7)	-0.11
H_2O control	22.93 (0.4635)	1.23 x 10^8 (3.17 x 10^7)	
Concentration of DNA = 3.5 x 10^6 CFU/ml (ANOVA p-value = 0.2826[e])			
LCP85-384	27.77 (0.2285)	6.66 x 10^6 (9.54 x 10^5)	0.31
Ho95-988	27.75 (0.1127)	6.72 x 10^6 (4.60 x 10^5)	0.29
HoCP85-845	27.64 (0.0764)	7.18 x 10^6 (3.18 x 10^5)	0.18
H_2O control	27.46 (0.2950)	8.10 x 10^6 (1.42 x 10^6)	
Concentration of DNA = 3.5 x 10^4 CFU/ml (ANOVA p-value = 0.1952[e])			
LCP85-384	38.15 (0.2511)	1.34 x 10^4 (2.02 x 10^3)	0.46
Ho95-988	37.63 (0.4579)	1.87 x 10^4 (5.44 x 10^3)	-0.06
HoCP85-845	37.58(0.0896)	1.89 x 10^4 (1.00 x 10^3)	-0.11
H_2O control	37.69 (0.5424)	1.82 x 10^4 (5.56 x 10^3)	

[a] CT or threshold cycle is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
[b] SD or standard deviation of the CT values mean. All the experiments were performed in triplicate.
[c] SD or standard deviations of the concentration in CFU/ml. The value in parenthesis reflects the SD of the concentrations means. All the experiments were performed in triplicate.
[d] Difference between the CT value means of the different treatments and the CT mean of the water control consisting of bacteria diluted in sterile, distilled water.
[e] ANOVA or analysis of variance calculated from the concentration values of the different treatments. The results did not show differences between the treatments in the concentrations evaluated.

Table 2.6. Inhibition of TaqMan qPCR amplification by DNA extracts of HoCP89-846[a] with three DNA concentrations of *Xanthomonas albilineans*

DNA source and concentration	Mean of CT value[b] (SD[c])	Concentration in CFU/ml (SD[d])	Difference of the CT value (ΔCT value)[e]
Concentration of DNA: 3.5 x 10^8 CFU/ml			
HoCP89-846	23.82 (0.6191)	2.07 x 10^8 (8.10 x 10^7)	-0.61
H_2O control	24.43 (0.2060)	1.24 x 10^8 (1.92 x 10^7)	0.00
Concentration of DNA: 3.5 x 10^6 CFU/ml			
HoCP89-846	28.41 (0.2303)	6.85 x 10^6 (3.51 x 10^6)	0.17
H_2O control	28.24 (0.6366)	7.26 x 10^6 (1.25 x 10^6)	0.00
Concentration of DNA: 3.5 x 10^4 CFU/ml			
HoCP89-846	35.95 (0.8650)	2.61 x 10^4 (1.60 x 10^4)	-0.01
H_2O control	35.96 (0.2926)	2.31 x 10^4 (5.31 x 10^3)	0.00

[a] HoCP89-846 analysis was performed in a different PCR plate due to space limitation in the PCR plate.
[b] CT or threshold cycle is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
[c] SD or standard deviation of the CT values mean. All the experiments were performed in triplicate.
[d] SD or standard deviations of the concentration in CFU/ml. The value in parenthesis reflects the SD of the concentrations means. All the experiments were performed in triplicate.
[e] Difference between the CT value means of the different treatments and the CT mean of the water control consisting of bacteria diluted in sterile, distilled water.

Table 2.7. Inhibition of SYBR Green qPCR amplification by DNA extracts of extracts of cultivars LCP85-384, Ho95-988 and HoCP85-846 with three concentrations of *Xanthomonas albilineans*

DNA source and concentration	Mean of CT value[a] (SD[b])	Concentration in CFU/ml (SD[c])	Difference of the CT value (ΔCT value)[d]
Concentration of DNA: 3.5 x 10^8 CFU/ml (ANOVA p-value = 0.1849[e])			
LCP85-384	21.89 (0.2491)	3.74 x 10^7 (7.09 x 10^6)	0.53
Ho95-988	22.03 (0.3365)	3.41 x 10^7 (7.64 x 10^6)	0.67
HoCP89-846	21.87 (0.4713)	3.89 x 10^7 (1.31 x 10^7)	0.51
H_2O control	21.36 (0.4574)	5.59 x 10^7 (1.66 x 10^7)	0.00
Concentration of DNA: 3.5 x 10^6 CFU/ml (ANOVA p-value = 0.0565[e])			
LCP85-384	23.22 (0.5221)	1.50 x 10^7 (6.01 x 10^6)	0.26
Ho95-988	23.03 (0.0819)	1.64 x 10^7 (9.17 x 10^5)	0.07
HoCP89-846	23.09 (0.1723)	1.60 x 10^7 (1.21 x 10^6)	0.13
H_2O control	22.96 (0.0666)	1.72 x 10^7 (8.25 x 10^5)	0.00
Concentration of DNA: 3.5 x 10^4 CFU/ml (ANOVA p-value = 0.6064[e])			
LCP85-384	32.75 (0.2409)	1.58 x 10^4 (2.81 x 10^3)	0.18
Ho95-988	33.27 (0.4657)	1.11 x 10^4 (3.72 x 10^3)	0.70
HoCP89-846	33.42 (0.4579)	9.94 x 10^3 (2.89 x 10^3)	0.85
H_2O control	32.57 (1.1915)	2.17 x 10^4 (1.44 x 10^4)	0.00

[a] CT or threshold cycle is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
[b] SD or standard deviation of the CT values mean. All the experiments were performed in triplicate.
[c] SD or standard deviations of the concentration in CFU/ml. The value in parenthesis reflects the SD of the concentrations means. All the experiments were performed in triplicate.

[d] Difference between the CT value means of the different treatments and the CT mean of the water control consisting of bacteria diluted in sterile, distilled water.
[e] ANOVA or analysis of variance calculated from the concentration values of the different treatments. The results did not show differences between the treatments in the concentrations evaluated.

Table 2.8. Inhibition of SYBR Green qPCR amplification by DNA extracts of extracts of cultivars Ho95-988 and HoCP85-845 with three concentrations of *Xanthomonas albilineans*

DNA source and concentration	Mean of CT value[a] (SD[b])	Concentration in CFU/ml (SD[c])	Difference of the CT value (ΔCT value)[d]
Concentration of DNA: 3.5 x 10^8 CFU/ml (ANOVA p-value = 0.3282[e])			
Ho95-988	21.68 (0.1007)	2.49 x 10^8 (1.72 x 10^7)	0.18
HoCP85-845	21.70 (0.0115)	2.44 x 10^8 (1.73 x 10^6)	0.20
H_2O control	21.50 (0.2627)	2.82 x 10^8 (4.87 x 10^7)	0.00
Concentration of DNA: 3.5 x 10^6 CFU/ml (ANOVA p-value = 0. 0949[e])			
Ho95-988	27.14 (0.2042)	5.83 x 10^6 (7.81 x 10^5)	0.18
HoCP85-845	27.43 (0.1980)	4.76 x 10^6 (6.65 x 10^5)	0.47
H_2O control	26.96 (0.2386)	6.62 x 10^6 (1.12 x 10^6)	0.00
Concentration of DNA: 3.5 x 10^4 CFU/ml (ANOVA p-value = 0.2860[e])			
Ho95-988	34.99 (0.4309)	2.69 x 10^4 (8.48 x 10^3)	0.30
HoCP85-845	35.38 (0.4027)	2.06 x 10^4 (5.80 x 10^3)	0.15
H_2O control	34.76 (0.3161)	3.11 x 10^4 (6.61 x 10^3)	0.00

[a] CT or threshold cycle is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction.
[b] SD or standard deviation of the CT values mean. All the experiments were performed in triplicate.
[c] SD or standard deviations of the concentration in CFU/ml. The value in parenthesis reflects the SD of the concentrations means. All the experiments were performed in triplicate.
[d] Difference between the CT value means of the different treatments and the CT mean of the water control consisting of bacteria diluted in sterile, distilled water.
[e] ANOVA or analysis of variance calculated from the concentration values of the different treatments. The results did not show differences between the treatments in the concentrations evaluated.

2.4 DISCUSSION

Garces (2011) developed molecular techniques (qPCR) for the amplification of a sequence in the albicidin toxin gene cluster that can be used for the quantification of *Xanthomonas albilineans*, the causal agent of leaf scald of sugarcane. Experiments comparing Xa populations in two highly resistant and two highly susceptible cultivars suggested that the qPCR could be a reliable method to evaluate leaf scald resistance (commonly assessed by the disease severity). This approach was based on the previous report that the *X. albilineans* population in the shoot apex was highly correlated with disease severity (Rott, *et al.*, 1997).

However, additional steps were needed to validate the ability of the qPCR to accurately quantify in-plant Xa populations before generalized use in epidemiological and host plant resistance studies. These steps included demonstration of the specificity of target organism amplification, pathogen extraction efficiency, and lack of PCR inhibitors in the amplification mixture.

Specificity was demonstrated by the complete lack of amplification of different species of bacteria and a fungus associated with the sugarcane or other bacteria. The results show that the qPCRs based on TaqMan detection or SYBR green are very specific and only detect Xa. These results support the use of both qPCRs for the quantification of Xa as a method for the evaluation of resistance to the pathogen.

A simple, inexpensive DNA extraction method is desirable for a technique that is intended to be used for testing large numbers of samples. However, the possibility of PCR inhibitors in the samples needs to be evaluated for a direct, absolute quantification PCR assay. It should be emphasized that DNA extracts containing no inhibitors to PCR is critical for precise comparison of bacterial DNA quantities in leaves, stem and meristem samples (Gao, *et al*. 2004). The lack of any differences in CT values for low, medium, and high Xa concentrations suspended in leaf extracts from four different cultivars compared to bacteria suspended in water controls indicate the absence of inhibitors in the samples. The CT value used to determine concentration has exponential behavior. The difference in the CT values of 1 was selected from a previous study (Gao, *et al*. 2004). Statistically, the data did not show differences among the different treatments for each Xa concentration evaluated. There was no trend evident for lower Xa quantification values when bacteria were amplified from samples containing leaf extracts. This indicates that both types of qPCR evaluated, SYBR Green and TaqMan, can be used to accurately quantify Xa concentrations in sugarcane leaf tissue in an absolute manner.

Another factor that might affect the evaluation of the DNA concentration in samples prepared from plant tissue is the possibility that the quantity of bacteria released to the water can be limited by plant and infection characteristics (such as biofilm formation on the host tissue). Pathogen extraction efficiency could affect the evaluation of resistance based on quantification of bacteria present in the sample if the quantity extracted does not accurately reflect the quantity present in the tissues. The comparison of bacteria in the initial extraction and remaining in the leaf tissue using TaqMan qPCR for quantification demonstrated that bacteria were efficiently extracted by the leaf disc diffusion method.

The study results indicate high specificity for Xa detection with SYBR Green and TaqMan qPCRs, efficient extraction of bacteria from leaf tissues, and an absence of amplification inhibitors in sugarcane extracts. This supports the use of the boiling-lysis method for the extraction of DNA from sugarcane leaf diffusates and absolute quantification of Xa concentrations with qPCR. The accurate quantification of Xa in infected leaves could be used to evaluate resistance to leaf scald in sugarcane.

CHAPTER 3: QUANTITATIVE PCR OF *XANTHOMONAS ALBILINEANS* IN SUGARCANE TISSUES AS METHODOLOGY FOR MONITORING RESISTANCE

3.1 INTRODUCTION

Leaf scald is a major disease of sugarcane with worldwide distribution caused by the bacterium, *Xanthomonas albilineans* (Ashby) Dowson (Rott and Davis, 2000). The disease can be a serious problem due to high losses in tons of cane per hectare and reduced juice quality (Hoy and Grisham, 1994; Ricaud & Ryan, 1989; Rott & Davis, 2000). *Xanthomonas albilineans* can cause three different phases of infection and symptomatology on sugarcane: latent (no symptoms), chronic, and acute (Ricaud & Ryan, 1989; Rott & Davis, 2000; Saumtally & Dookun, 2004). Symptom expression and severity are associated with the level of cultivar resistance, environmental conditions, and pathogen aggressiveness.

The chronic phase is characterized by symptoms that vary in severity, including bleached or chlorotic longitudinal streaks along leaf veins termed ‘pencil lines’, leaf chlorosis and/or bleaching, leaf necrosis, development of abnormal side shoots exhibiting symptoms on stalks, reddish discoloration of vascular bundles at the node level, stunting, wilting, and death (Ricaud & Ryan, 1989; Rott & Davis, 2000; Saumtally & Dookun, 2004). The acute phase occurs as a sudden wilting of plants resulting in death, with little or no previous symptom expression. Large areas of a field planted with a highly susceptible cultivar may be affected in this manner following a period of drought stress (Ricaud & Ryan, 1989; Rott & Davis, 2000; Saumtally & Dookun, 2004). The latent phase occurs and ends for reasons which are unknown (Rott & Davis, 2000). Detection of the disease is difficult when infection is latent, and this resulted in worldwide spread of leaf scald during sugarcane germplasm exchanges (Daugrois, *et al*., 2003).

Host plant resistance is the most important leaf scald control method (Ricaud & Ryan, 1989; Rott and Davis, 2000). Resistance levels are determined for clones in selection programs by assigning a numerical rating based on the severity of systemic infection symptoms following inoculation. The decapitation method, in which young shoots are cut above the apical meristem and bacterial inoculum is applied to the cut surface, is used for inoculation (Koike, 1965). However, accurate evaluation of resistance levels in sugarcane clones is difficult since reactions obtained from field inoculations are erratic. In addition, subjective rating systems based on symptom severity can be affected by variability among raters.

Resistance has been associated with the extent of bacterial colonization (Rott, *et al*., 1994; Rott, *et al*., 1997). Disease severity and bacteria concentration in the shoot apex were found to be correlated (Rott, *et al*., 1997). Susceptible cultivars were always extensively colonized in the apex and lower part of the stalk, whereas *X. albilineans* (Xa) populations in the shoot apex were low in cultivars considered resistant based on phenotype evaluation. These results suggested a method to accurately detect and compare bacterial populations in different sugarcane genotypes might provide an alternative method for resistance screening.

Real-time, quantitative PCR (qPCR) is a sensitive, reproducible and accurate method that is used for qualitative and quantitative analysis of nucleic acids (Ginzinger, 2002; Higuchi, *et al*., 1993). Real-time PCR assays have been developed for the detection of pathogens causing other systemic sugarcane diseases, including yellow leaf, caused by *Sugarcane yellow leaf virus* (Korimbocus, *et al*., 2002; Yun, *et al*., 2010), and ratoon stunt, caused by *Leifsonia xyli* subsp. *xyli* (Grisham, *et al*., 2007). The potential for high sensitivity and specificity could make qPCR a superior method for reliable detection and quantification of Xa.

Previously, a quantitative polymerase chain reaction (qPCR) was developed with demonstrated potential for leaf scald resistance screening (Garces, *et al.*, 2014). TaqMan and SYBR Green PCR assays were developed utilizing primers from the bacterium-specific albicidin toxin gene cluster. The qPCR assays for Xa detection were faster and more sensitive than conventional PCR (Garces, *et al.*, 2014). However, only six cultivars with extreme reaction against the disease (three susceptible and three resistant) were compared. Therefore, additional research was needed to demonstrate the utility of this method for determining and comparing the resistance levels of larger more diverse clone populations in selection and resistance studies. The determination of the best time after artificial inoculation to sample, the plant tissue best able to distinguish differences in bacterial population among clones with variable resistance levels, a comparison of composite versus single sample collection, the comparison between field and greenhouse inoculations, and finally, the comparison of multiple qPCR results with the visual rating system were all study objectives with the overall goal of determining whether qPCR can provide a more reliable alternative leaf scald resistance screening method.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial isolation and plant inoculation

Bacteria were isolated from a longitudinal section of leaf with a pencil-line symptom. Tissue was surface-sterilized with NaOCl (0.5%) for 30 s and rinsed with water. The leaf sections were dried, cut in small pieces, and placed in a 1.5 ml microcentrifuge tube (Eppendorf, Hauppauge, NY) containing 1 ml of sterile, distilled water. The tubes were incubated overnight at 4°C. A loop of bacterial suspension was transferred to semi-selective XAS medium (Davis, *et al*., 1994) and incubated at 28°C. After 5-8 days, single colonies were selected and streaked to obtain pure cultures on solid XAS medium without antibiotics (Davis, *et al*., 1994). Pure cultures

were incubated at 28°C, and after 48 h, 5 ml of sterile water were added to each plate. The bacterial suspension was diluted to obtain 10^8 CFU/ml based on spectrometric absorbance (0.18 optical density at 590 nm). The bacterial suspension was used for standard curve construction in the qPCR and for inoculation of plants in the field and greenhouse.

For inoculations in the greenhouse and field, the bacterial suspension was kept on ice prior to inoculation. In both greenhouse and field experiments, approximately 2-month-old plants were inoculated using the decapitation method (Koike, 1965). Plants were inoculated by placing 100 µl of bacterial suspension with a micropipette on the surface of a cut made above the apical meristem with scissors dipped in the inoculum suspension. Twenty plants per cultivar were inoculated in the field experiments and four plants per cultivar were inoculated in the greenhouse. Inoculations were done at the end of the day at about sunset.

The greenhouse experiment was performed between November of 2011 and February of 2012. Field inoculations were performed in the summer of 2011 and the summer of 2012. In the summer of 2011, two different inoculations were performed using the same sugarcane planting; the first inoculation was performed on 16 June and the second inoculation was on 1 July. The inoculation in the summer of 2012 was performed on 28 May.

3.2.2 Plant material and sample collection

Thirtyone sugarcane clones were included in field and greenhouse experiments (Table 3.1) to compare quantification of Xa by TaqMan qPCR and rating resistance based on symptom severity (Figure 3.1). The clones included three known leaf scald resistant clones, LCP 85-384, Ho 95-988, and HoCP 96-540, and two susceptible clones, HoCP 85-845 and HoCP 86-849, as checks. To determine the best source of tissue for distinguishing resistant and susceptible clones

by qPCR, different plant tissues were sampled and compared. The base of the second youngest leaf above the youngest fully emerged leaf that is known as the top visible dewlap (TVD) leaf designated as TVD – 2 was collected at 4, 8, and 12 weeks after inoculation (WAI). The leaf tissue was collected individually (one leaf per sample) or as a composite sample of three TVD - 2 leaves each collected from a different plant. Stem sections containing the apical meristem were collected 12 WAI; the stem base also was collected at 12 WAI. Meristem and stem base samples were collected in plastic bags and leaf sections in 50 ml centrifuge tubes then placed on ice and kept at 4°C until processing in the laboratory. Between samples, scissors and shears were surface sterilized by dipping in 95% ethanol and flaming.

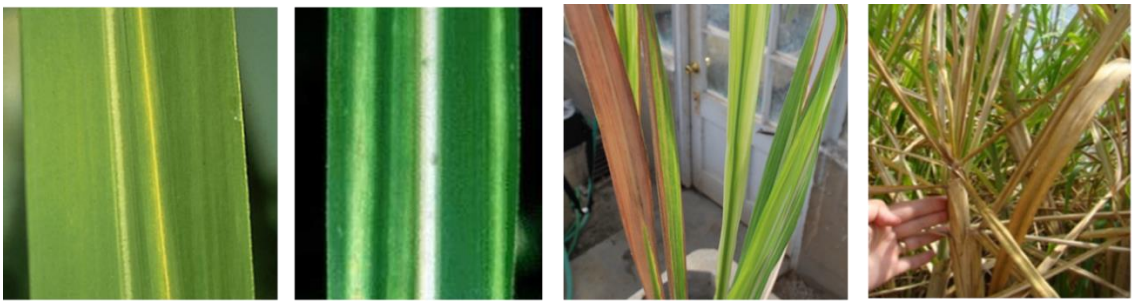
Leaf diffusates were obtained by immersion of 20 discs of tissue 6 mm in diameter in sterile distilled water overnight. Sap for the base stem tissue was collected by centrifugation of a small cylinder of stem tissue (1 cm in height and 0.8 cm in diameter) in 1.5 ml microcentrifuge tubes. Leaf diffusates and sap samples were transferred to 1.5 ml tubes, and the product was centrifuged at 9000 g for 5 min, the supernatant was discarded, and bacterial pellets were suspended in 100 µl of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl, 1% Tween 20, pH 8.3). Genomic DNA for PCR was prepared by lysing the cells at 95°C – 100°C for 15 min and immediately incubating the samples on ice for 10 min (Jacobs, *et al*. 2008). The DNA from the meristem was extracted by macerating 100 mg of tissue in a mortar containing 1.5 ml of AP1 buffer from the Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) then following the manufacturer's instructions.

3.2.3 Disease evaluation

Disease severity in the greenhouse and field experiments was evaluated according to the type of symptoms observed in a determined number of stalks (three to four in the greenhouse and

10 to 15 in the field experiments). Disease severity was assessed at different time points in field experiments: 2 WAI (or 4 WAI in the 2012 inoculation), 8 WAI, and 12 WAI. Rating of visual symptoms in the field and greenhouse was performed using a 1, 3, 5, 7 and 9 rating system (Figure 3.1) for which 1-3 would be considered resistant, 4-6 would be rated moderately susceptible, and 7-9 would be rated highly susceptible. However, at 2 or 4 WAI when disease could only be assessed on inoculated leaves, severity was assessed with a rating scale of 1 to 5 designed to reflect the range of different symptoms in inoculated leaves. For the inoculated leaf scale, 1 was local necrosis at ends of inoculated leaves, 2 was local necrosis plus one or two pencil lines, 3 was the presence of multiple pencil lines, 4 was multiple pencil lines plus extensive necrosis, and 5 was near total leaf necrosis. The assessment was performed using the TVD - 2 leaf, and a rating mean was calculated for each clone. For the greenhouse experiment, assessment was done at 8, 10 and 12 WAI.

Figure 3.1. Leaf scald resistance rating system using a 1(no symptoms), 3, 5, 7 and 9 rating scale for field and greenhouse systemic resistance evaluation 4 to 12 weeks after inoculation.



3.2.4 TaqMan qPCR conditions

Previously, Garces (2011) designed a set of primers for TaqMan qPCR from the Xa genome sequence using the program Beacon Designer (Premier Biosoft International, Palo Alto,

CA). The gene cluster of albicidin bio-synthesis corresponding to the *alb*I gene was targeted for Xa specific primers. A TaqMan Double-Quenched ProbeTM (5'FAM/ZEN/3'ABkFQTM) with two quenchers, ZEN and ABkFQ, and the FAM reporter was developed following the manufacturer instructions (IDT Integrated DNA Technologies, Skokie, IL).

From each sample of DNA, 2 µl were mixed with 10 µl of TaqMan universal master mix (Roche, Basel, Switzerland), 1 µl of each forward and reverse primers (10 uM), and TaqMan double-quenched probe XaQ (2 µM) (IDT Integrated DNA Technologies), 5 µl of sterile, distilled water for a final volume of 20 µl. The conditions of amplification were as follows: an initial step at 50°C for 10 min., a second step of 95°C for 2 min, followed by 35 cycles of DNA denaturation at 94°C for 15 s, and annealing-polymerization at 60°C for 1 min.

Positive control samples for all PCR experiments were diffusates from leaves collected from plants showing symptoms of leaf scald that previously tested positive for *X. albilineans*. Negative control samples were diffusates from known non-infected plants. A no-template sample (NTS) consisting of purified water was always included. All controls were added to the reaction plate in triplicate wells for all experiments.

A standard curve was constructed using the same concentration of bacteria used for the inoculation in the field (3.5×10^8 CFU/ml) with a five dilution series to 3.5×10^4 CFU/ml. The standard curve constructed from the dilution series was used for the determination of the Xa concentration in the samples.

3.2.5 Comparison between disease severity and bacterial population

Disease severity (visual rating scale) and bacterial populations (calculated indirectly using TaqMan qPCR) were compared using the Spearman's rank correlation coefficient. The

correlation was calculated separately for 2 or 4, 8, and 12 WAI in the three field experiments performed. For the greenhouse experiment, the correlations were performed only at 10 WAI. The selection of the Spearman's rank correlation coefficient (ρ) was based on the non-parametric nature of the data where two different methods (with different measure units) were compared and a linear relationship was not expected between the data sets. In addition, the correlation between different inoculations using the same method for resistance evaluation to leaf scald was compared using the Pearson correlation coefficient (r), a coefficient used for data with an expected linear relationship. All the statistical analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA).

3.3 RESULTS

3.3.1 Visual ratings of disease resistance

The results of the field inoculations (Table 3.1) and greenhouse inoculation (Table 3.2) for disease assessment using visual symptoms were variable among clones and showed the importance of time after inoculation and tissue sampled for disease resistance evaluation. In the field inoculations, even clones considered resistant (based on previous disease severity evaluations) showed symptom development in inoculated leaves. For example, the resistant cultivar LCP 85-384 showed mean ratings of 3.0 and 2.3 at 2 WAI in the first and second inoculations of 2011, respectively, while the susceptible cultivar HoCP89-846 showed similar ratings of 3.3 and 2.7 in the same inoculations, respectively (Table 3.1). These results showed the generalized symptom expression in inoculated leaves of all the cultivars and the impossibility of distinguishing variable resistance levels in different clones.

Table 3.1. Summary of the field results from three inoculations for all clones evaluated for leaf scald resistance using the mean of the visual rating for disease assessment and the mean of the bacterial population quantified by qPCR

Cultivars	Visual rating[a]				Bacterial population[c]						
	2 WAI[b]	4 WAI	8 WAI	12 WAI	2 WAI[d]	4 WAI	8 WAI SS[e]	8 WAI CS[f]	12 WAI[g]	Stalk base[h]	Meristem[h]
First inoculation 2011											
CP 65-357	3.33	ND	4.29	4.43	1.03E+08	4.26E+08	8.07E+07	1.02E+07	2.33E+07	2.72E+06	2.25E+07
CP 70-321	3	ND	2.67	2.47	7.64E+07	1.11E+05	7.38E+08	7.47E+06	6.11E+07	1.84E+07	2.26E+06
CP 73-351	3	ND	2.36	5.13	8.34E+07	4.40E+07	1.34E+08	1.66E+07	2.29E+07	1.73E+07	1.22E+07
LCP 82-89	3.33	ND	3.78	2.38	6.83E+07	1.11E+07	9.78E+06	2.66E+07	1.51E+07	2.78E+06	7.60E+06
LCP 85-384	3	ND	1.82	2	1.36E+07	4.20E+06	8.80E+05	0.00E+00	0.00E+00	1.11E+06	0.00E+00
HoCP 85-845	3.33	ND	4.4	6.2	8.83E+07	7.01E+07	1.18E+09	3.46E+07	4.45E+06	1.90E+06	1.87E+06
CP 89-2143	3	ND	3.44	3.57	6.40E+07	2.58E+06	0.00E+00	2.81E+06	0.00E+00	8.26E+06	0.00E+00
HoCP 89-846	3.33	ND	3.8	7.63	1.91E+07	8.66E+07	1.16E+09	1.35E+08	6.95E+06	7.47E+05	3.50E+06
Ho 95-988	3	ND	1.76	1.92	4.96E+07	4.77E+07	5.20E+08	2.12E+07	0.00E+00	5.33E+06	0.00E+00
HoCP 96-540	2.33	ND	1.69	1.8	2.77E+07	7.10E+05	3.13E+04	0.00E+00	0.00E+00	5.30E+05	0.00E+00
L 97-128	3.33	ND	6.78	5.36	1.79E+07	3.79E+03	2.62E+04	1.86E+04	0.00E+00	4.07E+06	0.00E+00
L 99-226	3	ND	3.67	4.33	1.56E+08	2.95E+07	5.98E+08	1.81E+06	2.97E+04	2.10E+07	0.00E+00
L 99-233	3.33	ND	3.86	3.08	2.72E+07	3.49E+07	1.70E+08	3.07E+07	1.14E+06	4.90E+06	0.00E+00
HoCP 00-950	3.67	ND	3.21	2.07	6.80E+08	1.22E+08	2.02E+05	6.36E+07	1.46E+05	3.53E+06	0.00E+00
L 01-283	3	ND	2.24	3.19	9.71E+07	3.97E+05	1.14E+08	2.71E+06	0.00E+00	4.59E+06	0.00E+00
L 01-299	3	ND	3.22	3.17	1.98E+07	2.48E+07	8.90E+08	1.60E+06	1.31E+07	6.31E+05	7.24E+06
L 03-371	3	ND	2.43	2.57	4.29E+07	2.55E+08	4.23E+04	0.00E+00	0.00E+00	2.35E+06	0.00E+00
HoCP 04-838	3.67	ND	5.42	6.14	1.15E+08	2.95E+07	3.70E+07	9.85E+07	9.27E+07	2.47E+07	6.14E+07
Ho 05-961	3.67	ND	3.5	2.67	4.90E+07	4.17E+07	0.00E+00	1.24E+07	8.48E+06	3.25E+07	7.25E+06
L 07-57	3	ND	2.89	2.16	3.61E+07	7.46E+03	0.00E+00	0.00E+00	0.00E+00	4.42E+06	0.00E+00
HoCP 07-613	3.33	ND	2.73	4.29	6.33E+07	1.08E+07	0.00E+00	1.56E+04	0.00E+00	8.95E+06	0.00E+00
Ho 08-706	3	ND	4.4	6	5.09E+07	6.75E+06	3.03E+07	2.87E+07	6.53E+06	4.43E+05	6.88E+05
Ho08-709	3.67	ND	3.29	4.2	7.63E+07	3.21E+07	7.63E+07	1.88E+05	7.13E+04	7.14E+06	0.00E+00
Ho 08-711	3.33	ND	3	1.77	6.26E+07	1.91E+04	0.00E+00	2.68E+04	0.00E+00	4.67E+06	0.00E+00
Ho08-717	2.67	ND	1.7	2.91	3.93E+07	7.75E+06	2.37E+05	0.00E+00	0.00E+00	1.54E+07	0.00E+00

(Table 3.1 continued)

Cultivars	Visual rating[a]				Bacterial population[c]						
	2 WAI[b]	4 WAI	8 WAI	12 WAI	2 WAI[d]	4 WAI	8 WAI SS[e]	8 WAI CS[f]	12 WAI[g]	Stalk base[h]	Meristem[h]
HoL 08-723	3.33	ND	2.94	3	1.06E+08	5.34E+06	0.00E+00	1.01E+04	0.00E+00	2.40E+06	0.00E+00
HoCP 08-726	3	ND	1.76	3.77	6.59E+07	6.64E+07	2.57E+04	2.90E+04	0.00E+00	2.84E+06	0.00E+00
L 08-75	3	ND	1.52	3.44	2.68E+07	2.26E+03	9.73E+03	7.23E+03	0.00E+00	2.06E+07	0.00E+00
L 08-88	2.67	ND[i]	1.13	1.2	2.75E+07	2.98E+03	8.97E+04	0.00E+00	0.00E+00	1.70E+06	0.00E+00
L 08-90	3	ND	2.65	2.85	1.27E+08	3.57E+07	9.10E+07	1.50E+08	1.23E+07	1.23E+06	1.37E+04
L 08-92	2.67	ND	3	2.56	4.41E+07	1.59E+07	4.14E+07	1.09E+07	8.03E+06	3.90E+06	0.00E+00
Mean	3.13	ND	3.08	3.49	8.15E+07	4.55E+07	1.89E+08	2.11E+07	8.91E+06	7.45E+06	4.08E+06
Second inoculation 2011											
CP 65-357	3	ND	2.4	2.25	2.44E+08	ND	7.09E+03	3.13E+08	7.17E+05	7.19E+06	0.00E+00
CP 70-321	2.33	ND	1.25	3	9.93E+07	ND	0.00E+00	0.00E+00	0.00E+00	1.47E+07	0.00E+00
CP 73-351	2	ND	1.67	1.57	1.38E+08	ND	0.00E+00	0.00E+00	3.14E+07	2.07E+07	3.06E+07
LCP 82-89	2.67	ND	3	3.21	1.27E+08	ND	2.36E+08	0.00E+00	3.90E+06	3.14E+06	4.11E+05
LCP 85-384	2.33	ND	1.19	1.42	1.85E+07	ND	0.00E+00	0.00E+00	6.87E+03	3.50E+06	0.00E+00
HoCP 85-845	2.67	ND	4	4.82	8.91E+07	ND	2.65E+04	3.01E+08	2.53E+07	3.48E+07	5.83E+06
CP 89-2143	2.33	ND	1.63	2.25	2.43E+08	ND	9.73E+04	0.00E+00	0.00E+00	2.44E+07	0.00E+00
HoCP 89-846	2.67	ND	3	5.93	3.68E+07	ND	0.00E+00	1.11E+09	4.69E+07	2.73E+06	4.30E+07
Ho 95-988	2.67	ND	1.17	1.43	3.47E+08	ND	7.47E+03	0.00E+00	0.00E+00	4.67E+07	0.00E+00
HoCP 96-540	1.67	ND	1.4	2	5.24E+07	ND	0.00E+00	0.00E+00	0.00E+00	2.66E+05	0.00E+00
L 97-128	1.67	ND	1.2	2.71	3.60E+07	ND	1.49E+04	0.00E+00	3.07E+04	0.00E+00	0.00E+00
L 99-226	2.33	ND	2.71	3.46	4.76E+07	ND	1.00E+09	1.03E+05	0.00E+00	2.91E+06	0.00E+00
L 99-233	2.67	ND	2.4	2.33	7.73E+07	ND	2.70E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00
HoCP 00-950	3	ND	3	4.8	2.97E+08	ND	1.16E+07	0.00E+00	0.00E+00	1.38E+06	0.00E+00
L 01-283	2.33	ND	2.47	3.17	3.27E+06	ND	0.00E+00	4.60E+03	0.00E+00	1.80E+06	0.00E+00
L 01-299	2	ND	2.54	2.5	9.08E+07	ND	1.44E+08	7.10E+03	4.90E+03	1.85E+07	0.00E+00
L 03-371	2.67	ND	1.33	2.09	5.45E+07	ND	0.00E+00	2.77E+08	0.00E+00	7.00E+06	0.00E+00
HoCP 04-838	3	ND	2	3.43	3.27E+08	ND	0.00E+00	9.73E+04	0.00E+00	4.10E+06	0.00E+00
Ho 05-961	ND	ND	1	5.18	1.62E+04	ND	0.00E+00	0.00E+00	0.00E+00	3.75E+07	0.00E+00

(Table 3.1 continued)

Cultivars	Visual rating[a]				Bacterial population[c]						
	2 WAI[b]	4 WAI	8 WAI	12 WAI	2 WAI[d]	4 WAI	8 WAI SS[e]	8 WAI CS[f]	12 WAI[g]	Stalk base[h]	Meristem[h]
L 07-57	2	ND	1.22	2.75	3.64E+07	ND	ND	2.60E+08	0.00E+00	0.00E+00	0.00E+00
HoCP 07-613	1.33	ND	1.67	7.8	2.31E+07	ND	0.00E+00	1.45E+04	0.00E+00	9.93E+05	0.00E+00
Ho 08-706	ND	ND	1.73	1.8	ND	ND	0.00E+00	1.49E+04	0.00E+00	1.04E+06	0.00E+00
Ho 08-709	1.33	ND	2.09	2.78	6.71E+07	ND	0.00E+00	0.00E+00	6.49E+06	1.48E+07	8.77E+06
Ho 08-711	2	ND	1.36	1.89	1.37E+08	ND	1.08E+04	2.70E+08	0.00E+00	6.01E+06	0.00E+00
Ho 08-717	2.67	ND	1.75	3	3.54E+07	ND	4.48E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00
HoL 08-723	2	ND	1.33	3.2	1.02E+08	ND	7.10E+03	2.36E+08	2.91E+03	0.00E+00	0.00E+00
HoCP 08-726	2.67	ND	2.2	3.14	5.44E+07	ND	0.00E+00	0.00E+00	0.00E+00	3.16E+04	0.00E+00
L 08-75	2	ND	1.36	1.5	3.25E+07	ND	1.03E+05	1.16E+07	0.00E+00	5.94E+06	0.00E+00
L 08-88	2.33	ND	1	1.22	5.24E+07	ND	0.00E+00	0.00E+00	0.00E+00	4.40E+07	0.00E+00
L 08-90	2.67	ND	1.57	3.4	8.52E+07	ND	0.00E+00	0.00E+00	0.00E+00	3.16E+03	0.00E+00
L 08-92	2.33	ND	2	3.88	2.48E+08	ND	2.77E+08	1.38E+05	1.06E+06	1.07E+06	0.00E+00
Mean	2.32	ND	1.89	3.03	1.07E+08	ND	6.48E+07	8.95E+07	3.74E+06	9.85E+06	2.86E+06
Inoculation 2012[j]											
CP 65-357	ND	3	4.25	5	ND	8.17E+06	3.75E+07	1.09E+08	1.40E+08	ND	ND
CP 70-321	ND	2.22	2.82	1.67	ND	0.00E+00	1.06E+08	4.30E+06	1.76E+08	ND	ND
CP 73-351	ND	4	4.85	4.83	ND	0.00E+00	5.02E+08	3.92E+08	3.67E+06	ND	ND
LCP 82-89	ND	2.4	2.78	4	ND	0.00E+00	4.81E+07	9.13E+07	3.77E+06	ND	ND
LCP 85-384	ND	1.5	1	1	ND	0.00E+00	9.50E+02	0.00E+00	0.00E+00	ND	ND
HoCP 85-845	ND	4.47	5.63	6.38	ND	2.92E+07	4.21E+08	8.14E+07	1.05E+08	ND	ND
CP 89-2143	ND	3.29	5.33	5.4	ND	1.33E+08	7.86E+05	1.05E+08	3.01E+07	ND	ND
HoCP 89-846	ND	6.33	7.8	6.85	ND	5.76E+07	1.93E+09	1.03E+09	8.27E+06	ND	ND
Ho 95-988	ND	3.63	3.11	7.15	ND	1.38E+07	1.64E+07	2.01E+08	1.10E+06	ND	ND
HoCP 96-540	ND	2.17	2	1	ND	0.00E+00	1.09E+07	1.80E+07	0.00E+00	ND	ND
L 97-128	ND	1.46	1.91	5.33	ND	0.00E+00	1.70E+03	1.00E+06	6.90E+02	ND	ND
L 99-226	ND	2.33	3	5.31	ND	0.00E+00	7.13E+08	2.70E+08	5.33E+06	ND	ND
L 99-233	ND	4.14	5.33	5.6	ND	1.21E+06	1.46E+08	1.60E+07	5.91E+07	ND	ND

(Table 3.1 continued)

Cultivars	Visual rating[a]				Bacterial population[c]						
	2 WAI[b]	4 WAI	8 WAI	12 WAI	2 WAI[d]	4 WAI	8 WAI SS[e]	8 WAI CS[f]	12 WAI[g]	Stalk base[h]	Meristem[h]
HoCP 00-950	ND	3.2	5.46	1.5	ND	0.00E+00	3.95E+07	6.33E+08	1.14E+08	ND	ND
L 01-283	ND	1.71	2.85	2.43	ND	4.40E+04	7.00E+02	0.00E+00	0.00E+00	ND	ND
L 01-299	ND	4.08	3.5	4.85	ND	1.92E+07	1.86E+08	2.79E+08	6.13E+02	ND	ND
L 03-371	ND	3.2	4.17	2.75	ND	3.31E+03	5.16E+07	6.88E+07	6.80E+02	ND	ND
HoCP 04-838	ND	4.69	5.17	6.64	ND	5.13E+05	3.29E+08	3.59E+08	1.85E+08	ND	ND
Ho 05-961	ND	2.69	4	1	ND	1.61E+04	4.76E+05	4.82E+05	4.93E+06	ND	ND
L 07-57	ND	1.77	1.92	1.89	ND	0.00E+00	0.00E+00	0.00E+00	0.00E+00	ND	ND
Ho 08-706	ND	3	3.25	3.8	ND	5.23E+05	1.87E+05	1.07E+08	1.08E+06	ND	ND
Ho 08-709	ND	2.87	3.13	3.62	ND	4.57E+05	1.93E+08	6.86E+07	7.03E+06	ND	ND
Ho 08-711	ND	1.55	3	2.45	ND	0.00E+00	1.64E+03	6.87E+07	0.00E+00	ND	ND
Ho 08-717	ND	1.67	3	3.15	ND	0.00E+00	3.13E+02	1.84E+05	0.00E+00	ND	ND
Ho 08-723	ND	1.75	3.18	6.82	ND	7.85E+05	1.95E+07	7.69E+07	1.52E+03	ND	ND
HoCP 08-726	ND	1.73	1.8	2	ND	1.49E+07	0.00E+00	1.04E+08	4.44E+04	ND	ND
L 08-75	ND	2.8	1.75	5.2	ND	0.00E+00	1.81E+05	2.79E+04	5.37E+03	ND	ND
L 08-88	ND	1.92	1.77	2.6	ND	0.00E+00	4.41E+03	2.06E+07	0.00E+00	ND	ND
L 08-90	ND	2.71	4.57	1.2	ND	6.83E+03	1.11E+09	7.84E+06	1.38E+07	ND	ND
L 08-92	ND	2.69	3	1.2	ND	9.58E+05	1.59E+08	3.24E+08	1.21E+05	ND	ND
Mean	ND	2.83	3.51	3.75	ND	9.34E+06	2.01E+08	1.48E+08	2.86E+07	ND	ND

[a] Visual rating based on severity of symptoms expressed by inoculated plants at 2 or 4, 8, and 12 weeks after inoculation (WAI) using a 1, 3, 5, 7 and 9 rating system.
[b] Disease severity was rated at 2 weeks after inoculation (WAI) using a 1 to 5 rating system.
[c] Bacteria populations were calculated indirectly by qPCR in leaf extracts at different time intervals after inoculation.
[d] Bacteria population at 2 weeks after inoculation (WAI) was determined from a single inoculated leaf.
[e] SS = single systemically infected leaf sample.
[f] CS = composite sample of three systemically infected leaves per sample.
[g] Composite leaf samples were collected at 12 weeks after inoculation (WAI).
[h] The stalk base and the apical meristem were collected from inoculated plants 12 weeks after inoculation.
[i] ND = no data
[j] For the 2012 inoculation, clone 07-613 was not included.

In the field experiments, the highest variation in symptoms observed within and between cultivars was recorded at 8 and 12 WAI based on systemic infection symptoms (Table 3.1). Visual ratings for the resistant cultivars across the three inoculations ranged from 1.0 - 1.8 at 8 WAI and 1.0 - 2.0 at 12 WAI for LCP 85-384, from 1.2 – 3.0 at 8 WAI and 1.4 – 7.2 at 12 WAI for Ho 95-988, and from 1.4 – 2.0 at 8 WAI and 1.0 – 1.8 as 12 WAI for HoCP 96-540 (Table 3.1, Figure 3.2, Figure 3.3, and Figure 3.4). In contrast, susceptible cultivar ratings ranged from 4.0 – 5.6 at 8 WAI and 4.8 – 6.4 at 12 WAI for HoCP 85-845 and from 3.0 – 7.8 at 8 WAI and 5.9 – 7.6 at 12 WAI for HoCP 89-846 (Table 3.1, Figure 3.2, Figure 3.3, and Figure 3.4). The susceptible check cultivars had higher disease severity in the three different inoculations; although, the visual ratings were in the range of moderately resistant to moderately susceptible at 8 WAI in the two 2011 inoculations. One of the resistant check cultivars, Ho 95-988, had erratic results based on disease severity assessed visually with a 7.2 rating at 12 WAI in the 2012 inoculation. The second inoculation performed in 2011 showed less severe symptoms across all cultivars at 8 WAI compared with the other inoculations with ratings ranging from 1.0 – 4.0 and a mean of 1.9 (Table 3.1, Figure 3.3).

For the greenhouse experiment, the variation among clones was lower compared with the field results, and symptom expression was not able to provide adequate separation between the cultivars evaluated (Table 3.2). The highest variation of the greenhouse data was observed at 10 WAI; however, symptom expression in the greenhouse was erratic, and cultivars previously reported as susceptible based on disease severity in different field evaluations did not show symptoms associated with susceptibility. The rating for both susceptible check cultivars (HoCP 85-845 and HoCP 89-849) was 2.5. Based on the greenhouse results, only four cultivars, CP 65-357, CP 89-2143, L 99-226 and L 03-371, were rated susceptible (rating of 4.0 or higher). The

ratings for cultivars HoCP 85-845 and HoCP 89-846 were still 3.0 and 3.5, respectively,

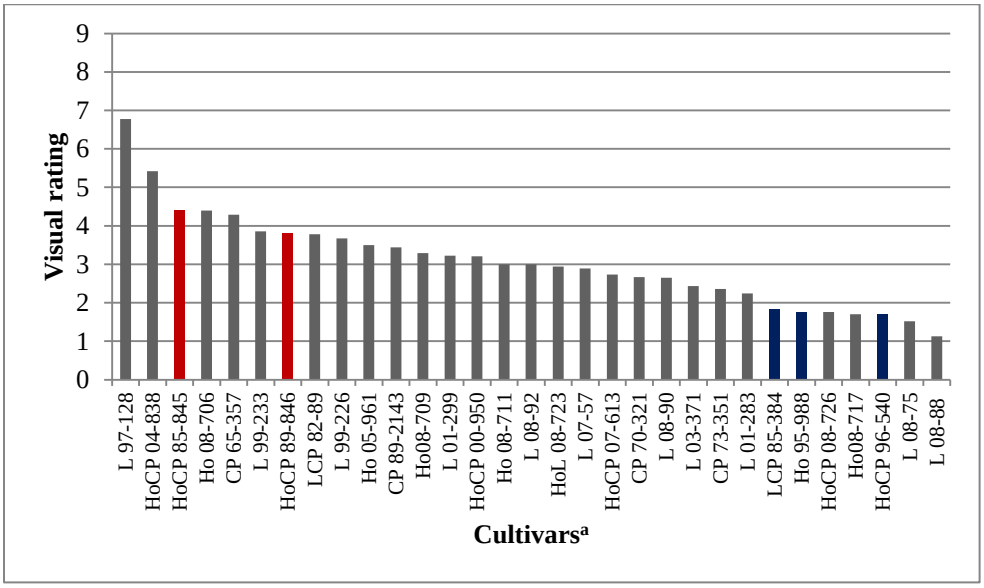
(moderately resistant) at 12 WAI (Table 3.2).

Table 3.2. Leaf scald resistance ratings based on visual evaluation of symptom severity and the bacterial population based on qPCR results in the greenhouse experiment performed in 2011-2012

Cultivars[a]	**Visual rating**[b]				**Bacteria population**[c]
	2 WAI	**8 WAI**	**10 WAI**	**12 WAI**	**10 WAI**
CP 65-357	1.8	4	6.5	5	8.20E+07
CP 70-321	1.5	2	2	2.5	4.34E+06
CP 73-351	1	1	2	1	0.00E+00
LCP 82-89	2.3	2	2	1.5	0.00E+00
LCP 85-384	1.8	1	1	1.5	2.38E+05
HoCP 85-845	2.3	2.5	2.5	3	6.88E+03
CP 89-2143	2.5	3.5	5	5.5	6.21E+06
HoCP 89-846	3.5	3	2.5	3.5	1.00E+08
Ho 95-988	1.3	1.5	2	1.5	1.77E+04
HoCP 96-540	1.8	2.5	1.5	2	8.33E+03
L 97-128	2	2.5	2	2	9.53E+05
L 99-226	2	1	3	2.5	1.14E+08
L 99-233	1.3	1	1	2	1.36E+04
HoCP 00-950	1	1	1	1	0.00E+00
L 01-283	1.3	2	2.5	2.5	3.03E+04
L 01-299	3.3	1	1	2	2.22E+07
L 03-371	2.5	2.5	4	3.5	5.14E+04
HoCP 04-838	3.8	2	1.5	2.5	9.49E+05
Ho 05-961	1	2	2	2	0.00E+00
Ho 08-706	2	1.5	1.5	1.5	0.00E+00
Ho 08-709	1	2	2	2	0.00E+00
Ho 08-711	3.3	2	1.5	1.5	0.00E+00
Ho 08-717	2.5	1	1	2.5	6.21E+07
HoL 08-723	2	1.5	2	2.5	3.73E+03
HoCP 08-726	2.5	2	1.5	1.5	0.00E+00
L 08-75	1	1	1	1	5.28E+03
L 08-88	1	1	1.5	1	0.00E+00
L 08-90	2.5	1	1	1	0.00E+00
L 08-92	3.3	1	1.5	2	6.46E+06
Mean	2.05	1.82	2.07	2.23	1.43E+07

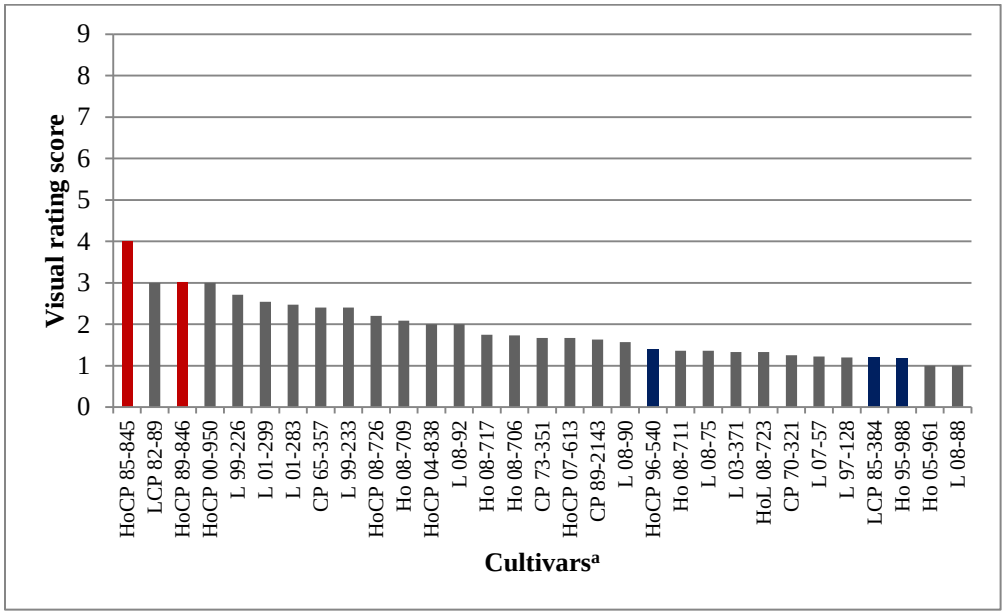
[a] For the greenhouse experiment, clones L 07-57 and Ho 07-613 were not included.
[b] Visual rating based on severity of symptoms expressed by inoculated plants at 8, 10, and 12 weeks after inoculation (WAI) using a1, 3, 5, 7 and 9 rating system. Rating at 2 weeks after inoculation used a 1 -5 rating scale.
[c] Bacteria populations were calculated indirectly by qPCR in leaf extracts at 10 weeks after inoculation (WAI) from a single inoculated leaf

Figure 3.2. Visual ratings at 8 weeks after inoculation for the different cultivars evaluated in the first inoculation during 2011.



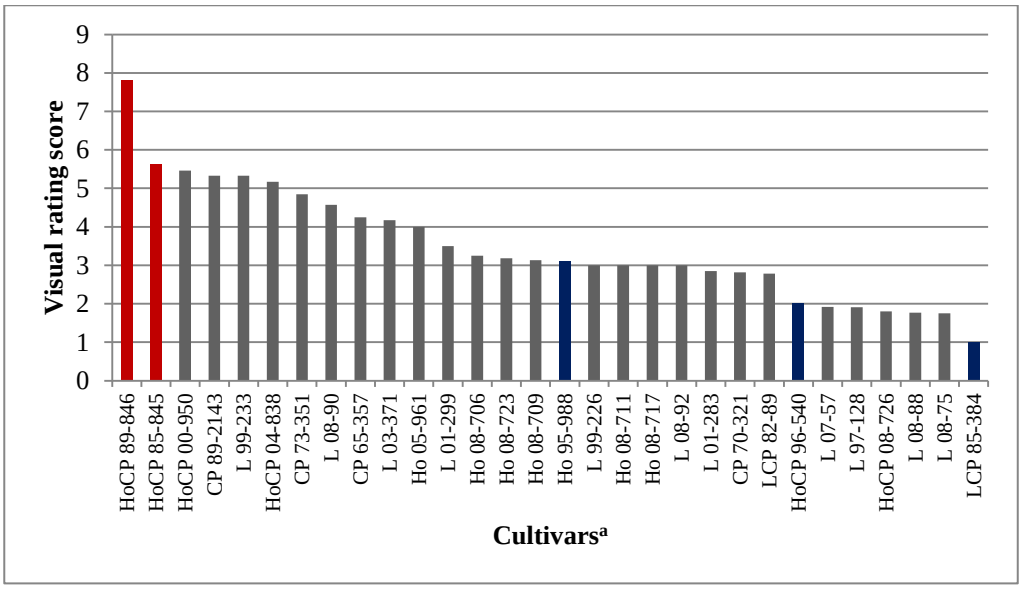
[a] The red bars correspond to the susceptible check cultivars and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

Figure 3.3. Visual ratings at 8 weeks after inoculation for the different cultivars evaluated in the second inoculation during 2011.



[a] The red bars correspond to the susceptible check cultivars and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

Figure 3.4. Visual ratings at 8 weeks after inoculation for the different cultivars evaluated in the inoculation during 2012.



[a] The red bars correspond to the susceptible check cultivars and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

3.3.2 Bacterial populations determined by qPCR

The Xa population quantified by qPCR showed variation among the clones within the different inoculations and the different time points analyzed in the field inoculations (Table 3.1) and the greenhouse inoculation (Table 3.2). At 2 WAI in the field inoculations, all the clones had a high concentration of bacteria in the inoculated leaves, and no clone tested negative for Xa, making it difficult to discriminate variable levels of resistance among clones (Table 3.1). Variation among clones became evident at 4 WAI in samples that included systemically infected leaf tissue (Table 3.1). The bacterial population at 8 WAI showed high variation among cultivars (Table 3.1). The Xa populations for the susceptible check cultivars ranged from $3.8 \times 10^7 - 3.0 \times 10^8$ CFU/ml for HoCP 85-845 and from $1.4 \times 10^8 - 1.1 \times 10^9$ for HoCP 89-846 across the three inoculations at 8 WAI (Table 3.1, Figure 3.5, Figure 3.6, and Figure 3.7). Two of the three resistant check cultivars gave variable results for Xa populations at 8 WAI. Bacteria were not

detected in all three inoculations for LCP 85-384 (Table 3.1, Figure 3.5, Figure 3.6, and Figure 3.7). However, populations of 2.1 x 10^7 and 2.0 x 10^8 CFU/ml were detected for Ho 95-988 in the first 2011 inoculation and 2012 inoculation, respectively, and a population of 1.8 x 10^7 CFU/ml was detected for HoCP 96-540 in 2012.

At 12 WAI, bacterial populations in leaf tissue were highly variable among cultivars (Table 3.1). The Xa populations for the susceptible check cultivars ranged from 4.45 x 10^6 – 1.05 x 10^8 CFU/ml for HoCP 85-845 and from 6.95 x 10^6 – 4.69 x 10^7 for HoCP 89-846 across the three inoculations (Table 3.1). The resistant check cultivars showed negative results, except LCP 85-384 in the second inoculation of 2011 (6.87 x 10^3 CFU/ml, a value near to the qPCR threshold) and Ho 95-988 in the 2012 inoculation (1.10 x 10^6 CFU/ml, a concentration similar to the susceptible check cultivar HoCP 89-846) (Table 3.1). At 12 WAI in the first inoculation of 2011, 45% of the cultivars were negative for Xa based on qPCR, and in the second inoculation, 65% of the cultivars were negative. However, only 23% of the cultivars were negative for Xa in the 2012 inoculation.

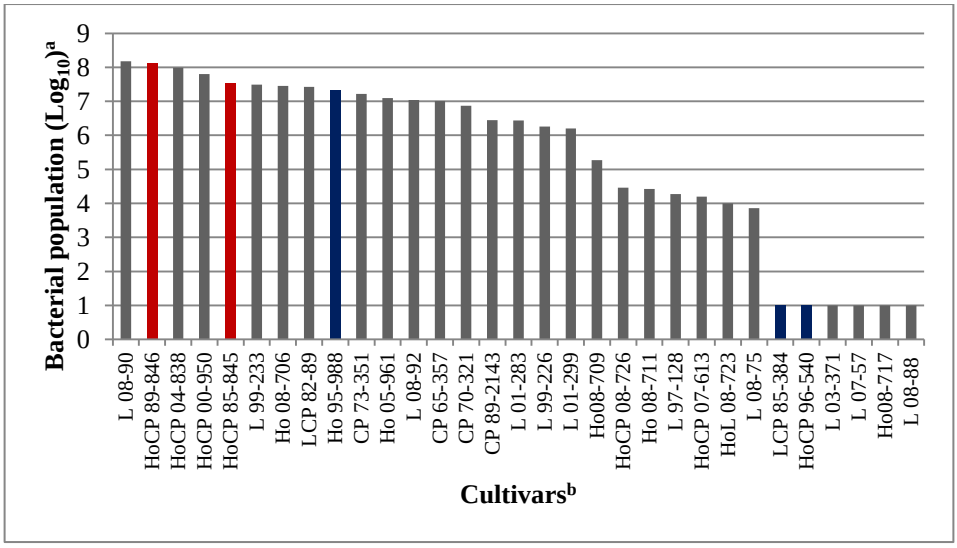
Xanthomonas albilineans populations quantified by qPCR at 12 WAI in three different plant tissues had similar overall population means (Table 3.3) but showed variable results among cultivars (Table 3.1). In the stalk base, the bacterial population was high in all cultivars evaluated in the first inoculation of 2011 preventing discernment among them for degree of resistance (Table 3.1). For the second inoculation of 2011, five cultivars tested negative for Xa, while the others exhibited variation in the concentration of bacteria (Table 3.1). However, the differences in bacterial populations did not distinguish the known resistant and susceptible cultivars. The population of bacteria in the apical meristem varied among clones and between inoculations with 63% testing negative for Xa in the first inoculation and 81% testing negative in the second

inoculation (Table 3.1). One cultivar, L 08-709 was negative in the first but positive in the second inoculation. Compared with the leaf tissue results at 12 WAI, the apical meristem results showed more agreement (53% in the first inoculation and 78% in the second inoculation) than the stalk base (34% in the first inoculation and 16% in the second inoculation) (Table 3.1). All the positive clones for Xa in the apical meristem were positive in the leaf tissue; however, some samples with positive results in the qPCR from leaf tissue were negative for the apical meristem evaluation.

Table 3.3. *Xanthomonas albilineans* populations in stalk base, apical meristem, and leaf samples determined by qPCR at 12 weeks after inoculation in two field experiments during 2011

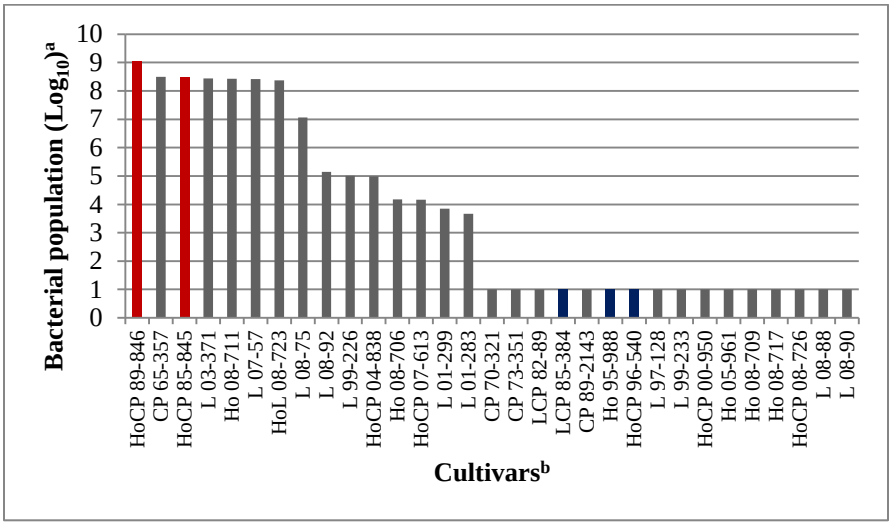
Experiment	Tissue evaluated	Mean	Min	Max	Median
First inoculation	Leaf (composite)	8.91×10^6	0.00	9.27×10^7	2.97×10^4
	Stalk base	7.45×10^6	4.43×10^5	3.25×10^7	4.07×10^6
	Apical meristem	4.08×10^6	0.00	6.14×10^7	0.00
Second inoculation	Leaf (composite)	3.74×10^6	0.00	4.69×10^7	0.00
	Stalk base	9.85×10^6	0.00	4.67×10^7	3.14×10^6
	Apical meristem	2.86×10^6	0.00	4.30×10^7	0.00

Figure 3.5. Bacterial populations determined by qPCR at 8 weeks after inoculation of the different cultivars evaluated in the first inoculation of 2011.



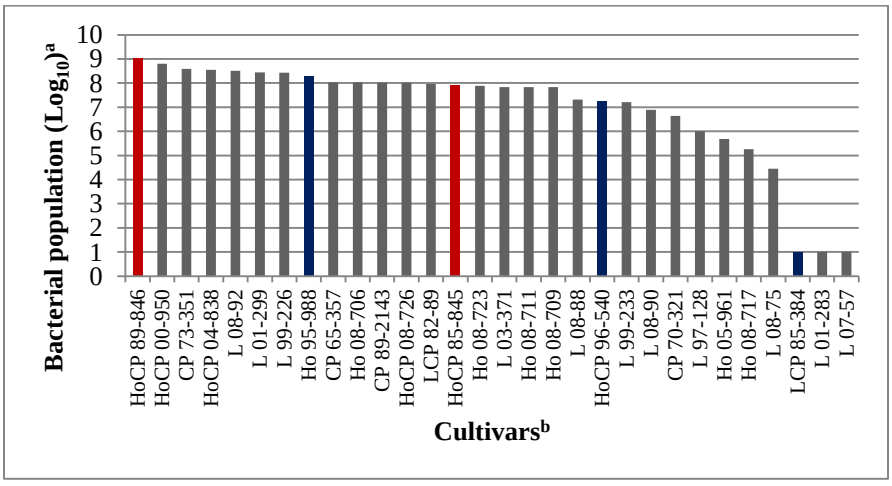
[a] For samples without detectable bacteria by qPCR, the assigned value was one in analogy with the visual rating system where the number one is assigned to plants without symptoms.
[b] The red bars correspond to the susceptible check cultivars, and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

Figure 3.6. Bacterial populations determined by qPCR at 8 weeks after inoculation of the different cultivars evaluated in the second inoculation of 2011.



[a] For samples without detectable bacteria by qPCR, the assigned value was one in analogy with the visual rating system where the number one is assigned to plants without symptoms.
[b] The red bars correspond to the susceptible check cultivars and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

Figure 3.7. Bacterial populations determined by qPCR at 8 weeks after inoculation of the different cultivars evaluated in the inoculation performed in 2012.



[a] For samples without detectable bacteria by qPCR, the assigned value was one in analogy with the visual rating system where the number one is assigned to plants without symptoms.
[b] The red bars correspond to the susceptible check cultivars and the blue bars are the resistant check cultivars. The susceptibility or resistance of these cultivars to leaf scald was determined previously in field evaluations.

In the greenhouse, the evaluation of the bacterial population was performed at 10 WAI due to the lower plant growth rate compared with the field experiment. Symptom expression was less in the greenhouse as reflected by the low visual symptom severity ratings (Table 3.2). A high number of clones (34%) tested negative for Xa, and check cultivars had unexpected results. The resistant cultivars LCP 85-384, Ho 95-988, and HoCP 96-540 had Xa populations of 2.4 x 10^5, 1.8 x 10^4, and 8.3 x 10^3 CFU/ml, respectively, with visual ratings of 1.0, 2.0, and 1.5, respectively, while the susceptible checks, HoCP 85-845 and HoCP 89-846, had resistant visual ratings of 2.5 each and Xa populations of 6.9 x 10^3 and 1.0 x 10^8 CFU/ml, respectively (Table 3.2).

The evaluation of cultivars with known resistance or susceptibility to leaf scald showed some differences between the visual ratings and the bacteria populations calculated by qPCR at 8 WAI. The susceptible check cultivars all had moderately resistant or moderately susceptible visual severity ratings ranging from 3.0 to 4.4 in the two 2011 inoculations but high Xa populations determined by qPCR (Table 3.1). For the resistant check cultivars, Ho 95-988 had comparable populations of bacteria to HoCP 85-845 for the first 2011 inoculation and 2012 inoculation with visual severity ratings of 1.8 and 3.1, respectively, and the high bacterial population detected in HoCP 96-540 during 2012 was paired with a visual rating of 2.0 (Table 3.1). However, the bacterial population of HoCP 96-540 at 8 WAI was lower than the populations present in the susceptible checks, and the evaluation performed at 12 WAI did not show detectable bacteria.

3.3.3 Correlation between visual disease severity ratings and bacterial populations quantified by qPCR

The comparison between visual severity ratings and Xa populations showed low to medium positive correlation values at 2 WAI for the first (ρ=0.38; *P*=0.036) and second (ρ=0.47; *P*=0.011) inoculations of 2011. However, resistance evaluation based on symptomatology and bacterial populations at 2 WAI did not distinguish known resistant and susceptible cultivars (Table 3.1). For that reason, the 2 WAI concentration data was not obtained for the inoculation performed in 2012. The correlation at 4 WAI in the 2012 inoculation was higher (ρ=0.57; *P*=0.001) than the correlation obtained at 2 WAI in the 2011 inoculations; however, comparing the two inoculations is not appropriate because the time points were different and the sampling methods used for the bacteria quantification were different (composite sampling was used at 4 WAI in the 2012 experiment).

Two different sampling methods were evaluated at 8 WAI. The Spearman's rank correlation coefficients between visual severity ratings and Xa populations determined by qPCR for single leaf samples were lower than for composite samples (Table 3.4). The composite leaf sample correlations at 8 WAI for all three field experiments were higher than the correlations obtained with any other sampling method or time after inoculation.

Table 3.4. Correlation between visual symptom severity ratings and *X. albilineans* populations determined by qPCR in systemically infected leaves at 8 weeks after inoculation among three experiments

Experiment	Single leaf sample[a]		Composite leaf sample[b]	
	ρ^c	*P*	ρ^c	*P*
2011 first inoculation	0.27	0.145	0.58	<0.001
2011 second inoculation	0.37	0.045	0.48	0.006
2012 inoculation	0.56	< 0.001	0.62	< 0.001

[a] One TVD – 2 leaf by sample, three samples by clone.

[b] Three TVD – 2 leaves each from a different plant by sample, three samples by clone.
[c] ρ = Spearman's rank correlation coefficient.

At 12 WAI, the correlations between visual severity ratings and Xa populations in systemically infected leaves were not significant (Table 3.5). A comparison of severity ratings and Xa populations in other plant tissues at 12 WAI found low correlation for stalk base and apical meristem samples for the 2011 inoculations. Correlation results for the stalk base were not significant, but a significant correlation was found for the apical meristem in one of two inoculations (Table 3.5).

Table 3.5. Correlation between visual symptom severity ratings and *X. albilineans* populations determined by qPCR in composite leaf, apical meristem and stalk base samples at 12 weeks after inoculation among three experiments

Experiment	Leaf tissue[a]		Stalk base		Apical meristem	
	ρ[b]	*P*	ρ[b]	*P*	ρ[b]	*P*
2011 first inoculation	0.32	0.0795	0.28	0.123	0.39	0.027
2011 second inoculation	0.13	0.472	0.06	0.764	0.20	0.279
2012 inoculation	0.33	0.071	ND[c]	ND	ND	ND

[a] Leaf tissue collected from three TVD – 2 leaves in composite sample.
[b] ρ = Spearman's rank correlation coefficient.
[c] ND = No data.

3.3.4 Correlation among inoculations for each resistance evaluation method

Correlation of the data from different inoculation experiments varied among time intervals after inoculation and resistance evaluation method. Resistance evaluations using visual severity ratings of inoculated leaves at 2 WAI were not correlated for the two 2011 field inoculations (r = 0.12, *P* = 0.529) and with the greenhouse inoculation (r = 0.03, *P* = 0.8722 and r – 0.23, *P* = 0.2551, respectively). When the Xa population data at 2 WAI were compared, the positive correlation between the first and the second inoculations of 2011 was higher (r = 0.42; *P* = 0.0207). The Xa populations were not determined at 2 WAI in the 2012 inoculation based on

the results obtained in the two field inoculations performed in 2011 that showed few differences between the cultivars evaluated.

The data for the bacterial population at 4 WAI was not correlated between the 2011 first inoculation and 2012 inoculation (r=0.00, *P* =0.999). For the other inoculations, the Xa populations were not determined.

The data for resistance evaluation using visual ratings at 8 WAI showed low to medium positive correlations among the different experiments (Pearson correlations ranged between 0.14 and 0.53). However, only half of the coefficients were significant (Table 3.6). Two of three field inoculation experiments were correlated, while only one of three comparisons between field and greenhouse experiments was correlated.

Table 3.6. Pearson correlation of the visual symptom severity rating data at 8 weeks after inoculation among the different experiments in 2011 and 2012

Experiment	**2011 first inoculation**		**2011 second inoculation**		**2012 inoculation**	
	r[a]	*P*	r[a]	*P*	r[a]	*P*
2011 first inoculation	1					
2011 second inoculation	0.33	0.0679	1			
2012 inoculation	0.38	0.0388	0.53	0.0026	1	
Greenhouse inoculation	0.39	0.0382	0.14	0.4576	0.31	0.1066

[a] r = Pearson product moment correlation coefficient.

The bacterial populations determined by qPCR at 8 WAI were compared among different experiments and sampling methods. Data from single leaf samples were not correlated between the two different inoculations performed in 2011 in the field, but both field data sets were correlated with the greenhouse data (Table 3.7). In contrast, the composite sample data showed medium to high correlation among the three field inoculation comparisons (Table 3.7) with

positive correlation coefficients ranging from 0.55 to 0.77. The results for comparisons of the field data sets with the greenhouse data were variable with no correlation in the comparison with the first inoculation of 2011 and medium positive correlation with the second inoculation in 2011 and the 2012 inoculation (Table 3.7). When the single leaf sampling data were compared with the composite sampling data of the same year, the correlation coefficients were low and not significant for the 2011 inoculation experiments (Table 3.7). However, the comparison between the single and composite leaf sampling of 2012 was high and significant (r = 0.67, $P < 0.0001$).

Table 3.7. Pearson correlation of the *X. albilineans* population data at 8 weeks after inoculation among experiments and sampling methods in 2011 and 2012

		2011 first inoculation		**2011 second inoculation**		**2012 inoculation**	
Experiment	**Sampling**	Single[a] (*P*)	Composite[b] (*P*)	Single (*P*)	Composite (*P*)	Single (*P*)	Composite (*P*)
2011 first inoculation	Single	1					
	Composite	0.29 (0.1117)	1				
2011 second inoculation	Single	0.20 (0.2808)	-0.10 (0.6031)	1			
	Composite	0.49 (0.0047)	0.55 (0.0014)	-0.13 (0.4922)	1		
2012 inoculation	Single	0.45 (0.0125)	0.56 (0.0013)	0.11 (0.5749)	0.58 (0.0007)	1	
	Composite	0.58 (0.0008)	0.77 (<0.0001)	0.19 (0.3319)	0.65 (<0.0001)	0.67 (<0.0001)	1
Greenhouse	Single[c]	0.41 (0.0253)	0.16 (0.4048)	0.52 (0.0039)	0.51 (0.0048)	0.44 (0.0148)	0.54 (0.0027)

[a] Single = one TVD -2 leaf by sample. Three samples were taken for all clones.
[b] Composite = three leaves each from a different plant by sample. Three samples were taken for all clones.
[c] For the greenhouse experiment, four samples were taken per clone.

The Pearson correlation analysis results for visual symptom rating data at 12 WAI were variable among the different inoculations performed in the field and greenhouse between 2011 and 2012 (Table 3.8). Only one of six data sets was significantly correlated. For the bacterial population data, the first inoculation of 2011 and the 2012 inoculation were correlated (Table 3.9). For the visual rating method, the correlation was positive and medium (r = 0.60; *P*=

0.0005), whereas it was higher (r = 0.75, P<0.0001) for the bacterial population. However, the results obtained 12 WAI showed fewer correlations among data obtained in different seasons, making evaluation of resistance at 12 WAI unreliable for visual rating or bacterial population.

The Xa populations present in the stalk base and apical meristem evaluated at 12 WAI were not correlated between the first and second inoculations of 2011. For the stalk base, the Pearson's correlation coefficient was 0.1934 (P = 0.2966). For the apical meristem, the correlation coefficient was 0.0536 (P = 0.7746).

Table 3.8. Pearson correlation of visual symptom severity rating data at 12 weeks after inoculation among experiments in 2011 and 2012

Experiment	**2011 first inoculation**		**2011 second inoculation**		**2012 inoculation**	
	r[a]	*P*	r[a]	*P*	r[a]	*P*
2011 first inoculation	1					
2011 second inoculation	0.35	0.0501	1			
2012 inoculation	0.60	0.0005	0.02	0.9299	1	
Greenhouse inoculation	0.32	0.0939	0.17	0.3675	0.34	0.0743

[a] r = Pearson product moment correlation coefficient.

Table 3.9. Pearson correlation of *X. albilineans* population data at 12 weeks after inoculation among experiments in 2011 and 2012

Experiment	**2011 first inoculation**		**2011 second inoculation**		**2012 inoculation**	
	r[a]	*P*	r[a]	*P*	r[a]	*P*
2011 first inoculation	1					
2011 second inoculation	0.03	0.8604	1			
2012 inoculation	0.75	< 0.0001	0.0002	0.9991	1	
Greenhouse inoculation	-0.03	0.8871	0.32	0.0901	0.04	0.8210

[a] r = Pearson product moment correlation coefficient.

3.4 DISCUSSION

A correlation demonstrated between *X. albilineans* populations in the shoot apex and disease severity (Rott, *et al*., 1997) suggested a method to accurately quantify bacteria could provide an improved method for resistance screening, which traditionally has been done by rating erratic symptom expression following inoculation (Rott and Davis, 2000). A qPCR technique for the indirect quantification of *X. albilineans* by DNA concentration was developed that could clearly differentiate a limited number of clones with either high leaf scald resistance or susceptibility (Garces, *et al*., 2014). However, additional research was needed to optimize the method and more extensively compare the performance of qPCR to the traditional method using a larger population of clones with variable levels of leaf scald resistance.

The results from this study demonstrated that quantification of Xa by qPCR can distinguish differences among a population of clones with variable levels of resistance to leaf scald. Young systemically infected leaves were confirmed to be the best tissue for detecting differences between clones (Garces, *et al*., 2014). In addition, the results demonstrated that a composite leaf sample will provide more consistent quantification of bacterial populations by qPCR. Leaf sampling is easy and relatively non-invasive, and DNA extraction does not require special methods or kits.

The time after inoculation for DNA extraction was found to be an important factor affecting the results in field experiments. As expected, the use of data from inoculated leaves (2 and 4 WAI) did not result in good separation between the clones evaluated. Even resistant genotypes become infected following inoculation with a high concentration of bacteria, but resistance expression results in reduced Xa colonization during systemic infection (Rott, *et al*., 1997). Data taken 8-12 weeks after inoculation showed variation among clones that was

associated with resistance and susceptibility in cultivars with known reactions against the disease. The visual ratings for the highly susceptible check cultivars ranged from moderately resistant to moderately susceptible at 8 WAI, whereas high Xa populations were detected by qPCR. At 12 WAI, the visual ratings for the susceptible cultivars were more accurate. However, the correlation among the 12 WAI data collected using the same evaluation method in different field and greenhouse inoculations was not significant. In addition, data collected 12 WAI would be too late for annual input into the breeding program in Louisiana.

The data collected at 8 WAI showed the highest correlation in the three different field experiments for both visual ratings and Xa populations. In addition, Spearman's rank tests comparing the data for visual ratings and bacterial populations in leaf tissue showed the highest correlation at 8 weeks after inoculation. Early or later evaluation data were not as consistent for the study population with either method.

In order to measure the reliability of each evaluation method for accurately determining the susceptibility of a clone to leaf scald in different seasons, the data collected in different experiments with variable environmental conditions were tested for correlation. The results showed that quantification of the bacterial population was more highly correlated and therefore more stable and repeatable than resistance evaluation based on rating symptom severity, the traditional method. However, a severe inoculation with Xa resulted in successful systemic colonization of known resistant clones in some cases. Therefore, multiple inoculations will still be needed to identify all resistant genotypes by qPCR during cultivar selection.

The cultivars used as resistant checks had varied results in the inoculations test at 8 WAI. LCP 85-384 had a low visual rating score and low bacterial population in leaf tissue 8 WAI in all

three inoculations as expected; however, Ho 95-988 and HoCP 96-540 had a high Xa population in systemically infected leaves in at least one of the different inoculations. Ho 95-988 showed a high visual rating in 2012 which was one of the two inoculations that resulted in successful systemtic colonization by Xa. The results revealed differences between cultivars that have shown resistance in multiple field evaluations performed in different seasons suggesting different mechanisms of resistance. With leaf scald, all sugarcane genotypes, even resistant ones, can be infected by Xa. Apparently, the cultivar screening inoculation can overcome resistance to systemic infection in some resistant genotypes. The wounding and exposure to a very high concentration of bacteria during the screening inoculation may be rare under natural infection conditions.

Environmental conditions can strongly affect leaf scald symptom severity (Rott and Davis, 2000). It was uncertain how much environmental conditions would affect Xa populations in systemic infections, and this was a major consideration in comparing inoculations at different times during the growing season and two different seasons. Inoculation resulted in severe symptom development for some cultivars in all three field inoculations, but the results suggested environment had an effect. The first inoculation of 2011 and the 2012 inoculation were both conducted at the beginning of summer, whereas the second 2011 inoculation was done during hotter weather conditions with larger plants. The highest correlations were found between the early summer inoculations at both 8 and 12 WAI. Since environmental conditions can affect Xa colonization, a consistent seasonal timing of resistance screening tests would improve reliability.

Data collected from the greenhouse experiment were not well correlated with field experiment data for either resistance evaluation method. Disease symptoms were less severe and transitory in the greenhouse. In a previous study, plant recovery and low bacterial concentrations

in systemically infected leaves of known susceptible clones occurred (Garces, *et al*., 2014). These results suggest that leaf scald resistance evaluations should be conducted under field conditions.

Sugarcane breeding programs must be able to accurately determine leaf scald resistance levels during selection. Preliminary research demonstrated the potential of quantifying Xa populations with qPCR for resistance evaluation (Garces, *et al*., 2014). The results of this study found higher reliability for Xa quantification compared to rating visual symptom severity following inoculation with a larger population of clones with variable resistance levels. These results confirm that qPCR can provide an improved method for the evaluation of the resistance to leaf scald during screening and resistance studies.

CHAPTER 4: GENERAL CONCLUSIONS AND PROSPECTS FOR FURTHER ANALYSIS

4.1 General conclusions

- Both types of qPCR (SYBR Green and TaqMan) were highly specific for the detection of *Xanthomonas albilineans*, the causal agent of leaf scald, in sugarcane tissues and growing on culture media.
- The DNA samples extracted from leaf tissue did not show the presence of inhibitors in both qPCR assays.
- The DNA extraction method showed reliable results in terms of bacterial DNA extraction efficiency, always with a higher concentration of bacterial DNA in the initial DNA extraction than from bacteria remaining in the tissue.
- The correlation between the leaf scald resistance evaluation based on the severity of visual symptoms and the *X. albilineans* populations present in systemically infected leaf tissue of inoculated plants was highest at 8 weeks after inoculation.
- The use of composite samples (three single leaves each from a different plant) for determining the *X. albilineans* population by qPCR showed better correlation with disease severity ratings and greater reliability comparing inoculations performed at different times.
- The low correlation between the symptom severity and the bacteria population in leaf tissue, inability to distinguish known resistant and susceptible clones and the low correlation among the results obtained in the field experiments and the results obtained in the greenhouse suggest that greenhouse trials are less reliable for leaf scald disease resistance evaluation.

- Two other plant tissue sources, stalk base and apical meristem, showed generally low correlation between symptom severity and bacteria population and therefore should not be used for resistance evaluation by qPCR.
- Correlations were higher in comparisons of the same resistance evaluation method across experiments under variable environmental conditions for quantification of *X. albilineans* by qPCR than for rating symptom severity and highest at 8 weeks after inoculation.
- The results support the use of qPCR as an improved method for the evaluation of resistance to leaf scald in the field.

4.2 Prospects for further research

Bacterial quantification by qPCR is a suitable method for the routine evaluation of the leaf scald resistance in selection programs and provides a precise method for evaluating the resistance in different clones for additional purposes. For example, qPCR could be used for the evaluation of susceptibility in the detection of quantitative trait loci (QTLs) associated with leaf scald resistance. However, the demonstrated effect of environment even on qPCR results indicates that multiple evaluations will be needed to accurately detect QTLs.

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VITA

Andres Felipe Gutierrez was born in Cali, Colombia, in 1984. In 2000, he started his bachelor studies in Biology with emphasis in genetics in the Universidad del Valle in Cali, Colombia. After his graduation, he was a young investigator in CIDEIM (International Center of Training and Medical Research) sponsored by COLCIENCIAS (Administrative Department of Science, Technlogy and Innovation of Colombia). In CIDEIM, he worked on projects associated with drug resistance surveillance and molecular characterization of the resistance in *Plasmodium falciparum*, causal agent of malaria. In 2008, he started to work with sugarcane in Cenicana (Colombian Sugarcane Research Center) as a Research Associate. In Cenicana, he worked on projects to develop molecular markers associated with resistance to *Sugarcane Yellow Leaf Virus* and the molecular characterization of *Puccinia melanocephala*, the causal agent of brown rust in sugarcane. In the spring of 2011, he was admitted to Louisiana State University to earn a Master's degree in the Department of Plant Pathology and Crop Physiology with the guidance of Dr. Jeffrey Hoy, and a Ph.D. degree in the School of Plant, Environment and Soil Sciences (SPESS) with the guidance of Dr. Niranjan Baisakh.