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Comparative proteome and qPCR analysis of the sugarcane reaction to leaf scald caused by Xanthomonas albilineans

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COMPARATIVE PROTEOME AND QPCR ANALYSIS OF THE SUGARCANE REACTION TO LEAF SCALD CAUSED BY *XANTHOMONAS ALBILINEANS*

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DEDICATION

This dissertation is dedicated to my wife Pilly for her unconditional love, support and advice and to my daughters Anamaria and Maria Paula for that everyday unstoppable energy and happiness that gave me joy during these years. To my parents Roberto and Arbelia for their guidance, support, and example of life that help me to excel in life.

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LIST OF ABREVIATIONS

albD	albicidin detoxification gene
ATPase	adenosine triphosphate synthase
avr	avirulence genes
CP	Canal point
C_T	threshold cycle value
CFU	colony forming units
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CyP	cyclophilin
ddH2O	distilled, deionized water
DAMPs	damage associated molecular patterns
DNA	deoxyribonucleic acid
DS	disease severity
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
ET	ethylene
FKBP	FK506 binding protein
GLP	germin-like protein
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
Ho	Houma
HR	hypersensitive response
hrp	hypersensitivity response and pathogenicity genes

IEF	isoelectric focusing
IPG	immobilized pH gradient
ISR	induced systemic resistance
JA	jasmonic acid
L	Louisiana
LC-ESI MS	liquid chromatographic separation and electrospray ionization mass spectrometry
LRR	leucine rich repeat
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
NAD-MDH	NAD-dependent malate deshydrogenase
NCBI	National Center for Biotechnology Information
NRPS	non-ribosomal polyketide synthase
PAMPs	pathogen associated molecular patterns
PCR	polymerase chain reaction
pI	isoelectric point
PPIase	peptidyl-prolyl cis-trans isomerase
PR	pathogenesis-related
PVP	polyvinylpyrrolidone
QTL	quantitative trait locus
qPCR	real-time, quantitative polymerase-chain-reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SA	salicylic acid

SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
tAPOD	thylacoid ascorbate peroxidase
TCA	trichloroacetic acid
TCTP	translationally controlled tumor protein
TOF	time of flight
TR	thioredoxin
TVD	top visible dewlap
TVD-2	second younger leave above the TVD
TVD+3	third older leave below the TVD
Xa	*Xanthomonas albilineans* (Ashby) Dowson
2-D gel	two-dimensional electrophoresis gel

ABSTRACT

Leaf scald is an important disease of sugarcane caused by *Xanthomonas albilineans* (Xa). Leaf scald is controlled by the development and planting of resistant cultivars. However, erratic symptom expression makes conventional screening for resistance difficult. In addition, the mechanisms of resistance to leaf scald are not completely understood.

Real-time, quantitative polymerase-chain-reaction (qPCR) assays were developed utilizing SYBR Green for a highly sensitive detection method or a TaqMan probe to quantify Xa populations in infected plants. Xa populations detected by qPCR followed similar trends to disease severity ratings and vascular infection results for two resistant and two susceptible cultivars under greenhouse and field conditions. Low bacterial populations were found in newly emerged, systemically infected leaves of resistant cultivars. The results suggest that Xa population quantification by qPCR has the potential to be used as an alternative method for leaf scald resistance screening.

A comparative proteomic analysis identified differentially expressed proteins that suggested mechanisms for the sugarcane resistance response to Xa infection. Protein expression was compared for inoculated and mock-inoculated plants of two resistant and two susceptible cultivars during a time-course encompassing the responses to initial and systemic infection. Differential expression also was compared across cultivars with and without Xa infection. The number of up- and down-regulated proteins increased in the resistant cultivars during systemic infection. Identified, differentially expressed proteins were mostly in the chloroplast (67%), and 48% were involved in photosynthesis. Identified proteins were homologous to cyclophilin, translationally controlled tumor protein (TCTP), thylakoid ascorbate peroxidase (tAPOD), germin-like protein (GLP), and thioredoxins. All are proteins that have been associated with

induced defense responses. Down-regulation of APOD and the thioredoxins and up-regulation of GLP could result in accumulation of reactive oxygen species, particularly H_2O_2, in the cytoplasm and the apoplast. In addition, proteins involved in ethylene biosynthesis, another key signaling molecule in induced systemic resistance (ISR), were differentially expressed in resistant cultivars. Differences found between the two resistant cultivars indicate that resistance mechanisms can vary between genotypes, but the suggested resistance mechanisms for both were inducible responses. Finally, some differentially expressed proteins were involved in primary metabolism that could represent a plant fitness mechanism to provide energy needed for ISR.

The differences in Xa populations detected by qPCR and comparative proteomic analysis both suggest the existence of an induced mechanism of resistance against Xa during systemic infection. Multiple lines of evidence from the proteomic analysis suggest a triggering of ISR that would result in the limited Xa colonization of the new xylem developing above the apical meristem and lack of symptom development that is evident in leaf scald resistant sugarcane genotypes.

CHAPTER 1:
GENERAL INTRODUCTION

1.1 LEAF SCALD OVERVIEW

Leaf scald, caused by *Xanthomonas albilineans* Ashby (1928) Dowson 1943, is one of the most important bacterial diseases affecting sugarcane around the world. The disease has been reported in more than 66 countries (Rott and Davis, 2000). Losses of 10-34% in tons of cane per hectare and up to 30% in juice quality have been reported (Hoy and Grisham, 1994; Ricaud and Ryan, 1989; Rott and Davis, 2000; Rott et al., 1995; Saumtally and Dookun, 2004; Victoria et al., 1995). Sudden, widespread death of previously symptomless immature and mature plants has been associated with the acute form of the disease, in which whole fields planted with a susceptible cultivar can be destroyed in a few months (Ricaud and Ryan, 1989; Rott, 1993).

Xanthomonas albilineans (Xa) is a xylem-inhabiting bacterium that can cause three different types of infection and symptomatology: latent (no symptoms), chronic, and acute (Ricaud and Ryan, 1989; Rott and Davis, 2000; Saumtally and 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 to chlorotic longitudinal streaks along veins, general leaf chlorosis or more typically bleaching, leaf wilting, abnormal development of side shoots on stalks, reddish discoloration of vascular bundles at the node level, cavity formation in nodal and inter-nodal regions near the stalk apex, stunting, wilting, and death (Birch, 2001; Ricaud and Ryan, 1989; Rott and Davis, 2000; Saumtally and Dookun, 2004). Symptoms could be caused by bacterial xylem blockage and by the metabolic wastes produced (Rich, 2001), while bleaching, chlorosis and

necrosis are associated with changes in the cells caused by phytotoxins produced by the pathogen, especially albicidin which inhibits DNA replication and blocks plastid development.

The acute phase occurs as a sudden wilting of plants resulting in death, with little or no prior symptom expression. Large areas of a field may be affected in this manner (Ricaud and Ryan, 1989; Rott and Davis, 2000; Saumtally and Dookun, 2004; Hoy, personal communication). The acute phase was observed in a highly susceptible cultivar after a period of drought stress following rainy conditions (Ricaud and Ryan, 1989). The latent phase occurs and ends for reasons which are as yet unknown (Rott and 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 and 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; Saumtally and Dookun, 2004).

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 et al., 1994). In cultivar CP74-383, yield was adversely affected by leaf scald causing reductions in harvestable stalk number and stalk sucrose factors (Hoy and Grisham, 1994). During the same survey, extensive death of plants was observed in some fields planted with CP 74-383. An indirect but significant loss caused by the elimination of promising clones in cultivar selection programs also has continuously occurred in Louisiana and other countries (Hoy and Grisham, 1994; Ricaud and Ryan, 1989). About 20% of the promising new sugarcane clones are

rejected annually due to leaf scald susceptibility in Australia, even though crosses between susceptible parents are avoided (Birch, 2001).

This bacterium belongs to the *Xanthomonadaceae*. 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 and Ryan, 1989; Shaad et al., 2001). It can be isolated on a modified, selective Wilbrink's medium (Davis et al., 1994). The colonies are buff yellow but non-mucoid, with optimal growth at 25 ^{o}C and maximum at 37 ^{o}C (Ricaud and Ryan, 1989). The bacteria are slow growing and appear after 4-6 days as minute, circular, moist and shiny but non-mucoid, transparent honey-yellow colonies (Ricaud and Ryan, 1989). Loss of aggressiveness in artificial culture has been observed after several sub-culturings (Dattamajunder, 2004). This bacterium also is unusual because it apparently does not possess avirulence (*avr*) genes or hypersensitivity or pathogenicity (*hrp*) genes that encode a Type III secretion system typically found in phytopathogenic bacteria (Champoiseau et al., 2006; Genoscope, 2008).

The disease is spread and increased locally mainly by the use of infected cuttings for planting and contaminated tools used at harvest (Ricaud and Ryan, 1989; Rott and Davis, 2000; Victoria et al., 1995). Transmission is greater when the cut is made above the growing point, rather than a cut at the base of the stalk (Antoine and Ricaud, 1962). Cyclonic conditions also have been associated with disease spread (Ricaud and Ryan, 1989; Hoy and Grisham, 1994). In Louisiana, the distribution and incidence of leaf scald was high in areas close to the Gulf of Mexico where in 1992 a severe hurricane passed. Aerial transmission was reported in Guadeloupe (Klett and Rott, 1994), where the bacterium was exuded from the leaf hydathodes and then spread by aerial means. Daugrois et al. (2003) found bacteria in leaf water droplets 13 weeks after transplanting sugarcane plants. The maximum epiphytic

population and stalk infection was positively correlated with rainfall conditions, especially with tropical storms during three sugarcane crop seasons, and the proximity of the contaminated fields was a determinant of the phyllosphere contamination (Champoiseau et al., 2009). This study indicated that normal rainy conditions could be responsible for short distance field to field transmission. Maize and several weeds have been reported to be naturally infected by the bacterium (Rott and 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, such as quaternary ammonium, roguing of infected shoots, and quarantine measures during germplasm exchanges are additional methods used to prevent and control the disease (Ricaud and Ryan, 1989; Rott and Davis, 2000; Victoria et al., 1995). Another proposed control measure consists of the development of transgenic plants with a gene (*alb*D) from *Pantoea dispersa* for detoxification of albicidin (Zhang et al., 1997).

Considerable molecular biology information concerning leaf scald has been generated for the bacterium and pathogenesis (Champoiseau et al., 2006a; Rott et al., 1997). This includes albicidin antibiotic biosynthesis, activity, and detoxification (Basnayake et al., 1995; Champoiseau et al., 2006b; Huang et al., 2000; Huang et al., 2001; Roger et al., 2004; Zhang et al., 1997; Zhang et al., 1998a; Zhang et al., 1999), the *Alb* genes-albicidin interaction (Weng et al., 2005; Zhang et al., 1998b), and albicidin production (Bostock et al., 2006; Hashimi and Huang, 2008; Vivien et al., 2007). The antipathogenesis approach for leaf scald proposed by Birch (2001) suggested that control efforts target and interfere with pathogenicity factors including toxins and other factors, such as signal molecules for quorum sensing or

compounds and enzymes of the pathogen that elicit in sugarcane responses favorable for infection. The antipathogenesis approach could lead to the design of novel genes and transformed plants that will resist or inactivate pathogen pathogenicity factors. This is the case of the *alb*D gene from *Pantoea dispersa* (Zhang et al., 1997). The *alb*D sequence encodes an albicidin hydrolase that prevented the release of albicidin from a genetically modified *X. albilineans* resulting in the inability to incite symptoms in a susceptible sugarcane cultivar (Zhang et al., 1997). This conferred a high level of resistance to chlorosis induction and bacterial systemic invasion in transgenic sugarcane plants (Zhang et al., 1999). However, Champoiseau et al. (2006a) demonstrated that variability in albicidin production among *X. albilineans* isolates was not correlated with the ability to incite disease indicating that, while albicidin is closely linked to disease symptom expression, there were other as yet unknown pathogenicity factors. A subsequent study identified additional pathogenicity factors associated with various membrane proteins (Rott et al., 2011).

1.2 SUGARCANE RESISTANCE TO LEAF SCALD

Cultivated sugarcane clones are the result of interspecific hybridization involving *Saccharum officiarum*, *S. barberi*, *S. sinense*, *S. spontaneum* and *S. robustum* (Julien et al., 1989; Wanderley, 2007). Because of its multispecies origin, sugarcane is thought to have one of the most complex plant genomes carrying highly variable chromosome numbers (generally 2n = 70-120) with a commensurately large DNA content. This complexity complicates the application of conventional genetics and plant breeding techniques for inclusion of a particular resistance trait into agronomically elite clones (Lacksumanan, 2005; SUCEST, 2008; Vettore et al., 2001). Despite this complexity, resistance to leaf scald is one of the most important control methods (Ricaud and Ryan, 1989). However, accurate evaluation of

resistance levels in sugarcane clones is difficult since reactions obtained from field inoculations are erratic. Lopes et al. (2001) established an alternative inoculation test in a greenhouse test using infectivity titration.

The most commonly used artificial inoculation method for resistance screening is the decapitation method, in which young shoots are cut above the apical meristem, and the inoculum is applied to the cut surface (Koike, 1965; Ricaud and Ryan, 1989). Resistance has been associated with the extent of bacterial colonization (Rott et al., 1994; Rott et al., 1997), but the mechanisms of resistance are unknown. Rott et al. (1997) found a correlation with disease severity and bacterium presence in the shoot apex in field and greenhouse experiments. Susceptible cultivars were always extensively colonized in the apex and lower part of the stalk, whereas Xa populations in the shoot apex were low in resistant cultivars.

These results suggest a method to accurately detect and compare Xa populations in different sugarcane genotypes might provide an alternative method for resistance screening. Real-time, quantitative PCR (qPCR) is a highly sensitive, reproducible and accurate method that is 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* (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 potential for high sensitivity and specificity could make qPCR a superior method for reliable detection of Xa. If the relationship between Xa population dynamics inside the plant, symptom severity, and resistance level is confirmed, Xa quantification with qPCR could

provide a more reliable method for determining resistance levels in sugarcane genotypes in inoculated tests.

Leaf scald causes several morphophysiological changes in young and mature cane (Rao et al., 2001). Madan et al. (1982) found that the activity of acid invertase was elevated in affected plant buds, the quantity of reduced sugars increased five-fold, and the protein content decreased. In infected leaves, the activity of nitrate reductase and the synthesis of protein and chlorophyll were decreased. Moreover, in old plants, nitrate reductase activity was inhibited, leaf tissue dehydration occurred, and protein and chlorophyll degradation increased. In sugarcane infected with Xa, the composition of polyamines and phenolic acids was increased (Fontaniella et al., 2007). Levels of ornithine descarboxilase were increased, and polyamine metabolism was increased. They proposed that the susceptibility of the C439-52 cultivar was linked to a decrease in the levels of free phenolic compounds and the massive accumulation of phenolic conjugates. Solas et al. (2003) found that xylem, phloem and mesophyll tissues in general were occluded by a xanthan-like polysaccharide, and the cell walls of the xylem were disintegrated. Zhang et al. (1999) analyzed the development of leaf scald in transgenic plants and suggested that albicidin-mediated disruption of plastid division and development could facilitate systemic invasion and disease development by weakening plant defense mechanisms.

Systemically infected resistant and susceptible cultivars expressing chronic symptoms were compared for differential gene expression using the macro-array technique based on the SUCEST expression profile of 3,575 ESTs from sugarcane (Dabbas et al., 2006). In a resistant cultivar, ESTs of proteins were induced with similarities to isopropanoid biosynthesis, ethylene biosynthesis, a transmembrane protein kinase with leucine rich repeat (LRR), a

leucine zipper, and a mutase/prephenato dehydratase associated with cell wall lignification for environmental stress adaptation. Conversely, an EST of a protein with similarities to a glutathione synthetase and ESTs for plant cellular expansion, detoxification and auxin transport were repressed. In Xa-infected plants of both the susceptible and resistant cultivar, a protein with similarity to an auxin transporter was repressed. In a susceptible cultivar, ESTs associated with plant defense response, ethylene biosynthesis, transcription regulation, and a protein with similarity to Zinc finger type C3H4 were repressed. An EST of a protein similar to an RNA helicase was induced in the resistant cultivar and repressed in the susceptible one. This protein is associated with plastid differentiation and cold tolerance. According to Dabbas et al. (2006), the differential induction of RNA helicase in the resistant cultivar may be one of the factors that confer resistance to Xa, and its repression could be the cause of susceptibility. They indicated that resistance could be associated with bacterial growth in the plant and with a repression or induction of metabolic pathways involved with resistance.

Vettore et al. (2001) indicated that the SUCEST project has 237,954 ESTs potentially derived from protein-encoding messenger RNA (mRNA). Unfortunately, SUCEST information is derived from a private project that does not have publicly available information. However, a total of 280 genes similar to R-genes were identified by Wanderley et al. (2007) from the EST sequences of SUCEST and are available in Genbank (NCBI). They found a class I kinase, a class II LRR-NBS-CC, a class IV LRR and a class V LRR kinase. A class V type gene was found in 15 of the clusters that corresponded to the LRR-kinase-Xa21 group, while five best matches were similar to Xa21 genes from *Sorghum bicolor, Oryza sativa* and *Zea mays*. These findings represent a valuable resource for the development of

markers for molecular breeding and development of resistance gene analogs or gene-specific markers specific for sugarcane and other related cereal crops (Wanderley et al., 2007).

Another approach to study the sugarcane reaction against *X. albilineans* could be proteomics, utilizing recent advances in two-dimensional electrophoresis to analyse the global protein pattern expression under differential conditions of infection, in combination with quantitative, real-time polymerase chain reaction (qPCR), to quantify the bacterial population. Two-dimensional gel electrophoresis combined with protein identification by mass spectrometry is currently most frequently employed method for proteomics (Gorg et al., 2004). Two-dimensional gel electrophoresis with immobilized pH gradients enables the separation of a complex mixture of proteins according to isoelectric point, molecular mass, and relative abundance (Gorg et al., 2004). The two-dimensional electrophoresis system was used in sugarcane to study protein variation during leaf de-differentiation (Ramagopal, 1993) and to identify a drought-inducible protein localized in the bundle sheath cells (Sugihario et al., 2002). Ramagopal (1989) pointed out that the two dimensional approach presents a unique opportunity to clone and characterize a large number of proteins expressed by the sugarcane genome.

A proteomic approach to study protein differential expression could provide a better understanding of the sugarcane reaction against infection by *X. albilineans*. The identification and characterization of differentially expressed proteins under different conditions related to resistance and susceptibility in different sugarcane clones or association with other factors affecting disease development, such as bacterial population, could help to understand and possibly allow monitoring of the reaction of sugarcane to leaf scald in time and specific tissues and elucidate molecular aspects of the expression of resistance. The information

generated could be integrated with sugarcane genomic data to improve our understanding of the nature of the sugarcane and bacterium interaction. In addition, it might be possible to utilize some of the information to develop molecular markers that could be used in marker-assisted selection for resistance to leaf scald.

CHAPTER 2:
DETECTION AND QUANTIFICATION OF *XANTHOMONAS ALBILINEANS* USING QPCR AND CHARACTERIZATION OF SUGARCANE RESISTANCE TO LEAF SCALD

2.1 INTRODUCTION

Leaf scald of sugarcane (inter-specific hybrids of *Saccharum* L.) is a systemic, vascular bacterial disease that can cause severe yield reductions, eliminate potential cultivars, and pose quarantine concerns for germplasm exchange. Leaf scald has been reported in more than 66 countries (Rott and Davis, 2000) and was first detected in Louisiana in 1993 (Hoy and Grisham, 1994). 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; Saumtally and Dookun, 2004). Latent symptoms in susceptible cultivars also can result in the distribution of infected seed-cane within sugarcane production regions.

Several methods have been developed to detect and identify the leaf scald causal agent, *Xanthomonas albilineans* (Ashby) Dowson, including selective media, microscopy, serological, and polymerase chain reaction (PCR) based techniques (Alvarez et al., 1996; Champoiseau et al., 2006; Davis et al., 1994; Guzman et al., 1997; Jaufeerally et al., 2002; Lin et al., 1994; Lopez et al., 2001; Pan et al., 1997; Pan et al., 1999; Wanga et al., 1999). These techniques have been used to detect infection, differentiate strains of *X. albilineans* (Xa), index seed-cane, and assay germplasm in quarantine for exchange (Alvarez et al., 1996; Champoiseau et al., 2006; Garces et al., 2005; Jaufeerally et al., 2002; Lopez et al., 2001; Victoria et al., 1998). However, detection of Xa in asymptomatic plants can be problematic with any of the detection methods.

Host plant resistance is the primary method of leaf scald control (Ricaud and Ryan, 1989; Rott and Davis, 2000). The most common method used to discriminate different levels of resistance is through inoculation of plants with the decapitation method (Koike, 1965), in which shoots are cut above the apical meristem and inoculum is applied to the cut surface. Cultivar reaction is then evaluated according to some combination of observed levels of disease incidence and severity. Different arbitrary rating systems have been proposed using qualitative information based on the type of symptoms and the quantity of plants with different symptom types (Rott et al., 1997). However, the erratic nature of leaf scald symptom expression even in inoculated tests makes screening for resistance a difficult task. Environmental stress conditions affect symptom severity in naturally infected plants, inoculation success, and the severity of symptom expression after inoculation (Ricaud and Ryan, 1989). Bacterial population at the shoot apex determined by quantification on semi-selective medium was associated with leaf scald resistance level in sugarcane clones varying in susceptibility (Rott, 1997). This suggests the possibility of a resistance screening method based on the quantification and comparison of Xa populations in different clones.

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* (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 potential for high sensitivity and specificity could make qPCR a superior method

for reliable detection of Xa in all of the situations described previously. If the relationship between Xa population dynamics inside the plant, symptom severity, and resistance level is confirmed, Xa quantification with qPCR could provide a more reliable method for determining resistance levels in sugarcane genotypes in inoculation tests. The objectives of this study were to develop a qPCR for Xa and determine its potential for detection, bacterial population quantification, and leaf scald resistance screening.

2.2 MATERIALS AND METHODS

2.2.1 DNA Extraction

Three different DNA extraction protocols were evaluated. The first included three freeze and thaw cycles of leaf vascular exudates and stem xylem sap samples in sterile, distilled, deionized water (sdH_2O) (Davis et al., 1997). Samples were frozen with liquid nitrogen for 2 min and thawed in a water bath at 60 ^{o}C for 10 min. The second consisted of a lysis boiling method described by Jacobs (2008) (Appendix 1) using the same exudate and sap samples. The last one employed the Qiagen DNeasy Plant Mini Kit for DNA extraction (Qiagen Inc., Valencia, CA). It was used initially for bacterial DNA from cultures and later for total DNA extraction from the apical meristem.

Leaf exudates and sap from the stem were transferred to 1.5 ml tubes for extraction with each method. After centrifugation at 9000 g for 5 min, the supernatant was discarded and the bacterial pellets were suspended in 100 µl of sdH_2O or lysis buffer, to follow the first or second DNA extraction protocol, respectively. The DNA from the meristem was extracted by macerating 100 mg of tissue in a universal BIOREBA extraction bag of 12x14 cm (BIOREBA AG, Switzerland) containing 1.5 ml of AP1 buffer from the Qiagen DNeasy plant mini kit. DNA was extracted using the kit according to the manufacturer's guidelines. Total DNA

extracted by each method was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and diluted to10 ng/µl for qPCR.

2.2.2 Primer and Probe Design

Six different sets of primers were designed from the Xa genome sequence (Renier et al., 2007; Royer et al., 2004) using the program Beacon Designer (Premier Biosoft International, Palo Alto, CA) (Table 2.1). The gene cluster of albicidin bio-synthesis corresponding to the *alb*I gene, the nonribosomal polyketide synthase NRPS -1/3 module, and the A4-A5 region (GenBank accession number AJ586576, Royer et al., 2004) was targeted for Xa specific primers. A TaqMan Double-Quenched Probe™ (5'FAM/ZEN/3'ABkFQ™) with two quenchers, ZEN and ABkFQ, and the FAM reporter was developed following the manufacture instructions (IDT Integrated DNA Technologies, Skokie, IL). After blasting the sequences, all the primer sets were tested using a 10-fold dilution series prepared from an initial suspension of Xa with $3.4x10^7$ colony-forming-units per ml (CFU/ml).

2.2.3 Conditions of Amplification for Conventional, SYBR Green, and TaqMan PCR

- **Conventional PCR**

Following the conventional PCR protocol of Davis et al. (1997), a 2 µl aliquot from each DNA extract was mixed with 12.5 µl of GoTaq master mix (2X) (Promega BioSciences, CA), 0.1 µl of each forward and reverse primer (Xalb2 primer set) (100 µM, IDT Integrated DNA Technologies), 10.16 µl of sdH2O, and 0.14 µl of BSA fraction V (100 µg/ul) for a final volume of 25 µl. The conditions of amplification were as follows: an initial step at 94 °C for 4 min, followed by 31 cycles of DNA denaturation at 94 °C for 30 sec, annealing at 55 °C for 30sec, polymerization at 65 °C for 3 min, and a final extension at 65 °C for 3 min.

- **SYBR Green PCR**

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) (IDT Integrated DNA Technologies), and 5.3 µl of sdH_2O. Six primer sets were designed and evaluated. 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 sec, and annealing-polymerization at 60 °C for 30 sec. 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 sec. A 10-fold dilution series of Xa DNA extracted from a 10^7 CFU/ml suspension was diluted five or six times to be used as standards with concentrations from 10^7 to 10^2 CFU/ml to determine the qPCR amplification efficiency, cycle threshold value (C_T), and concentration of the unknown samples.

- **TaqMan PCR**

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^{TM} probe XaQ (2 µM) (IDT Integrated DNA Technologies), 5 µl of sdH_2O 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 40 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 Xa. 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 all experiments.

2.2.4 Plant Material, Inoculation, and Sample Collection

- **Plant Material**

The sugarcane cultivars used in both greenhouse and field experiments were LCP 85-384, Ho 95-988, HoCP 85-845, and HoCP 89-846. Two cultivars, HoCP 96-540 and HoCP 00-950, were included only in field experiments. According to ratings determined from evaluation of symptom severity following inoculation in the field and field observations of natural infection, LCP 85-384, Ho 95-988, and HoCP 96-540 were resistant, HoCP 00-950 was moderately susceptible, and HoCP 85-845 and HoCP 89-846 were susceptible (J. Hoy, unpublished). A group of 15 different cultivars, including the cultivars listed above and CP 65-357, CP 70-321, CP 73-351, L 97-128, L 99-226, L 99-233, L 01-283, L 01-299, and Ho 01-12, were inoculated in additional experiments under greenhouse conditions. L 97-128 was rated as resistant to leaf scald. The other cultivars were rated as moderately susceptible exhibiting variable levels of symptom severity in different inoculation tests.

- **Bacterial Isolation and Plant Inoculation**

Bacteria were isolated from a longitudinal section of the leaf that included a pencil-line symptom. Tissue was surface-sterilized with NaOCl (10% v/v of commercial product) for 30 s and rinsed two times with sdH_2O. The leaf sections were dried in a laminar flow hood, cut in small pieces 1-2 mm wide, and placed in a 1.5 ml microcentrifuge tube (Eppendorf, Hauppauge, NY) containing 1 ml of sdH_2O. The tube with infected tissue was left overnight at 4 °C. A loop of bacterial suspension was transferred to XAS selective medium with antibiotics (Davis et al., 1994) (Wilbrink's medium that contains 5 g Bacto Peptone, 10 g sucrose, 0.5 g K_2HPO_4 $3H_2O$, 0.25 g $MgSO_4$ $7H_2O$, and 0.05 g Na_2SO_3 per liter supplemented with 25 mg cephalexin, 30 mg novobiocin, 50 mg kasugamycin, 100 mg cycloheximide, 2 mg benomyl,

and 5 g KBr). After 5-8 days, single colonies were selected to obtain pure cultures on solid XAS medium without antibiotics. Pure cultures were incubated at 28 °C, and after 48 h, 5 ml of sdH_2O was added to each plate. The suspended Xa colonies were diluted to obtain 10^8 CFU/ml based on spectrometric absorbance (0.18 optical density at 590 nm) and direct colony counting on XAS medium. The inoculum was kept at 4 °C in the dark prior to inoculation. In both greenhouse and field experiments, 2-month-old plants were inoculated using the decapitation method (Koike, 1965) (Figure 2.1).

Plants were inoculated by placing 100 µl of bacterial suspension 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 greenhouse experiments, and plants in single-row plots, 2 m in length with two replicates were inoculated in field experiments. Inoculations were done at the end of the day at about sunset. Humidity in the greenhouse was increased by wetting the floor below the bench, and the ventilation fan was turned off overnight to avoid desiccation of inoculum or inoculated tissue.

Figure 2.1. Decapitation method used for bacterial inoculation of sugarcane plants. (A) Cut made just below the top visible dewlap leaf (see detail in Figure 2.2), (B) Point of inoculation after cutting of apex leaves, (C) Emerging inoculated leaves, (D) Leaf chlorosis pattern 2 weeks after inoculation, indicating successful inoculation.

- **Sample Collection from Leaves**

Leaves collected from the greenhouse experiments were the youngest fully emerged leaf commonly designated in sugarcane as the top visible dewlap (TVD) leaf, the second younger leaf above the TVD (TVD -2) and the third older leaf below (TVD +3) (Figure 2.2). Leaves from the field were collected in 10 composite samples of five newly emerged leaves (TVD -2) with five composite samples collected from each 2 m plot. All samples from the greenhouse and field were collected in plastic bags and kept at 4 °C until processing. In both greenhouse and field experiments, 20 leaf discs were collected from each leaf using a 5-mm-hole paper punch, with nine discs from the lamina on each side of the midvein (Figure 2.3). The discs were placed together in a 1.5 ml microcentrifuge tube containing 1 ml sdH_2O. Between samples, the paper punches were surface sterilized by dipping in 95% ethanol and flaming.

- **Stalk Sample Collection from the Shoot Base and Apex (Including Meristem)**

A 7-cm-long internode section from the base of the stalk was excised using sterilized clippers (Figure 2.3). The vascular sap from the internode was extracted in a 50 ml tube by centrifugation at 3,500 rpm for 5 min and transferred to a 1.5 ml sterile microcentrifuge tube.

Samples from the meristem and surrounding tissue were collected from the shoot apex by peeling off fully emerged leaves and collecting 100 mg of tissue that included the apical meristem and not yet emerged apical leaf tissue (Figure 2.3). Samples were kept at 4 °C during the DNA extraction procedure to reduce tissue oxidation. For bacterial streaming, the tissue was chopped and transferred to a microcentrifuge tube, while for genomic DNA extraction, 100 mg of tissue was homogenized as described in the next section.

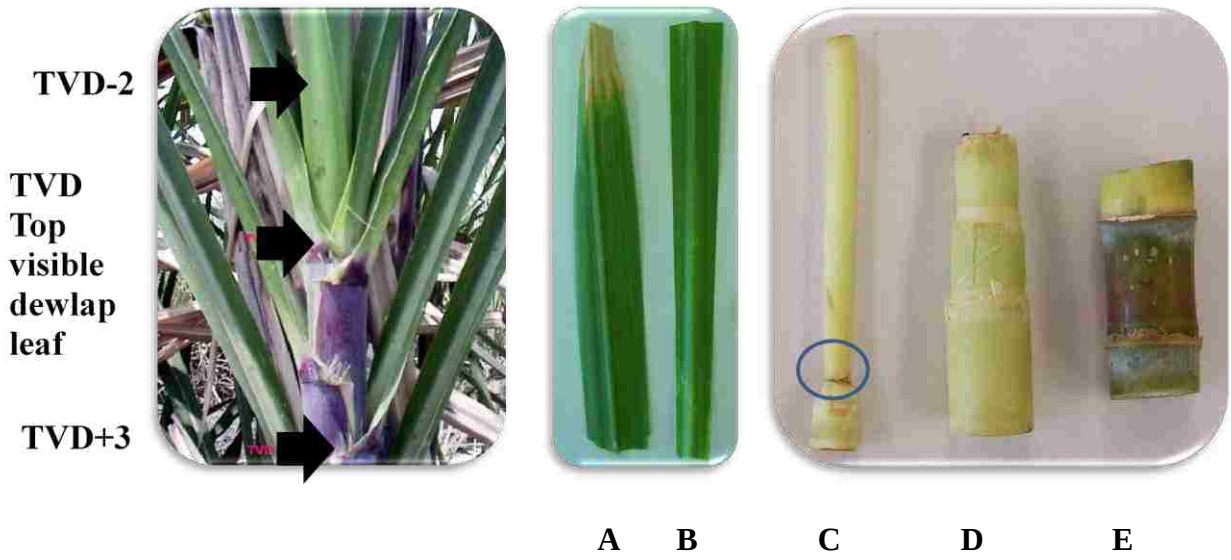


Figure 2.2. Samples collected for bacterial quantification. (A) Inoculated leaf, (B) Newly emerged, systemically infected leaf, (C) Apical meristem, (D) Shoot apex, (E) Basal stem internode. Top visible dewlap (TVD) leaf that is the youngest fully emerged leaf, second younger, emerged leaf above the TVD (TVD -2), and third older leaf below the TVD (TVD +3).

Figure 2.3. Sampling of leaf discs using a paper hole punch.

2.2.5 Disease Assessment

- **Disease Severity and Incidence**

Disease severity in the greenhouse and field experiments was evaluated according to the type of symptoms observed and number of stalks exhibiting different symptom types in each plant using the formula described by Rott, et al. (1997) 4 months after inoculation in the greenhouse and 2 months after inoculation in the field under Louisiana environmental conditions.

Severity was evaluated according to the type of symptom observed in each plant using the formula: DS = [(1 × FL + 2 × ML + 3 × CB + 4 × N + 5 × D)/5 × T] 100, where the 1 to 5 multiplication factors increasingly weight the number of stalks with more severe systemic symptom types as follows: FL = number of stalks with leaves exhibiting one or two narrow, white, pencil-line streaks, ML = number of stalks with more than two pencil-line streaks in leaves, CB = number of stalks with leaf chlorosis or bleaching, N = number of stalks with leaf necrosis, D = number of dead stalks or stalks with side shooting, and T = total number of stalks.

- **Vascular Colonization**

Samples of stalks from greenhouse experiments were collected to assess Xa vascular infection by stalk tissue printing on air-dried, selective XAS-medium (Appendix 2) (Figure 2.4). Basal stalk internode sections were cut with flamed clippers then briefly imprinted on the medium surface. Two days after blotting, vascular infection was evaluated according to the scale described by Champoiseau et al. (2006a) that used five levels of colony growth from 0 to 4, where 0 = no bacterial growth, 1 = 1-10 colonies in the stalk print, 2 = more than 10

colonies or confluent growth in less than 25% of the imprint, 3 = confluent growth in 25 to 75% of the imprint and 4 = more than 75% of confluent bacterial growth on the blotted area.

2.2.6 Data Analysis

A total of 20 individual stalk replicates were arbitrarily selected for each cultivar in two field experiments. Disease incidence and severity were analyzed using ANOVA and the minimal significant differences for means were established according to Tukey's at $P \leq 0.05$ (Infostat, National University of Cordoba, Argentina). Xa populations were quantified by qPCR through introgression of the positive samples average on the standard curve determined by linear regression of the known Xa concentration dilution series.

Figure 2.4. Stalk tissue printing on XAS medium with antibiotics for evaluation of *X. albilineans* vascular infection and estimation of the extent of colonization.

2.3 RESULTS

2.3.1 DNA Extraction and qPCR Optimization

After bacterial DNA quantification, the modified Jacob's lysis method yielded 4.2 x more DNA than the freeze and thaw (FT) method and 132 x more than the Qiagen kit protocol. However, the FT method combined with lysis buffer alkaline disruption similarly increased DNA yield 4 x compared to the FT method, indicating that lysis improved DNA

release from bacterial cells (Figure 2.5). Boiling and alkaline disruption with lysis buffer were selected for DNA extraction following bacterial diffusion from meristem and leaf tissue pieces and stem vascular sap. However, after the samples where left overnight at 4 ^{o}C in water before DNA extraction, the meristematic tissue had 4.6 x more phenolic compounds in suspension (Figure 2.6) as estimated by spectrometric absorbance at 400 nm, and the addition of polyvinylpyrrolidone (PVP 40,000 MW) did not reduce phenolics. Therefore, the total plant DNA from the meristem was extracted using the Qiagen DNeasy Plant Mini Kit to avoid any effects of phenolic compounds during PCR, using homogenization buffer instead of liquid nitrogen during the first step of sample preparation.

2.3.3 SYBR Green and TaqMan PCR Amplification of Xa

Primers used in conventional PCR, XaAlb2, and SYBR Green primer sets 1, 2, and 3 showed more than one peak of dissociation or melting curve after amplification, indicating amplification of more than one product (Table 2.1, Figure 2.7). Primer amplification efficiency and the melting curve showed that primer sets 4, 5, and 6 and the TaqMan XaQ primers had a high amplification efficiency and a high reproducibility between standard dilutions according to the calculated slope value of -3.3 and efficiency (Figures 2.7 and 2.8,Table 2.1). The highest amplification efficiency was observed with the TaqMan XaQ probe, then primer sets 2 and 6, followed by sets 5 and 4. The TaqMan XaQ probe and primer sets 5 and 6 showed threshold C_T values between 15 and 35 for the entire range of six, 10-fold standard dilutions of the known bacterial concentration. The standard curves of the SYBR Green primer sets 5 and 6 and the TaqMan XaQ probe showed a high reproducibility between replicates of the standards with C_T standard deviation values that ranged from 0.03 for the lower C_T value and 0.31 for the highest (Figures 2.9 and 2.10, Table 2.2).

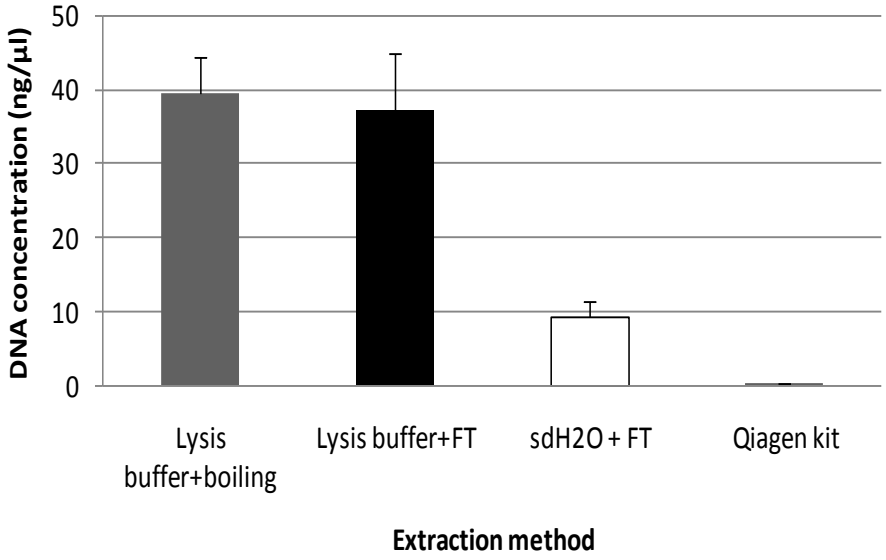


Figure 2.5. Comparison of total DNA extracted from *X. albilineans* suspension using different methods. FT = freeze and thaw, sdH2O = sterile, distilled water.

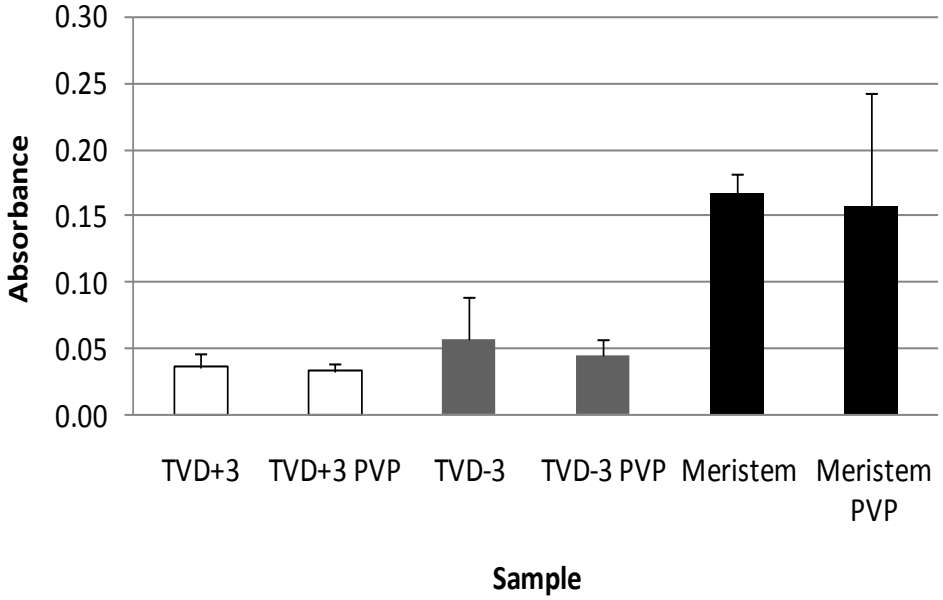


Figure 2.6. Estimation of phenolic compound content (absorbance at 400 nm) of exudates from different sugarcane tissue samples with and without the addition of polyvinyl pyrrolidone (PVP) (40,000 MW). TVD +3 is the third older leaf below the youngest fully emerged leaf or top visible dewlap leaf (TVD). TVD -3 is the third younger leaf above the TVD leaf.

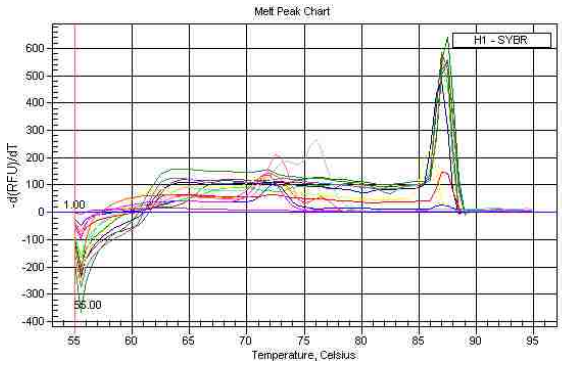
The single peak of fluorescence associated with primer sets 5 and 6 for Xa DNA fragments of 186 and 98 bp with the SYBR Green PCR showed a single melting point of 82 ˚C and 82.5 ˚C, respectively (Figures 2.7 and 2.8).

The optimal SYBR Green and TaqMan XaQ probe qPCR established were able to quantify and detect a low level of bacteria in a reaction. The amplification plots (Figure 2.9) and the standard curves (Figure 2.10) of the six 10-fold Xa dilutions with a range of 10^7 to 10^2 CFU/ml indicate successful detection for all dilutions using primer sets TaqMan probe XaQ and SYBR Green primer set 6. The standard curve y-intercept of primer set 6 and the TaqMan probe showed the highest value for the theoretical "1-copy" detection limit with 43.7, and 42.2 C_T values, respectively (Table 2.1). After selecting the best primers for Xa quantification, a 10-fold dilution series from 10^7 to 10^2 CFU/ml was used to compare TaqMan XaQ, SYBR Green primer set 6, and conventional PCR sensitivities. The TaqMan probe and SYBR Green primers had similar Ct values for all the bacterial dilutions ranging from 15.4-17.0 to 32.2-33.5 for the TaqMan and SYBR Green PCRs, respectively. In contrast, the conventional PCR did not detect the lowest dilution of 10^2 CFU/ml (Figure 2.10, Table 2.2).

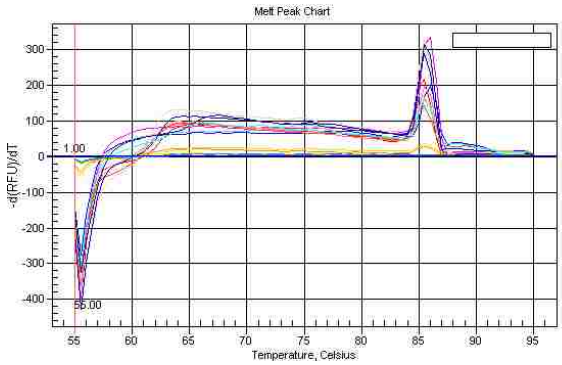
2.3.4 Disease Incidence and Severity

Symptoms developed initially in inoculated leaves of both resistant and susceptible cultivars. Inoculated leaves of HoCP 85-845, HoCP 89-846, and Ho 95-988 exhibited chlorosis, pencil-lines, yellow chlorotic streaks or necrosis of the tissue from the edge of a cut leaf toward the base of the leaf 2-3 wk after inoculation (Figure 2.11). LCP 85-384 exhibited only very short necrotic streaks at the cut leaf edge.

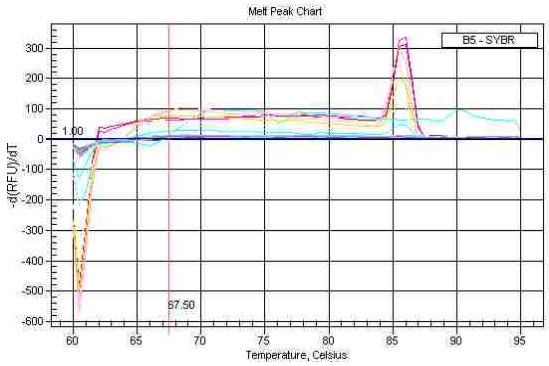
XaAlb2 set (Used in conventional PCR)



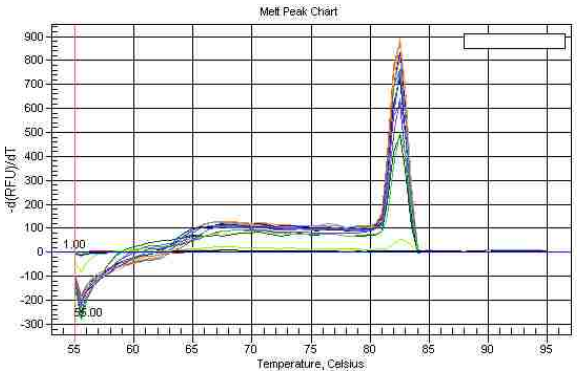
Primer set 3



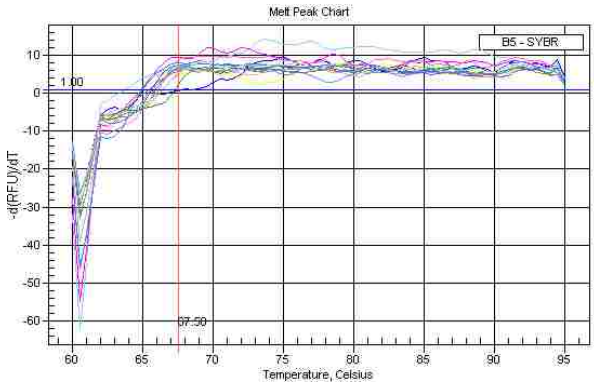
Primer set 1



Primer set 4



Primer set 2



Primer set 5

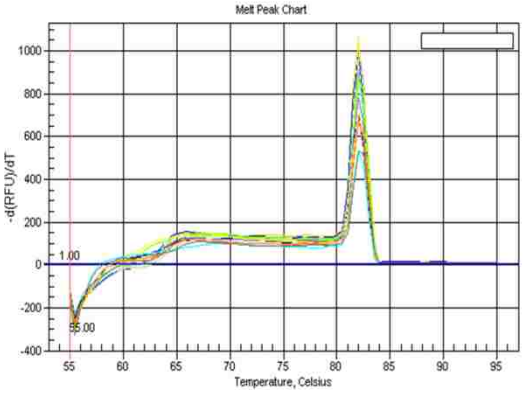


Figure 2.7. Melting point curve analysis following amplification with different sets of primers, XaAlb2 for conventional PCR and sets 1-5 for SYBR Green PCR, that amplified DNA targets associated with the gene cluster of albicidin synthesis in the bacterium, *Xanthomonas albilineans*, accession number AJ586576. Different colored lines indicate the melting point (fluorescence emission peak) for multiple double-stranded DNA amplicons.

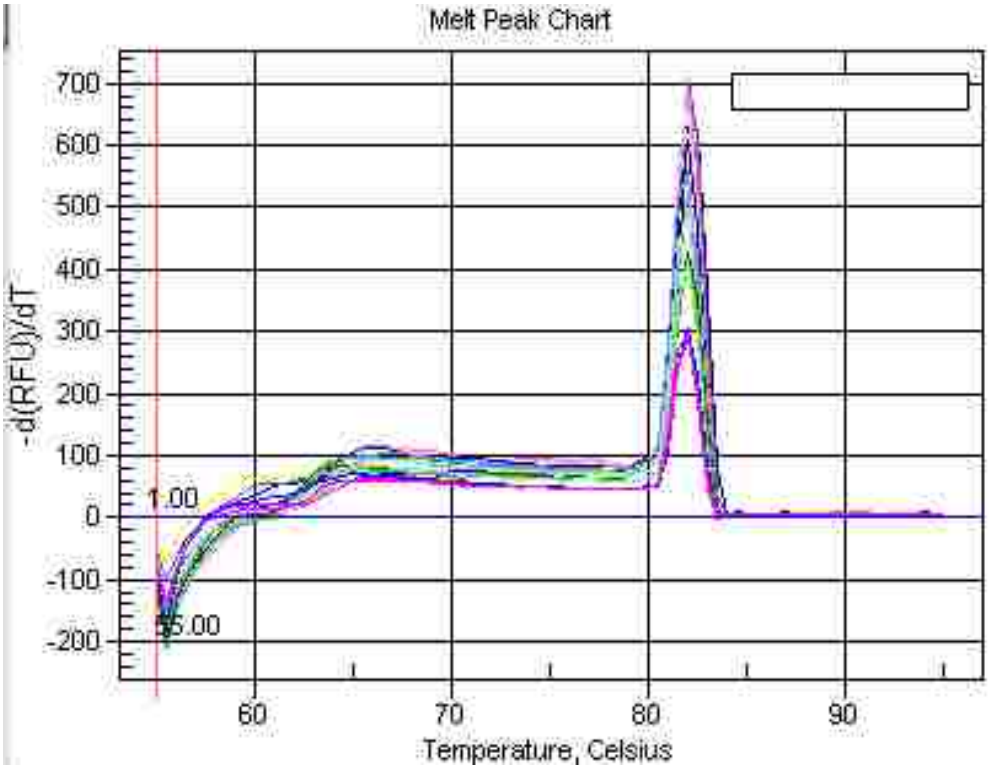
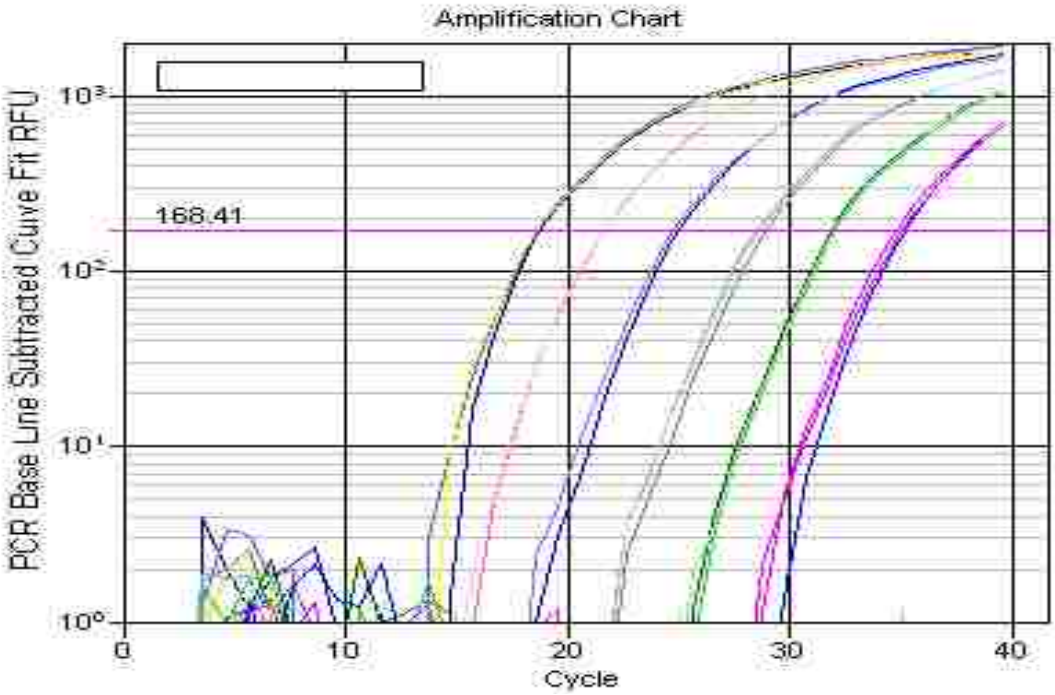


Figure 2.8. Melting point curve analysis for SYBR Green PCR primer set 6 that amplified one single fragment of the *X. albilineans* DNA target (Accession number AJ586576). Different colored lines indicate the melting point (fluorescence emission peak) for multiple double-stranded DNA amplicons.

A



B

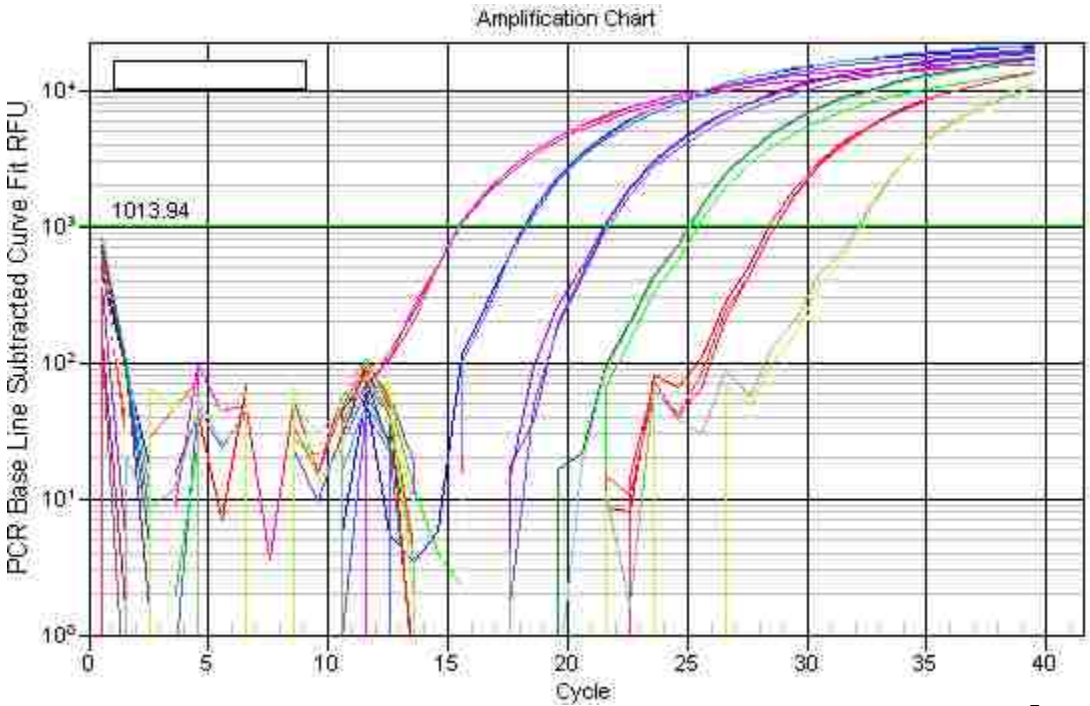


Figure 2.9. Amplification curves of a *X. albilineans* 10-fold dilution series from 10^7 to 10^2 colony forming units ml^{-1} for SYBR Green primer set 6 (A) and TaqMan XaQ ZEN Double-Quenched™ probe (B).

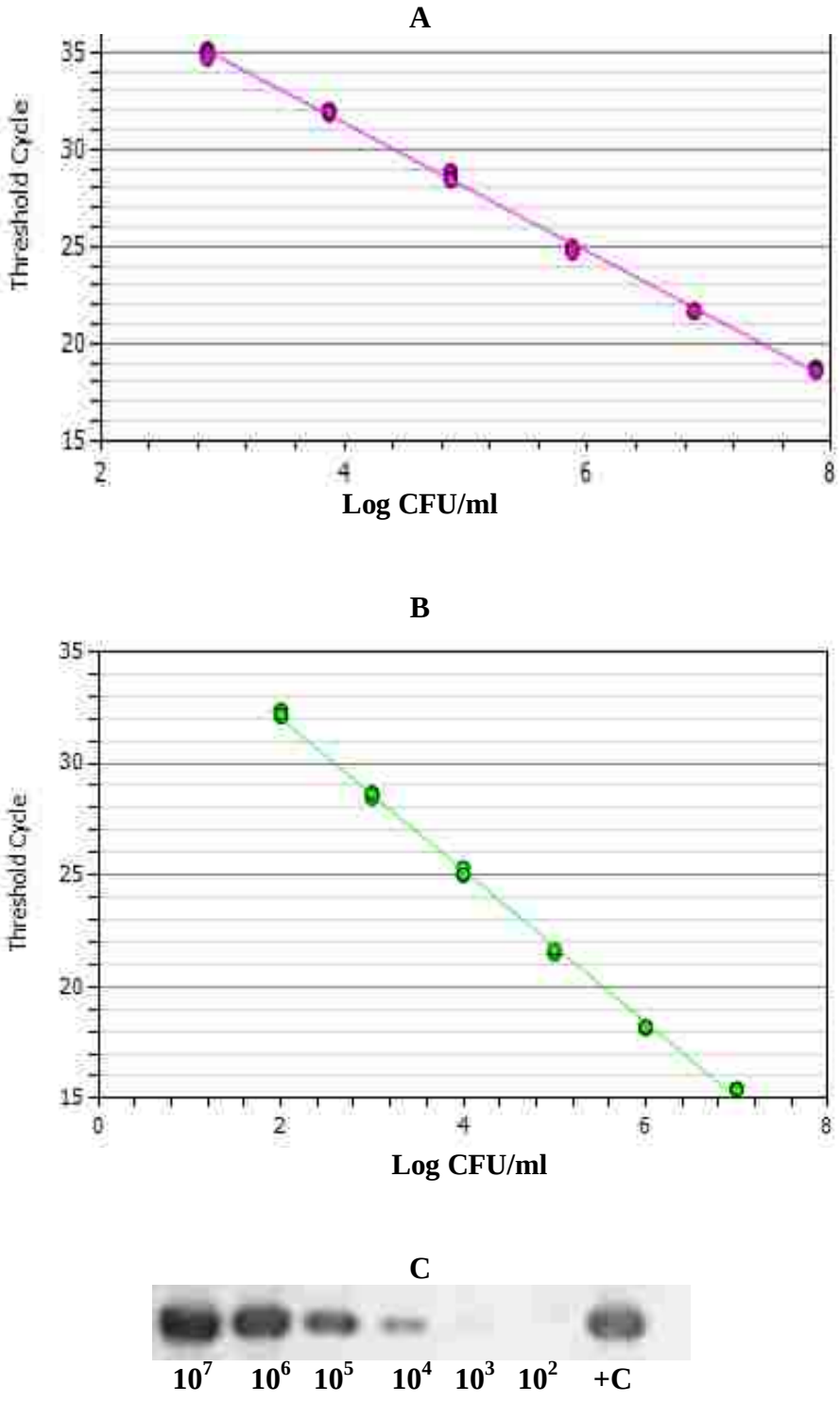


Figure 2.10. Standard curves of SYBR Green primer set 6 (A) and TaqMan probe (B) and end-point for conventional PCR (C) amplification of *X. albilineans* 10-fold dilution series from a suspension of 10^7 colony-forming-units per ml (CFU/ml). (TaqMan PCR efficiency = 97.1%, $R^2 = 0.998$, slope = -3.34)

Table 2.1. Primers for conventional, SYBR Green, and TaqMan PCR assays for the detection and quantification of *X. albilineans.*

Ida	Probe and primer oligonucleotide sequence 5' - 3'	Product (bp)[b]	Tm[c]	GC %[d]	PCR efficiency (%)	R squared	Slope[e]	y-Intercept[f]	Melting temperature[g]
FAM									
Taqman XaC	5'/56-FAM/CGT CAC CTG /ZEN/CCA TTG CTC AAT CGC C/31ABkFQ/ -3		64.4	60.0	97.1	0.998	-3.39	42.2	NA
XaQr	5'- GCG ATG GCA CTA GGT ACA GC -3'	148	58.1	60.0					
XaQf	5'- TTT GCG GTG TCG GTA AAG GAG -3'		57.6	52.3					
SYBR Green									
XaAlb2-f3	5'- CAC ACA CAC AAT ACA GCA TTG CGG -3' (Davis et al 1997)	440	59.1	50.0	54.5	0.995	-5.29	27.8	87, 74, 72, 65, 63.0
XaAlb2-r3	5'- CCC AAC TTA CTT GAG GCT ATG G -3'		55.2	50.0					
XAprF1	5'- CTT GCT GCT GAC CTT CTT C -3'	104	53.0	52.6	67.1	0.980	-4.40	31.9	87.3, 80.8, 73, 74.2
XAprR1	5'- CCT GCT TAC CTT GCT TGA G -3'		52.6	52.6					
XAprF2	5'- ATG GTG ATG ATA CGA CTA C -3'	182	47.9	42.1	NA	NA	NA	NA	NA
XAprR2	5'- GAT GAG ATG GAC GAC TTC -3'		48.4	50.0					
XAprF3	5'- TCC GAT GAT GCG TTT GAC -3'	191	52.0	50.0	147.7	1.000	-2.54	32.0	94, 92, 90, 86, 81.5
XAprR3	5'- ATG TCT GAC AGC GAA ATC C-3'		52.1	47.4					
XAprF4	5'- ATC CGA TGA TGC GTT TGA C -3'	186	52.0	50.0	96.7	0.997	-3.40	14.8	82.5
XAprR4	5'- GAC AGC GAA ATC CAG AAG G -3'		52.8	52.6					
XAprR5	5'- GAC AGC GAA ATC CAG AAG G -3'	98	52.8	52.6	98.1	0.999	-3.37	42.7	82.0
XAprF5	5'- GCG AGA TCA AGT TCA GTG AG -3'		52.8	52.6					
XAprF6	5'- CGA GCA GGT GAA GGA CAG -3'	98	55.5	61.1	100.5	0.999	-3.31	43.7	82.5
XAprR6	5'- GCG ATG GCA CTA GGT ACA G -3'		55.3	57.8					

[a]Primer/probe identification (ID) and fluorophore (FAM in TaqMan ZEN Double-Quenched probe and SYBR Green); [b]Amplification product size in base pairs; [c]Primer melting temperature; [d]Guanine and cytosine content (%); [e]Standard curve slope; [f]Y-intercept of standard curve, [g]Amplicon melting temperature, NA= not applicable.

Table 2.2. Cycle threshold (C_T) means of TaqMan and SYBR Green PCR amplifications for six, 10-fold dilutions of an *X. albilineans* suspension and end-point of detection with conventional PCR.

	TaqMan PCR		SYBR Green PCR		Conventional
Xa CFU/ml[a]	C_T mean[b]	C_T SD[c]	C_T mean	C_T SD	PCR
$3.4x10^7$	15.4	0.03	17.0	0.09	+
$3.4x10^6$	18.2	0.07	21.8	0.94	+
$3.4x10^5$	21.6	0.11	25.3	0.35	+
$3.4x10^4$	25.1	0.20	28.5	0.40	+
$3.4x10^3$	28.6	0.14	31.6	0.71	+
$3.4x10^2$	32.2	0.17	33.5	0.31	-

[a]CFU = colony forming units per ml of *X. albilineans* (Xa) 10-fold dilution series.
[b]C_T mean of three replicates.
[c]C_T standard deviation (SD).

At 8-16 wk after inoculation, the new, emerged leaves of different cultivars showed variable amounts of systemic chronic symptoms of infection, including pencil-lines, leaf chlorosis or bleaching, shortening of internodes, abnormal lateral shoot growth, leaf necrosis, wilting and curling of leaves, and death of shoots (Figure 2.11).

The types of symptoms observed were used to calculate a disease severity score that clearly differentiated the local cultivars into resistant and susceptible groups under both greenhouse and field conditions. Disease severity scores were higher for cultivars HoCP 85-845 and HoCP 89-846 compared to LCP 85-384 and Ho 95-988 (Table 2.3). Some variability was observed in the resistant cultivars. Ho 95-988 showed chlorotic and necrotic symptoms of infection in the inoculated leaves and a low frequency of minor systemic symptoms in newly emerged leaves. LCP 85-384 showed only local necrosis at the point of inoculation and no systemic symptoms.

Disease incidence and severity were both reduced in resistant cultivars in greenhouse and field experiments according to the frequency of systemic symptoms of infection in

emerged leaves (Table 2.3). Incidence and severity were generally lower in the field compared to the greenhouse experiments.

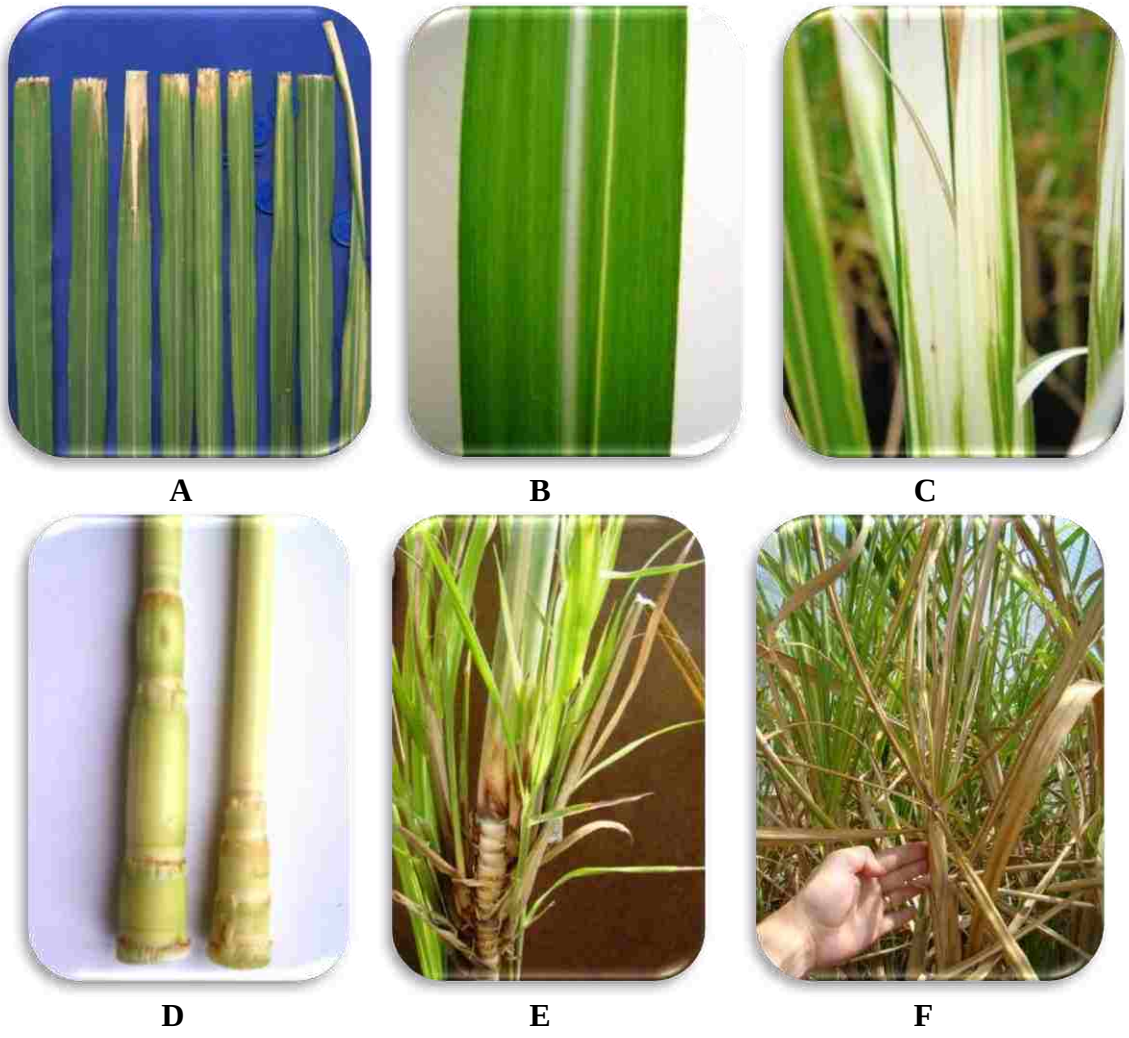


Figure 2.11. Leaf scald symptoms. (A) Initial local necrosis and chlorotic streaking symptoms in inoculated leaves. (B) Systemic "pencil-line" symptom. (C) Chlorosis or bleaching. (D) Shortening of internodes (right). (E) Abnormal lateral shoot growth with leaf symptoms. (F) Leaf necrosis and shoot death.

Table 2.3. Incidence and disease severity following inoculation of two resistant (R) and two susceptible (S) cultivars in greenhouse and field experiments.

	Greenhouse[a]		Field[a]	
Cultivar	Incidence	Severity	Incidence	Severity
LCP 85-384 (R)	0	0	2 a	1 ab
Ho 95-988 (R)	5	20	5 ab	2 b
HoCP 85-845 (S)	100	84	64 c	37 c
HoCP 89-846 (S)	90	88	39 bc	36 c

[a] Incidence (percentage) of shoots with systemic symptoms of infection. Disease severity evaluating systemic infection symptoms on a scale of 0-100. Disease parameter means withing a column followed by the same letter were not significantly different according to Tukey's at $P \leq 0.05$.

2.3.5 Comparison by qPCR of *X. albilineans* Populations in Different Tissues of Resistant and Susceptible Cultivars

The Xa population detected by qPCR varied in different tissues and between resistant and susceptible cultivars (Table 2.4). Bacterial populations were four orders of magnitude greater in leaves emerging after inoculation in the susceptible cultivars, HoCP 85-845 and HoCP 89-846, compared to the few positive samples from the resistant cultivars, LCP 85-384 and Ho 95-988 (Table 2.4).

The Xa population was high in leaves of three different ages and in the meristem and stalk of the susceptible cultivars, ranging from 10^5 to 10^9 CFU/ml (Table 2.4). More variation was detected among tissues for the resistant cultivars, LCP 85-384 and Ho 95-988, with populations ranging from 10^2 to 10^6 CFU/ml. The greatest difference between resistant and susceptible cultivars was detected in the new emerging, systemically-infected TVD -3 leaf with four to five orders of magnitude difference in population levels.

Table 2.4. Quantification of *X. albilineans* population by SYBR Green qPCR in different tissues of two leaf scald resistant and two susceptible sugarcane cultivars in the greenhouse.

	Resistant				Susceptible			
	LCP85-384		Ho95-988		HoCP89-846		HoCP85-845	
Tissue	C_t[e]	Cells/ml	Ct	Cells/ml	Ct	Cells/ml	Ct	Cells/ml
Meristem[a]	26.0	$3.6x10^4$	25.1	$7.9x10^4$	22	$1.7x10^6$	23.7	$7.3x10^5$
Leaf TVD-3[b]	34.8	$3.8x10^2$	32.1	$3.1x10^3$	19.4	$1.5x10^7$	17.6	$1.6x10^7$
Leaf TVD	34.0	$6.2x10^2$	26.5	$1.8x10^6$	20.3	$6.6x10^6$	19.3	$7.3x10^6$
Leaf TVD+3[c]	34.0	$6.3x10^2$	25.5	$8.6x10^5$	20.2	$5.5x10^6$	16.4	$1.6x10^7$
Stalk[d]	27.9	$2.1x10^6$	31.3	$1.0x10^4$	20.9	$1.3x10^9$	16.1	$2.9x10^7$
Average	31.3		28.1		20.6		18.6	

[a]Meristem tissue with primordial leaf tissue (100 mg).
[b] TVD -3, third leave above the top visible dewlap leaf (TVD).
[c] TVD +3, third leave below the top visible dewlap leaf.
[d]Xylem sap extracted from the second and third internodes at the base of the stalk.
[e] C_T, cycle threshold value.

2.3.6 Comparison of Xa Population Quantified with qPCR and Leaf Scald Incidence, Severity, and Systemic Infection in Resistant and Susceptible Cultivars

Lower bacterial population levels and fewer infected samples corresponded with low disease severity, incidence, and vascular colonization in the two resistant cultivars (Figure 2.12, Table 2.5). Ho 95-988 and LCP 85-384 had only 10 and 5% positive samples, with an average of 10^2 CFU/ml in the infected plants, no systemic symptoms in LCP 85-384, and some single pencil-line symptoms in Ho 95-988. In contrast, a high Xa population was detected by qPCR in both susceptible cultivars with a high frequency of infection and severe disease symptoms, including several chlorotic streaks that coalesced to form large chlorotic or bleached areas and necrosis. The variation in frequency and concentration of the Xa target in different samples was evident when plotted on the standard curve after qPCR amplification to determine CFU/ml (Figure 2.13). Xa was detected in many samples of the susceptible cultivar, HoCP 89-846, which also had high bacterial populations, whereas the resistant cultivar, Ho 95-988, had fewer samples with positive detection and a lower mean population in samples in which the bacterium was detected.

Similarly, a higher level of vascular colonization was detected in susceptible compared to resistant cultivars (Table 2.5). Vascular colonization was high in all tissues sampled, including the stalk base, apex, and meristem samples collected from the susceptible cultivars, whereas the greatest level of colonization in resistant cultivars occurred at the stalk base (Figure 2.12). No vascular colonization was detected in the resistant cultivars above the apical meristem in strong contrast to the susceptible ones.

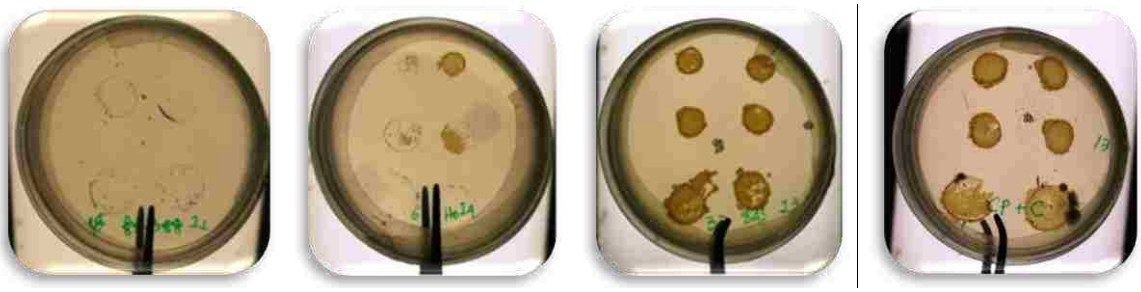


Figure 2.12. Bacterial growth from stalk base tissue prints on XAS medium for evaluation of *X. albilineans* vascular infection and extent of bacterial colonization for resistant (LCP 85-384 and Ho 95-988) and susceptible (HoCP 85-845 and HoCP 89-846) cultivars.

2.3.7 Quantification of *X. albilineans* by qPCR in Multiple Cultivars in Greenhouse and Field Experiments

In a greenhouse experiment in which 15 cultivars with a range of leaf scald susceptibility were inoculated with Xa, the susceptible cultivars had higher populations of Xa (Figure 2.14). However, no bacteria were detected in five cultivars, four of which exhibit variable levels of susceptibility in field inoculation experiments. In a second experiment, plants of all cultivars showed symptoms in the inoculated leaves but did not develop systemic symptoms of infection. Samples were tested by qPCR to attempt to detect Xa latent infection in different plant tissues (Table 2.6). Xa was detected by qPCR in the TVD +3 leaves (inoculated leaves) of 15 inoculated cultivars that did not exhibit any systemic infection symptoms 4 months after inoculation in the greenhouse, and high Xa populations were

detected in the base of the stem in 14 of 15 cultivars (Table 2.6). The leaves did not show any symptoms of infection in the TVD -3 in the emerged leaves for 14 of 15 cultivars, but latent infection of Xa was detected by conventional PCR, isolation on XAS-medium, and TaqMan PCR for one, three, and three cultivars, respectively. Xa was similarly detected in the TVD leaf of the same cultivars. The Xa population in these cultivars was estimated by qPCR to be below 10^4 CFU/ml.

Table 2.5. Comparison of *Xanthomonas albilineans* populations determined by SYBR Green qPCR in emerged leaves of two susceptible and two resistant cultivars with disease severity, incidence, and vascular infection in a greenhouse experiment.

	Bacterial population			Conventional screening	Bacterial colonization			
					Vascular infection[d]			
Cultivar[a]	C_T value[b]	Cells/ml by qPCR[c]	Positive samples	Disease severity	Meristem	Apex	Base	Average value
HoCP85-845 (S)	21.9	1.9×10^6	20/20	68	3.6	3.4	4.0	3.7
HoCP89-846 (S)	23.6	1.2×10^6	17/20	79	4.0	4.0	4.0	4.0
Ho95-988 (R)	33.2	8×10^2	2/20	20	0.0	0.5	2.6	1.0
LCP85-384 (R)	33.9	3×10^2	1/20	0	0.0	0.5	1.9	0.8

[a]Two leaf scald susceptible (S) and two resistant (R) cultivars were compared. Number of plants per cultivar = 20.
[b]Average SYBR Green PCR critical threshold (C_T) value for systemically infected leaves.
[c]Xa cells detected per ml according by qPCR.
[d]Vascular infection determined by stalk blotting on XAS medium where 0 = no bacterial growth, 1 = 1-10 colonies in the stalk print, 2 = more than 10 colonies or confluent growth in less than 25% of the imprint, 3 = confluent growth in 25 to 75% of the imprint and 4 = more than 75% of confluent bacterial growth on the blotted area. Meristem = apical meristem and primordial leaves; apex = internode of the stem below apical meristem; base = first-second internode above ground.

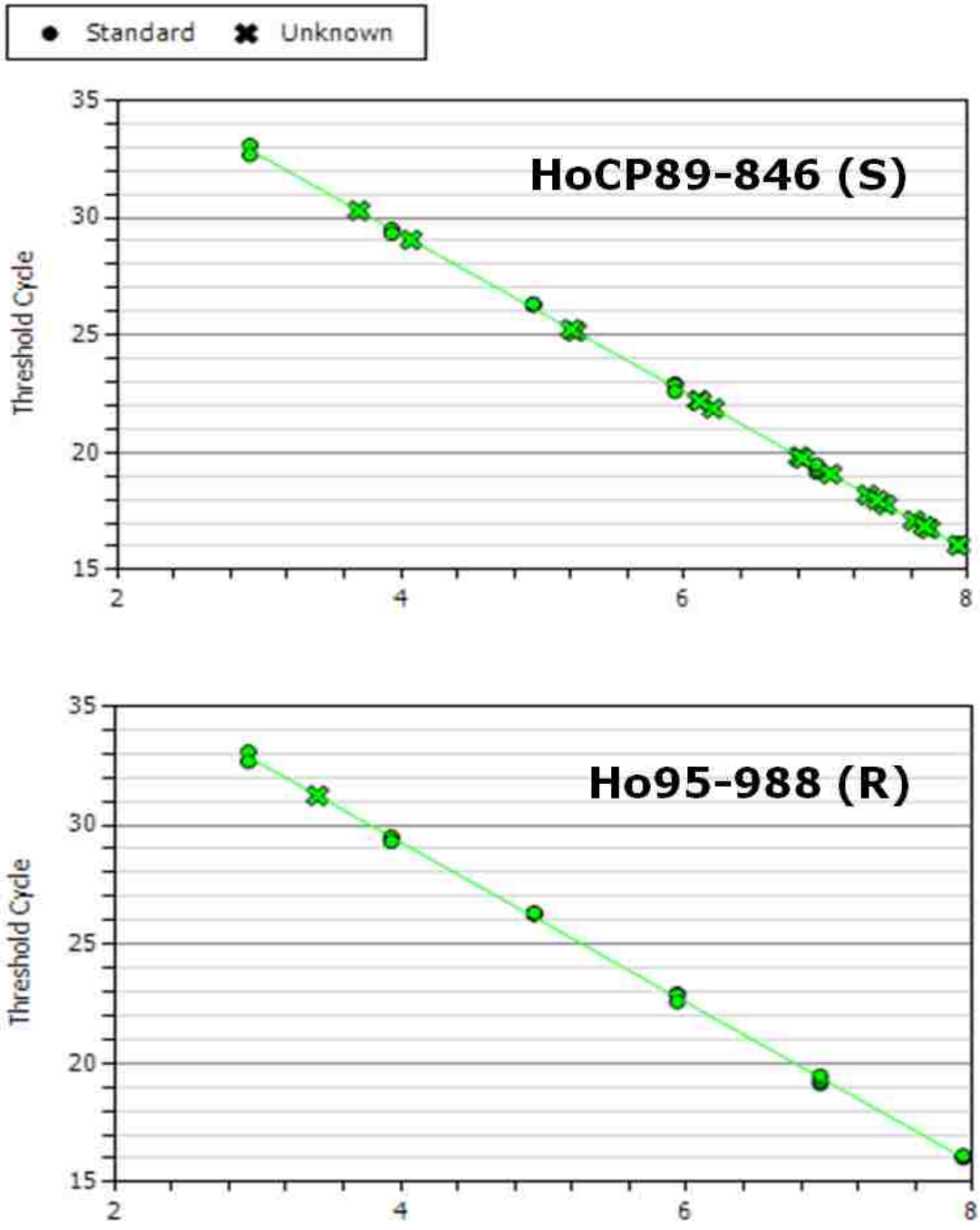


Figure 2.13. SYBR Green PCR amplification plots of *X. albilineans* in a susceptible (S) cultivar HoCP 89-846 (top) and a resistant (R) cultivar Ho 95-988 (bottom). High threshold cycle values (C_T) indicate low bacterial population, whereas low Ct values indicate high bacterial population. (PCR efficiency = 98.1%, R^2 = 0.999, slope = -3.37)

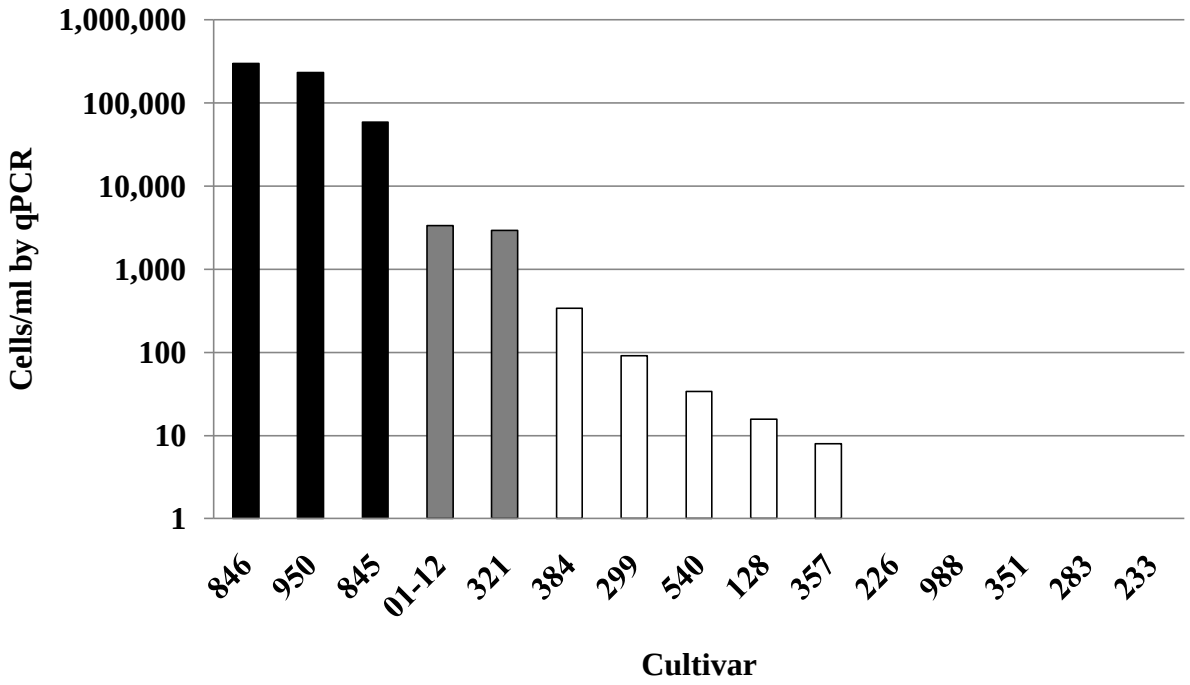


Figure 2.14. Population levels of *X. albilineans* detected by TaqMan-PCR in 15 different cultivars with variable levels of leaf scald resistance in a greenhouse experiment.

Table 2.6. Comparison of detection of *X. albilineans* (Xa) with three methods in three different plant tissues from 15 inoculated cultivars without symptoms of systemic infection.

	Number of Xa positive cultivars			
Sample [a]	Symptoms[b]	XAS-medium	TaqMan PCR[c]	PCR[d]
TVD -3	1	3	3	1
TVD	0	3	3	1
TVD +3	15	15	15	15
Stem	0	-[e]	14	-

[a] Plant tissue sampled. TVD -3 is the third leave above the youngest fully emerged leaf termed the top visible dewlap leaf (TVD). TVD +3 is the third older leave below the top visible dewlap leaf. The TVD +3 samples were inoculated leaves. Xylem sap was extracted from the second and third internodes at the base of the stem.
[b]Number of cultivars with plants exhibiting symptoms in inoculated leaves.
[c]TaqMan PCR with XaQ primers and TaqMan probe XaQ.
[d]Conventional PCR using primer set XaAlb2.
[e]Not tested

Xa populations detected by qPCR corresponded with disease incidence and severity in a comparison of three leaf scald resistant and three susceptible cultivars in an inoculated field

experiment (Table 2.7). Bacterial populations were three to four orders of magnitude higher in systemically infected leaves of the three susceptible compared to the resistant culivars that showed higher incidence and severity of disease.

Table 2.7. Comparison of *X. albilineans* populations determined by SYBR Green qPCR in systemically infected leaves of three susceptible and three resistant cultivars with disease severity and incidence in a field experiment.

	qPCR		Conventional screening			
Cultivar[a]	C_T value[b]	Xa/ml by qPCR[c]	Severity[d]		Incidence[d]	
HoCP 85-845 (S)	20.2	1.9×10^6	36.6	a	63.6	a
HoCP 89-846 (S)	23.6	4.0×10^6	35.7	a	39.6	a
HoCP 00-950 (S)	24.1	1.8×10^6	20.4	ab	38.1	a
Ho 95-988 (R)	32.1	6.1×10^3	2.2	b	5.4	b
LCP 85-384 (R)	34.3	1.2×10^3	1.1	b	2.3	b
HoCP 96-540 (R)	32.6	4.3×10^3	0.3	b	1.7	b

[a]Three leaf scald susceptible (S) and three resistant (R) cultivars were compared.
[b]Average SYBR Green PCR critical threshold (C_T) value for systemically infected leaves.
[c]*X. albilineans* (Xa) detected per ml according by qPCR.
[d]Disease parameter means within a column followed by the same letter were not significantly different according to Tukey's at $P \leq 0.05$.

2.4 DISCUSSION

TaqMan and SYBR Green qPCR assays were developed utilizing primers from the bacterium-specific albicidin toxin gene cluster for the detection and quantification of *Xanthomonas albilineans* in sugarcane. The qPCR assays for Xa detection were more sensitive than conventional PCR. For diagnostic purposes, the qPCR assays could detect Xa in samples with 10^2 CFU/ml, and qPCR only requires 3 h to complete compared to 1-2 days for conventional PCR used which include sample processing and DNA extraction, longer amplification time and visualization in agarose gel. The lower cost of SYBR Green PCR makes

this method suitable for larger scale testing. TaqMan PCR could be utilized in situations requiring the most sensitive detection capability possible, such as quarantine operations and healthy seed-cane foundation or breeder nurseries. The ability to quantify bacterial populations provides the potential for use in comparative studies.

The first detection of leaf scald in Louisiana in 1993 (Hoy and Grisham, 1994) was part of widespread outbreak of the disease in the Western Hemisphere (Davis et al., 1997). Studies to examine intraspecific variation in *X. albilineans* determined that isolates associated with the recent disease outbreak were all similar as indicated by serological variation (Serovar I) (Rott et al., 1994), pulsed-field gel electrophoresis group (haplotype B) (Davis et al., 1997), and rep-PCR (Lopes et al., 2001). Intraspecific variation was not detected in a large isolate group from Louisiana in the rep-PCR study, and biological variation, such as cultivar shifts from resistance to susceptibility, has not been detected in Louisiana. Only a few Xa isolates were tested in this study, but the low level of intraspecific variability detected in previous studies, and the fact that the XALB1sequences used in the primer design are conserved among isolates (Roger et al., 2003) suggest that the PCR primers developed will detect all Xa isolates currently occurring in Louisiana. Additional testing is needed to determine whether the qPCR assays will detect Xa isolates in other sero-groups or haplotypes.

The most effective method to prevent or control leaf scald is the development and planting of resistant cultivars (Rott and Davis, 2000). However, the erratic nature of symptom expression causes problems in accurately determining genotype resistance levels in inoculation tests conducted as part of a cultivar selection program. In addition, development of a useful resistance rating system based on qualitative traits is challenging.

Resistance to leaf scald is associated with the bacterial population at the shoot apex (Rott et al., 1997). Selective isolation of an antibiotic resistant mutant strain revealed that even resistant clones are infected and colonized by Xa; however, resistant genotypes exhibited a lower level of bacterial colonization, particularly in tissues at the shoot apex containing the apical meristem (Rott et al., 1997). This finding suggests that a qPCR assay could provide a less laborious, more rapid and accurate method to determine Xa populations in inoculated plants for comparing resistance levels in clones in cultivar selection programs.

In this study, the Xa population levels quantified by qPCR clearly distinguished known resistant and susceptible cultivars. These results agreed with the traditional measures for the assessment of resistance, disease severity and vascular colonization by the pathogen. As previously shown (Rott et al., 1997), the low and high bacterial populations detected in resistant and susceptible genotypes, respectively, were similar in inoculated plants in both greenhouse and field experiments. Comparisons of Xa populations quantified by qPCR in different tissues indicate young leaves emerging from the apical meristem are the best tissue for detecting differences in Xa populations among genotypes varying in level of resistance to leaf scald.

In one experiment, infection escapes or failures to successfully establish systemic infection following inoculation occurred. Known precautions for successful inoculation by the decapitation method were used, such as using a high inoculum concentration of an Xa isolate recently obtained from symptomatic, infected tissue and avoiding exposure of the inoculum on the cut surface to UV light (Koike, 1965; Lopes et al., 2001; Ricaud and Ryan, 1989). However, it appears additional research is needed to identify other factors, such as environmental conditions and plant nutrition prior to or following inoculation, that limit initial or systemic infection and the best timing of sampling after inoculation to maximize the detection of

differences. Field experiments offer the advantage of natural conditions for plant growth and disease development, but greenhouse experiments provide flexibility. Additional testing of more clones with differing levels of resistance under variable conditions in both the greenhouse and field is needed.

This study shows that qPCR is a more reliable method for the detection of *X. albilineans* that is capable of quantifying bacterial populations in infected sugarcane plants. The results suggest Xa quantification during systemic infection can provide a measure of the relative resistance/susceptibility to leaf scald among host genotypes. TaqMan PCR has the potential to serve as a powerful tool for understanding the basis of resistance and the effects of environmental conditions on development of bacterial populations, pathogen multiplication, disease development, and expression of resistance.

CHAPTER 3:
COMPARATIVE PROTEOMIC ANALYSIS OF SUGARCANE RESPONSE TO INFECTION BY *XANTHOMONAS ALBILINEANS,* THE CAUSAL AGENT OF LEAF SCALD

3.1 INTRODUCTION

Leaf scald, caused by *Xanthomonas albilineans* (Ashby) Dowson, is an important disease of sugarcane (inter-specific *Saccharum* hyrids) that has been reported in more than 66 countries (Rott and Davis, 2000). Losses in susceptible cultivars of 10-34% in tons of cane per hectare and up to 30% in juice quality have been reported (Hoy and Grisham, 1994; Ricaud and Ryan, 1989; Rott and Davis, 2000; Rott et al., 1995; Victoria et al., 1995; Saumtally and Dookun, 2004).

The disease is characterized by erratic symptom expression. It can occur as three phases: latent, chronic, and acute (Ricaud and Ryan, 1989; Rott and Davis, 2000; Saumtally and Dookun, 2004). A range of chronic symptoms, including diagnostic narrow, white to chlorotic longitudinal streaks along veins termed "pencil lines" that progressively become necrotic from the leaf margin, general bleaching or chlorosis, abnormal development of symptomatic side shoots on stalks, reddish discoloration of vascular bundles, cavity formation in nodal and inter-nodal regions near the stalk apex, stunting, wilting, and death (Birch, 2001; Ricaud and Ryan, 1989; Rott and Davis, 2000; Saumtally and Dookun, 2004). An acute phase can occur in which entire fields of previously asymptomatic mature plants suddenly die. Symptom severity is affected by host and pathogen genotype and by environmental conditions, particularly the occurrence of stressful conditions, such as drought.

The bacterial pathogen, *X. albilineans* (Xa), is a xylem-inhabiting xanthomonad that systemically colonizes the entire host plant. It is unusual in that it lacks the hypersensitive

response and *hrp* genes encoding the type III secretion system typical of plant pathogenic bacteria, but it has an animal-associated T3SS that is not important in pathogenesis (Pieretti et al., 2009; Marguerettaz et al., 2011), and it does not produce xanthan gum (Pieretti et al., 2009). These characteristics are similar to another important xylem-limited plant pathogenic bacterium, *Xylella fastidiosa* (Simpson et al., 2000). Pathogenicity is associated with the production of a phytotoxin, albicidin, that inhibits the production of proplastic DNA and blocks differentiation of chloroplasts (Birch and Patil, 1985 a and b; Birch and Patil, 1987 a and b). However, it was demonstrated that albicidin-deficient Xa mutants are still able to colonize sugarcane and produce symptoms (Champoiseau et al., 2006), and additional virulence factors important in symptom expression or vascular colonization were identified by insertion mutagenesis of the Xa genome (Rott et al., 2011). Loci encoding hypothetical proteins related to pathogenicity included an OmpA-family protein, an outer-membrane protein that may have an important role in adaptation to the nutrient poor xylem habitat, other membrane associated proteins, and an enzyme involved in lipopolisaccaride and exopolyssacharide biosynthesis homologous to the *rmlB* gene (dTDP-glucose 4,6-dehydratase) (Rott et al., 2011). Mutants of *rmlB* were not able to colonize the sugarcane stem and produce symptoms.

Breeding and selecting for host plant resistance has been the most important control measure for leaf scald (Rott and Davis, 2000; Ricaud and Ryan, 1989). Resistance is evaluated in host genotypes in inoculated field experiments. However, accurate evaluation of resistance levels in sugarcane clones is difficult since reactions obtained from field inoculations are erratic. Resistance has been associated with the extent of bacterial colonization, and disease severity and bacterium presence in the shoot apex were correlated in field and greenhouse experiments (Rott et al., 1994; Rott et al., 1997). Resistant host genotypes become infected by Xa but have

limited bacterial population development during systemic infection and no severe symptom expression.

The mechanisms by which resistant sugarcane genotypes limit bacterial population growth and prevent symptom development are not completely understood. Leaf scald causes several morphophysiological changes in young and mature cane (Rao et al., 2001). In Xa-infected sugarcane, the composition of polyamines and phenolic acids was altered, levels of ornithine descarboxilase were increased, and polyamine metabolism was increased (Fontaniella et al., 2007). Solas et al. (2003) found that xylem, phloem and mesophyll tissues in general were occluded by polysaccharide, and the cell walls of the xylem were disintegrated.

A differential gene expression study used macro-arrays based on the SUCEST expression profile of 3,575 ESTs from sugarcane to study the reaction of sugarcane with systemic Xa infection and expressing chronic symptoms (Dabbas et al., 2006). In a resistant cultivar, ESTs of proteins were induced with similarities to isopropanoid biosynthesis, ethylene biosynthesis, a transmembrane protein kinase with LRR, a leucine zipper, and a mutase/profenato dehydratase associated with cell wall lignification for environmental stress adaptation. Conversely, an EST of a protein with similarities to a glutation synthetase and ESTs for plant cellular expansion, detoxification and auxin transport were repressed. In both susceptible and resistant cultivars, a protein with similarity to an auxin transporter was repressed. In the susceptible cultivar, ESTs associated with plant defense response, ethylene biosynthesis, transcription regulation, and a protein with similarity to Zinc finger type C3H4 were repressed. An EST of a protein similar to an RNA helicase was induced in the resistant cultivar and repressed in the susceptible one. This protein is associated with plastid differentiation and cold tolerance. According to Dabbas et al. (2006), the differential induction of RNA helicase in the resistant cultivar may be one of the

factors that confer resistance to Xa, and its repression could be the cause of susceptibility. They indicated that resistance could be associated with bacterial growth in the plant and with a repression or induction of metabolic pathways involved with resistance.

Proteomic analysis offers another approach to potentially increase understanding of the molecular mechanisms of resistance to leaf scald. Sugarcane proteomic analyses have examined general protein polymorphisms (Ramagopal, 1989), protein expression changes after dedifferentiation of leaf tissue in callus culture (Ramagopal, 1994), and drought-stress responsive proteins (Jangpromma, et al., 2010; Sugiharto et al., 2002). The impact of bacterial pathogens on plant proteomes has been evaluated for *Psuedomonas syringae* pv. tomato DC3000 in *Arabidopsis* (Jones et al., 2004 and 2006), *P. aeruginosa* in tobacco (Mathesius et al., 2003), *P. savastinoi* pv. s*avastinoi* in olive (Campos et al., 2009), *Clavibacter michiganense* subsp. *michiganense* in tomato (Coaker et al., 2004), one xanthomonad, *Xanthomonas oryzae* pv. *oryzae,* in rice (Chen et al., 2007; Mahmood et al., 2006), and *Xylella fastidiosa* (Yang et al., 2011), a pathogen genetically and ecologically very similar to Xa.

The erratic expression of disease and resulting difficulty in identifying and selecting resistant cultivars by traditional means and the unusual characteristics of the pathogen gives impetus to efforts to improve understanding of the molecular mechanisms of leaf scald resistance. The objective of this study was to utilize a comparative proteomic analysis of the sugarcane response in resistant and susceptible cultivars under differential conditions of infection by Xa to identify proteins associated with resistance to leaf scald.

3.2 MATERIALS AND METHODS

3.2.1 Plant Materials, Bacterial Isolation, Inoculation and Sample Collection

Three sugarcane cultivars were included in a comparative time-course experiment: Ho 95-988 (leaf scald resistant), LCP 85-384 (highly resistant), and HoCP 89-846 (highly susceptible). An additional experiment was established to assess plant response 8 weeks after inoculation (wai) in the same cultivars and an additional susceptible cultivar, HoCP 85-845. The time-course experiment for Ho 95-988 and HoCP 89-846 was repeated once. The vegetative planting material was obtained from a leaf scald symptom-free field nursery plot. Single-node cuttings received a long hot water treatment (24 h with circulating cool water followed by 1 h at 51 °C). Cuttings were planted in Styrofoam trays in the greenhouse, and after 2 wk, plants were transplanted into 3.785 L plastic pots containing a silt-loam soil-sand mix at a 1:1 ratio. The plants were fertilized twice with 14:13:13 N:P:K and inoculated after producing 4-6 fully emerged leaves. Soil moisture content was adjusted to field capacity daily.

Xa was isolated from a longitudinal leaf section that contained a white pencil-line symptom. Tissue was surface-sterilized with NaOCl (10% v/v of commercial bleach) for 30 sec and rinsed two times with sterile, distilled, deionized water (sdH_2O). Leaf sections were dried under laminar flow, cut in small pieces 1-2 mm wide and placed in a 1.5 ml microcentrifuge tube (Eppendorf, Hauppauge, NY) containing 1 ml of ddH_2O. The tube with infected tissue was left overnight at 4 °C. A loop of bacterial suspension was inoculated by dilution streaking on XAS medium with antibiotics developed by Davis et al. (1994) (Wilbrink's medium that contains Bacto Peptone, 5 g; sucrose, 10 g; K_2HPO_4 $3H_2O$, 0.5 g; $MgSO_4$ $7H_2O$, 0.25; Na_2SO_3, 0.05 g per litre supplemented with 25 mg cephalexin, 30 mg novobiocin, 50 mg kasugamycin, 100 mg cycloheximide, 2 mg benomy, and 5 g KBr per litre. After 5 - 8 days, single colonies

were selected and dilution streak plated again on solid XAS medium without antibiotics to obtain pure cultures.

Pure Xa cultures were spread to form a lawn on XAS medium and incubated at 28 °C for 48 h. A cell suspension was prepared by adding 5 ml of sdH_2O to each plate and diluting to obtain 10^8 CFU/ml based on spectrometric absorbance (0.18 optical density at 590 nm). The inoculum was kept at on ice in the dark prior to inoculation, and 2-month-old plants were inoculated using the decapitation method (Koike, 1965) (Figure 3.1). A transverse cut with scissors dipped in the inoculum suspension was made above the apical meristem, and 100 µl of Xa suspension was added to the cut surface. Four plants of each cultivar were inoculated with Xa and four were mock-inoculated with only water. Inoculations were done at the end of the day during sunset, and the humidity in the greenhouse was increased by wetting the floor below the bench and turning fans off overnight to avoid desiccation of inoculum and inoculated tissue.

Leaf samples collected included tissues from the youngest fully emerged leaf commonly designated in sugarcane as the top visible dewlap (TVD) leaf, and all the emergent leaves in the leaf whorl above the TVD (Figure 3.1). Between samples, scissors were surface-sterilized by dipping in 95% ethanol and flaming. Mock-inoculated plants were sampled before inoculated ones. A composite sample of all the newly emerged leaves that included the foliar lamella and the midvein were cut in 2 cm long pieces and placed in a 50 ml conical tube at 4 °C before final storage at -80 °C.

For Xa quantification by TaqMan qPCR, 20 leaf discs were collected from the second leaf above the TVD in the leaf whorl using a 5-mm-hole paper punch, with nine discs from each laminar side, and two from the midvein. The discs were placed in a 1.5 ml microcentrifuge tube

containing 1 ml ddH_2O. Between samples, the paper punch was surface sterilized by dipping in 95% ethanol and flaming.

Composite leaf tissue samples were collected from individual plants at 2, 4, and 8 wai. The presence of a cut was used to distinguish leaves that had been inoculated from leaves emerging after inoculation. The 2 wk sample consisted exclusively of tissue from emerging, directly inoculated leaves. The 4 wk sample contained leaf tissue from inoculated leaves and from newly emerging, systemically infected leaves. The 8 wk sample contained only systemically infected leaf tissue.

3.2.2 Disease Assessment and Bacterial Quantification by qPCR

- **Disease Severity Assessment During Initial and Systemic Infection**

Disease severity was assessed with a scoring system utilizing a weighted quantification of qualitative symptom types. Initial infection symptom severity was assessed for the expanding inoculated leaves 2 wai, and severity during systemic infection was assessed for the emerged leaves 8 wai. Severity of both initial and systemic symptoms was scored according to the types of symptoms observed in each plant using the formula described by Rott, et al. (1997) in which disease severity = $[(1 \times S1 + 2 \times S2 + 3 \times S3 + 4 \times S2 + 5 \times S5)/5 \times T] 100$, where the 1 to 5 multiplication factors increasingly weight the number of stalks with more severe systemic symptom types as follows. For initial symptoms of infection: S1 = number of plants with leaves exhibiting local necrosis at the cut leaf margin, S2 = number of plants with leaves exhibiting short yellow streaks less than 5 cm long, S3 = number of plants with leaves exhibiting short yellow streaks more than 5 cm long, S4 = number of plants with white pencil-lines, S5 = number of plants necrotic or with dead leaves, and T = total number of plants. For systemic infection symptoms: S1 = number of plants with leaves exhibiting one or two narrow white

pencil-lines, S2 = number of plants with more than two pencil-lines in leaves, S3 = number of plants with leaf chlorosis or bleaching, S4 = number of plants with general leaf necrosis, S5 = number of dead plants or with symptomatic side shooting, and T = total number of plants.

- **DNA Extraction**

Xa populations were quantified by qPCR at the same time that leaf samples were collected for the time-course proteomics analysis. Bacterial genomic DNA was extracted using the lysis-boiling modified method described by Jacobs (2008) (Appendix 1) using exudates from the 20 leaf discs collected from each leaf sample. Bacterial streaming from leaf discs was allowed to occur for 24 h at 4 °C. Exudates were then transferred to 1.5 ml tubes. After centrifugation at 9000 x g for 5 min, the supernatant was discarded, and then bacterial pellets were suspended in 100 µl lysis buffer and incubated in a water bath at 95 ^{o}C for 10 min. The microcentrifuge tubes containing the samples were cooled in ice and then stored at -20 °C.

- **TaqMan PCR**

From each sample of DNA, 2 µl were mixed with 10 µl of TaqMan universal master mix (Roche), 1 µl of each forward and reverse primers (10 µM), and TaqMan double-quenched^{TM} probe (2 µM), 5 µl of sdH_2O for a final volume of 20 µl. The conditions of amplification were: an initial step at 50 °C for 10 min, a second step of 95 °C for 2 min, followed by 40 cycles of DNA denaturation at 94 °C for 15 sec, and annealing-polymerization at 60 °C for 1 min.

3.2.3 Protein Extraction

Figure 3.1 describes the workflow for the identification of differentially expressed proteins and peptide sequences. A protein extraction procedure that provides adequate protein quality and quantity is a critical step in comparative proteomics. Protein extraction was by the mini-protein extraction protocol described by Want (2006) adjusted with the protocol for larger

samples described by the University of Missouri (2002). The trichloroacetic acid (TCA) and phenol methods were combined and followed by ammonium acetate precipitation.

Samples were ground into a fine powder with a mortar and pestle under liquid nitrogen. Powder (2.5 g) was transferred into a 50 ml phenol resistant tube, and 10 ml of 10% TCA/acetone was added. Samples were placed on ice for 1 min vortexed for 1 min then centrifuged at 6000 x g for 15 min at 4 ºC. The supernatant was carefully removed by decanting. TCA and other contaminants (phenol) were washed with 10 ml of 100% methanol plus 0.1 M ammonium acetate kept at -80 ^{o}C. Vortex mixing for 1 min alternating with 1 min in ice three times was followed by centrifugation at 6000 x g for 15 min at 4 ºC. The supernatant was discarded, and the pellet was washed with 10 ml of 80% acetone with vortex mixing. After centrifugation at 6000 x g for 15 min at 4 ºC, the supernatant was discarded. Samples were air-dried in a laminar flow hood at room temperature for 30 min or incubated at 35 ºC with air flow for 15 min to remove residual acetone. Next, 2 ml of Tris-saturated phenol (pH 8.0) and 2 ml of sodium dodecyl sulfate (SDS) extraction buffer were added per each 1 g of starting material (6 ml of each solution/2.5 gm of leaf tissue). Samples were thoroughly vortexed (1 min in ice and 1 min of vortexing 15 times). The phenol phase was separated by centrifugation at 6000 x g for 15 min at 4 ºC. The upper phenol phase (3-4 ml) was transferred into a new 50 ml tube, taking care not to disturb the SDS white phase. After adding 20 ml of 100% methanol containing 0.1 M ammonium acetate, samples were stored at -30 ºC for 2 h to overnight. After centrifugation at 6000 x g for 30 min at 4 ºC and discarding the supernatant, a white protein pellet remained. The pellet was washed once with 10 ml 100% methanol. The pellet was disturbed with a spatula, vortexed for 3 min, centrifuged at 6000 x g for 20 min at 4 ºC, and the new pellet was washed once with 5 ml 80% acetone with vortexing for 3 min. The pellet was physically disturbed and

collected again by centrifugation at 6000 x g at 4º C for 20 min. Under air-flow, the proteins were air dried for 2-3 min, and the pellet was collected. Pellets were left inside the air flow chamber an additional 20 min to allow acetone evaporation. Dried pellets were stored at -30 ºC or dissolved in buffer (SDS buffer or isoelectrofocusing IEF rehydration buffer). All wash solutions were pre-cooled at -20 ºC for at least 1 h.

3.2.4. Protein Solubilization and Quantification

Protein pellets were re-suspended and solubilized at room temperature for 1 h without disturbing the pellet in 150 µl lysis buffer (8 M urea, 4% w/v of CHAPS, 40 mM dithiothreitol DTT, and 2% v/v IPG 3-10 pH non-linear buffer) (Gorg et al., 1998).

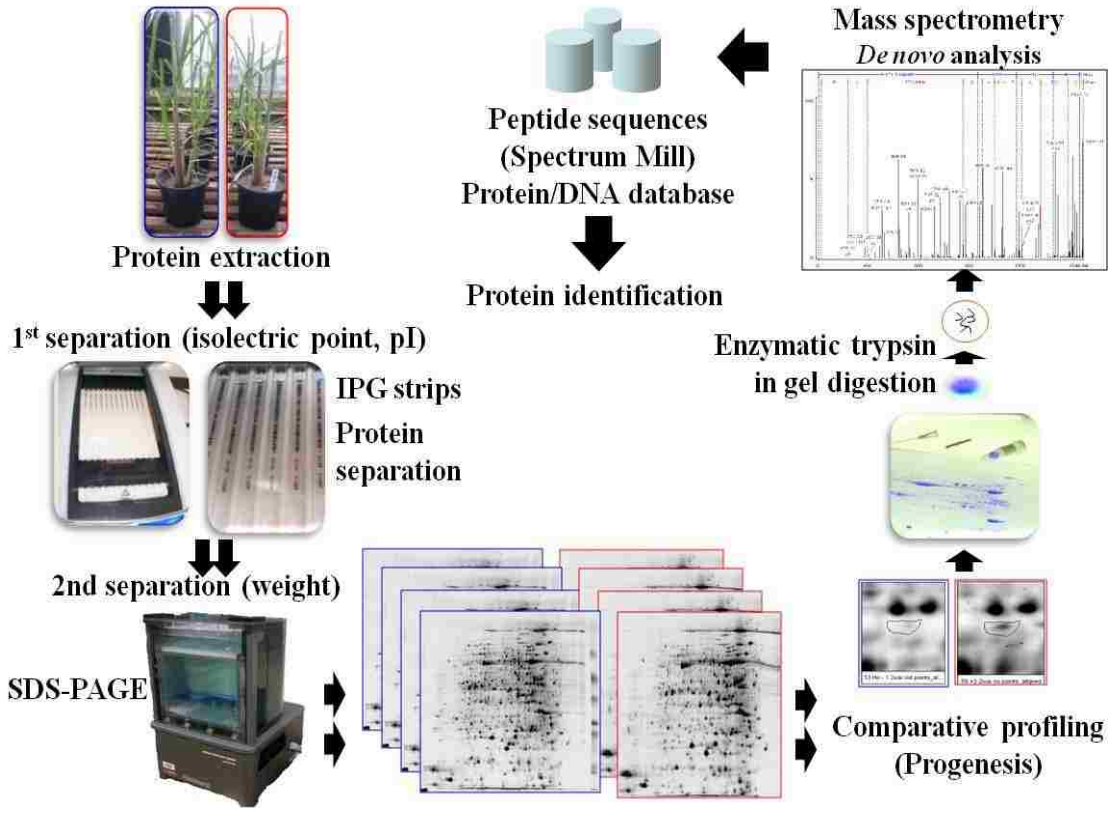


Figure 3.1. Workflow for comparative proteomic analysis using two-dimensional gel electrophoresis.

The pellet was ground with mortar and pestle until completed solubilized. Centrifugation followed at 14,000 rpm at room temperature for 10 min to precipitate non-soluble proteins. Protein concentration was determined by transferring 2 µl of the supernatant to a 2 ml spectrometric cuvette containing 1 ml 1x Bio-Rad protein assay dye reagent concentrate (BioRad, Hercules CA) and determining spectrophotometric absorbance at 595 nm as described by Bradford (1976). The soluble protein volume was adjusted to a final concentration of 150 µg in 340 µl of rehydration solution (8 M urea, 2% CHAPS, 20 mM DTT, bromophenol blue and 0.5% wt/vol IPG buffer).

3.2.5 Protein Separation by Two-Dimensional Electrophoresis

- **First Dimension**

The prepared protein sample in the rehydration solution was applied on a rehydration tray, and a Immobiline 3-10 pH non-linear 18 cm DryStrip (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was placed face down to allow sample protein absorption and gel rehydration. The gels and samples were each covered with 3 ml DryStrip cover fluid (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to avoid desiccation. The gels were left 12 h to overnight at room temperature. After removing the gels from the rehydration tray and removing excess dehydration buffer – DryStrip cover fluid, the Immobiline DryStrips were placed face-up on a IPGphor Manifold ceramic tray in an Ettan IPGphor 3 Isoelectric Focusing Unit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The Immobiline 3-10 pH non-linear 18 cm DryStrips rehydrated with the sample – buffer solution were subjected to isolectric focusing at 500 V for 1.5 h, 1000 V for 1.5 h and 8000 V during 5 h, followed by 40 min in SDS equilibration buffer with 1% DTT (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and 50 min in the same buffer with 2.5% Iodoacatamide (IAA) in place of DTT (Sigma, St Louis, MO).

- **Second Dimension**

The Immobiline IPG gel strips were placed on top of a second-dimensional SDS-polyacrylamide gel (13.5%) after equilibration. The polyacrylamide gel strips were sealed and embedded in a 1% agarose-200 ul bromophenol blue (1%) solution, after cooling down to 50 °C. The gel cassettes were transferred to an Ettan Dalt 2-D electrophoresis system (GE Healthcare Biosciences Corp., Piscataway, NJ) after filling the electrophoresis tank with 800 ml of a 10x SDS electrophoresis buffer solution (Tris-base 25 mM, Glycine 192 mM, SDS 0.1%, 1200 final volume) and 7.2 liters of ddH_2O to a final 1x SDS buffer concentration. After a total of 12 gel cassettes were loaded, 2 L of a 2X SDS solution (400 ml of 10x SDS buffer plus 1.6 L of ddH_20) was used to cover the gels to reduce local temperature increase near the negative electrode. The SDS-PAGE gels were run 1 h at 40 V followed by 110 V at 17 °C for 1800 Vh.

3.2.6. Gel Staining, Visualization, Imaging Analysis and Spot Selection

- **Analytical Silver Staining of Gels**

Following second dimension electrophoresis, gels were transferred to a plastic staining tray and incubated for 1 h in fixation solution (ethanol 40%, acetic acid 10%, and water 50% for a final volume of 10 L) to fix the protein-spots in a staining Dodeca Gel Stainers shaker (Bio-Rad, Hercules CA). The gels were then incubated in a sensitizing solution (potassium acetate 0.5 M, sodium thiosulphite 0.2%, and ethanol 30%) for 30 min. The gels were rinsed three times with ddH_2O (10 min each) followed by silver staining (0.2 M $AgNO_3$) for 30 min**.** The gels were rinsed again with ddH_2O for 1 min and incubated in developing solution (potassium carbonate 3% w/v, formaldehyde 0.02% v/v, and sodium thiosulphite 0.2% w/v) for 12 min until spots were visualized. The reaction was stopped by incubation in 20 L of acetic acid solution (5% v/v).

- **Preparative Gel Staining**

Preparative gels were stained with Coomassie Blue to recover protein spots from 2-D electrophoresis gels. The trays and surfaces, work areas, and the Dodeca Gel Stainers shaker were cleaned with ethanol and methanol before staining to reduce contamination. After the second dimension electrophoresis, gels were incubated for 2 h in staining solution (methanol 50%, acetic acid 10% in ddH_2O, and 0.25% Coomassie Blue R-250 previously filtered through Whatman No. 1 filter paper, for a 10 L final volume) with shaking at 20 rpm. Gels were then rinsed two times for 10 min each with ddH_2O and immersed in de-staining solution (methanol 50% and acetic acid 10% in ddH_2O for a 10 L final volume) for 6-8 h or until the spots and molecular weight markers were visible and background was clear. Finally, the preparative gels were incubated for 30 min in storage solution (glacial acetic acid 5% v/v) followed by a final rinse with ddH_2O for at least 2 h before spot recovery.

- **Imaging Analysis**

Stained gels were scanned and visualized with a PowerLook II scanner (UMAX data systems, Taiwan), and the Progenesis SameSpot software for 2-D analysis (Nonlinear Dynamic Ltd., Durham, NC) was used for imaging analysis. Comparative profiling and imaging analysis was according to Chen et al. (2007). The scanned gels were matched automatically by warping each biological replicate gel to a reference gel that was selected based on the total number of spots and the spot resolution and distribution. Some manual matching was done before automatic matching to avoid mismatches between gels. The comparative profiling of groups of gels was organized according to cultivar and treatment (inoculated and non-inoculated), and the normalized volume was used to compare protein expression levels between cultivars and treatments. Treatment groupings were separated by Principal Components Analysis (Progenesis,

Newcastle UK; Durham, NC) and clustering using Euclidian analysis (Infostat, National University of Cordoba, Argentina).

- **Spot Selection**

The differences between treatments in values for the normalized volume of spots were considered significant at a *P* value $\leq$0.05 when differences exceeded 1.4 fold. Some spots with a non-significant *P* value were selected if the fold difference was large. The up- or down-regulated spots were selected through comparative analysis of multiple experiments comparing inoculated and non-inoculated resistant and susceptible cultivars at different times after inoculation. The differentially expressed spots selected for further study were designated as Ho (spots selected from Ho 95-988), CP (selected from HoCP 89-846), and L (selected from LCP 85-384) and numbered sequentially. Those spots that showed more than one significant change in at least three of four biological replicates for LCP 85-384 and HoCP 85-845, or two of three biological repeats in Ho 95-988 and HoCP 89-846, over different experiments between and within cultivars were selected for sequencing.

3.2.7 Tryptic-in Gel Digestion

Selected protein spots were excised from the preparative gels under laminar flow to avoid keratin contamination. The spots selected were each recovered with the tip of a 1 ml pipette, using one tip per selected spot to avoid cross contamination. Gel plugs 1-2 mm wide were transferred to a 1 ml microcentrifuge tube to be washed in purified and deionized water, following the protocol described by the In-Gel Tryptic Digestion Kit (Thermo scientific, Rockford, lL) with incubation in 200 ul of de-staining solution (50% acetonitrile, 100 mM ammonium bicarbonate) for 12 h at 4 °C. The gels blocks were washed five more times with purified and deionized water and dried by adding 50 µl of acetonitrile. After 15 min at room

temperature, the acetonitrile (ACN) was removed, and the gels were allowed to air-dry for 5-10 min. Then, 10 µl of activated trypsin solution (0.1 µg/µl) were added to the microcentrifuge tube and incubated at room temperature for 15 min. Then, 25 µl of digestion buffer (provided by the manufacturer) were added to the tubes and incubated at 37 °C for 4 h. The total digestion mixture was transferred to a new tube and 10 µl of 1% formic acid solution was added and incubated for 5 min to stop the enzymatic digestion. The solution containing each digested protein was recovered and transferred to a new tube.

3.2.8 MS/MS Spectrometry, Database Searching and Protein Identification

Two groups of spots were selected for peptide sequence analysis. The first was sent to the Nevada Proteomics Center of University of Nevada-Reno, in Reno, NV, and the second group was sent to the USDA-ARS Southern Regional Research Center, Food and Feed Safety Research laboratories in New Orleans, LA. The samples sent to Reno were processed according to the procedures of the Nevada Proteomic Center. Samples were washed twice with 25mM ammonium bicarbonate (ABC) and 100% CAN, reduced and alkylated using 10 mM dithiothreitol and 100 mM iodoacetamide and incubated with 75 ng sequencing grade modified porcine trypsin (Promega) in 25 mM ABC for 6 hours at 37^{o} C using a modified protocol (Rosenfeld et al., 1992). Samples were separated on a Michrom Paradigm Multi-Dimensional Liquid Chromatography (MDLC) instrument, and run on Thermo Finnigan LTQ-Orbitrap - Xcalibur v 2.0.7. Spectra were collected using the full ion scan mode over m/z range 400-2000. Scaffold software (version 2.5.0, Proteome software) was used to analyses the peptide sequences samples.

At the USDA-ARS lab in New Orleans, the second group of peptide samples was sequenced by liquid chromatographic separation and electrospray ionization mass spectrometry

using the 6520 Accurate-Mass Q-TOF LC/Ms (Agilent Technologies, Santa Clara, CA). De novo sequences were analyzed with the Spectrum Mill MS Proteomics Workbench Software (Agilent Technologies). Peptide sequence homology analysis was performed using BLAST search against known plant proteins and expressed sequence tags (ESTs) in databases at the National Center for Biotechnology Information (NCBI).

3.2.9 Data Analysis

Bacterial populations quantified by qPCR were analyzed using a completely random design by ANOVA, and least significant differences were calculated by Tukey's Test. Differences in differential expression (normalized volumes) of spots between treatments were compared by the Student's t-test. Total proteome and differentially expressed spot data sets were analyzed by Principal Components Analysis using the Progenesis software (Progenesis, Newcastle UK; Durham, NC) and Euclidian cluster analysis (InfoStat, National University of Cordoba, Argentina).

3.3 RESULTS

The research combined comparative proteomic analysis and protein identification with monitoring of Xa populations by TaqMan qPCR in resistant and susceptible cultivars under differential conditions of infection (Figure 3.2) to identify proteins associated with the mechanisms of sugarcane resistance to leaf scald.

3.3.1 Xa Population Quantification by TaqMan qPCR and Disease Severity for Resistant and Susceptible Cultivars

The Xa population changed over time in infected plants of the resistant cultivar Ho 95-988 and the susceptible cultivar HoCP 89-846 (Figure 3.3).

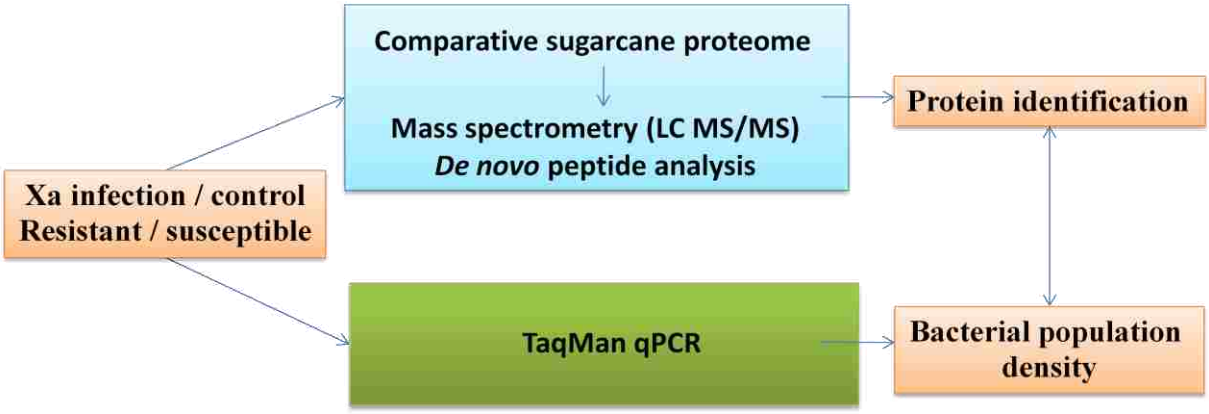


Figure 3.2. Research approach of the sugarcane proteomics analysis under differential conditions of infection by *Xanthomonas albilineans* with monitoring of bacterial populations by TaqMan qPCR.

The initial Xa population at 2 wai in the inoculated leaves was higher in the resistant than the susceptible cultivar. However, the bacterial population in Ho 95-988 decreased over time and was less than $8x10^2$ CFU/ml in systemically infected leaves at 8 wai. In contrast, the bacterial population in HoCP 89-846 increased following inoculation to $1.2x10^6$ CFU/ml in the systemically infected leaves at 8 wai. The increase in bacterial population in the new, emerged leaves in the susceptible cultivar and the decrease in the resistant one corresponded with disease severity scores. Severity scores were 27 and 79, respectively, for the inoculated and systemically infected leaves of HoCP 89-846 and 24 and 20, respectively, for the inoculated and systemically infected leaves of Ho 95-988. A reduction of plant height (30%) and the stem diameter (16%) comparing infected to mock-inoculated plants was observed 8 wai in resistant Ho 95-988. Highly resistant cultivar LCP 85-384 had disease severity scores of 21 and 0, respectively, whereas the susceptible cultivar HoCP 85-845 had severity scores of 54 and 85, respectively, for inoculated and systemically infected leaves. Similarly, the Xa populations detected in the new emerged leaves were $3x10^2$ CFU/ml in highly resistant LCP 85-384 and $1.9x10^6$ CFU/ml in susceptible HoCP 85-845. Cultivar HoCP 85-845 was not classified as

highly susceptible based on field observations of recovery from leaf scald symptom expression in ratoon crops (J. Hoy unpublished).

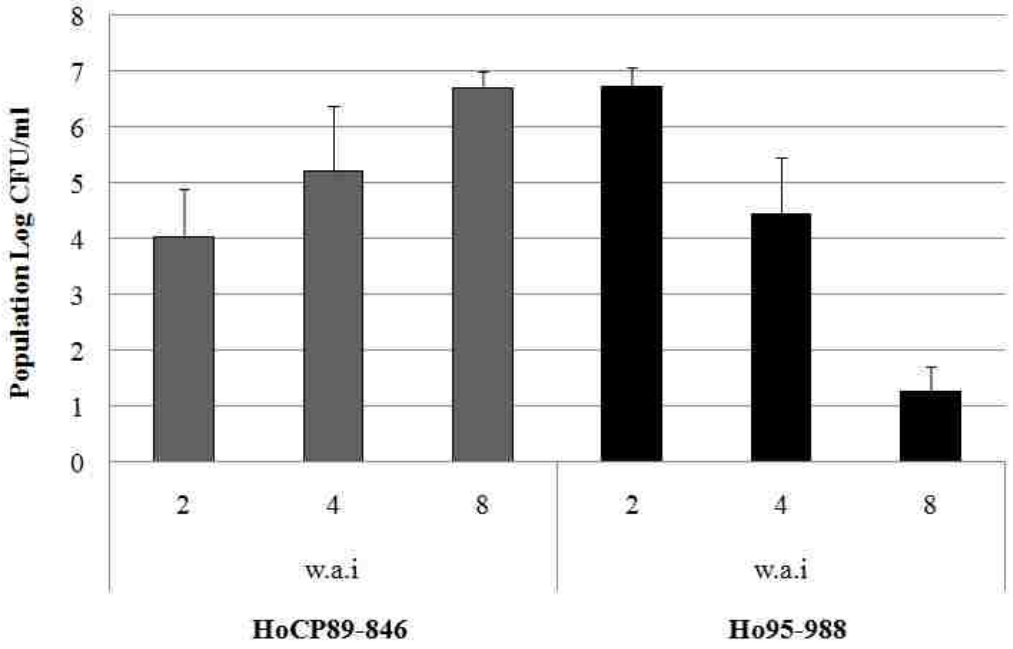


Figure 3.3. Comparison of *X. albilineans* populations quantified by TaqMan qPCR in a leaf scald highly susceptible (HoCP 89-846) and resistant (Ho 95-988) cultivar at 2, 4, and 8 weeks after inoculation (wai). Bacterial population in leaf samples expressed as the log of colony forming units (CFU) per ml.

3.3.2 Total Protein Extraction from Sugarcane Leaves and 2D-Gel Electrophoresis

The modified protein extraction protocol allowed the processing of large samples of leaves to obtain a high quality and quantity of the sugarcane proteins that resulted in good 2-D gel protein profiles. The profile of cultivar Ho 95-988 exhibited spots with limited vertical and horizontal streaking, no smearing, and good separation (Figure 3.4).

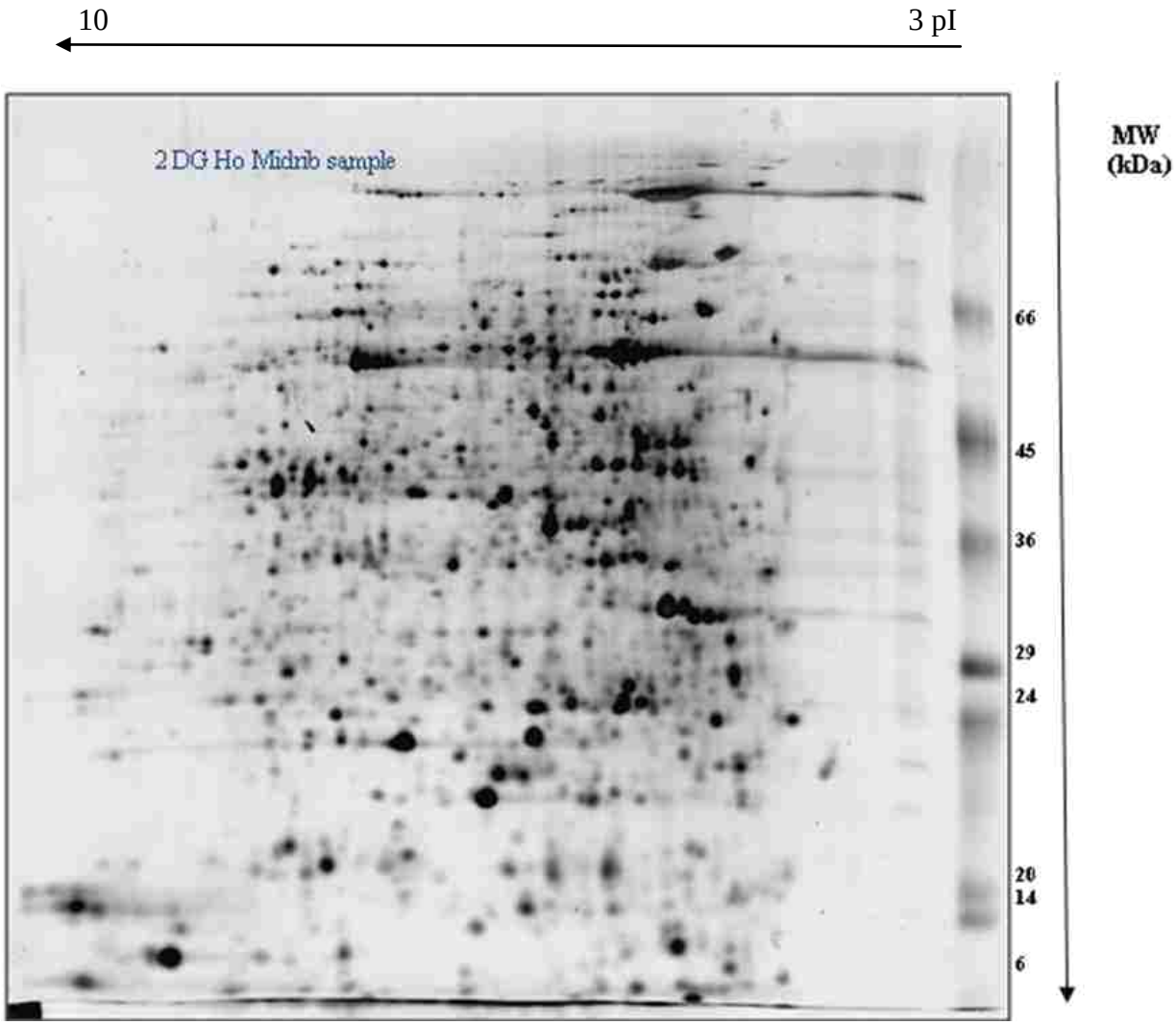


Figure 3.4. Protein profile of sugarcane cultivar Ho 95-988 obtained from leaf tissue with a combined trichloroacetic acid, phenol and ammonium acetate extraction protocol. MW = molecular weight; pI = isoelectric point, 3-10 (non-linear).

3.3.3 Time-Course Proteome Analysis of Susceptible And Resistant Cultivars Under Xa Infection

Up- and down-regulated spots were detected in the proteomic analyses comparing Xa-infected and mock-inoculated plants of leaf scald resistant Ho 95-988, highly resistant LCP 85-384, highly susceptible HoCP 89-846, and susceptible HoCP 85-845. The total number of spots in leaf samples collected from Xa-infected compared to mock-inoculated control plants was 889, 886, and 846 at 2, 4, and 8 wai, respectively, in the resistant cultivar, Ho 95-988; 993, 978, and 883 in highly susceptible cultivar, HoCP 89-846; 937 in the susceptible HoCP 85-845 8 wai, and 884 in highly resistant LCP 85-384 8 wai.

There were more up-regulated spots in the susceptible compared to the resistant cultivars in the Xa inoculated leaves at 2 wai and in the 4 wai samples that contained both inoculated and systemically infected leaves (Figure 3.5). The number of differentially expressed spots decreased over time in the systemically infected leaves of highly susceptible HoCP 89-846. In contrast, the number of differentially expressed spots increased in systemically infected leaves of resistant Ho 95-988, and there were more up- and down-regulated spots compared to the susceptible cultivar. More down-regulated spots were detected at 8 wai in systemically infected leaves of highly resistant LCP 85-384. The number of differentially expressed spots was only determined at one time point, 8 wai, for the susceptible cultivar, HoCP 85-845. There were 35 more up- and 49 more down-regulated spots for this cultivar compared to highly susceptible HoCP 89-846. At 8 wai, the total spots produced in the 2-D gels from four replicate plants that were Xa-infected or mock-inoculated and up- and down-regulated spots grouped for each cultivar under Principal Components Analysis.

3.3.4 Selection of Differentially Expressed Spots Associated with Sugarcane Response to Xa Infection for Protein Identification

The comparative time-course proteomics analysis resulted in the selection of two groups of spots differentially expressed in response to Xa infection in two resistant cultivars, Ho 95-988 and LCP 85-384. The expression of the selected spots was then analyzed between inoculated plants of the two resistant and two susceptible cultivars to compare the induction of expression. Finally, the spots were analyzed between mock-inoculated plants of the two resistant and two susceptible cultivars to identify possible constitutive protein expression.

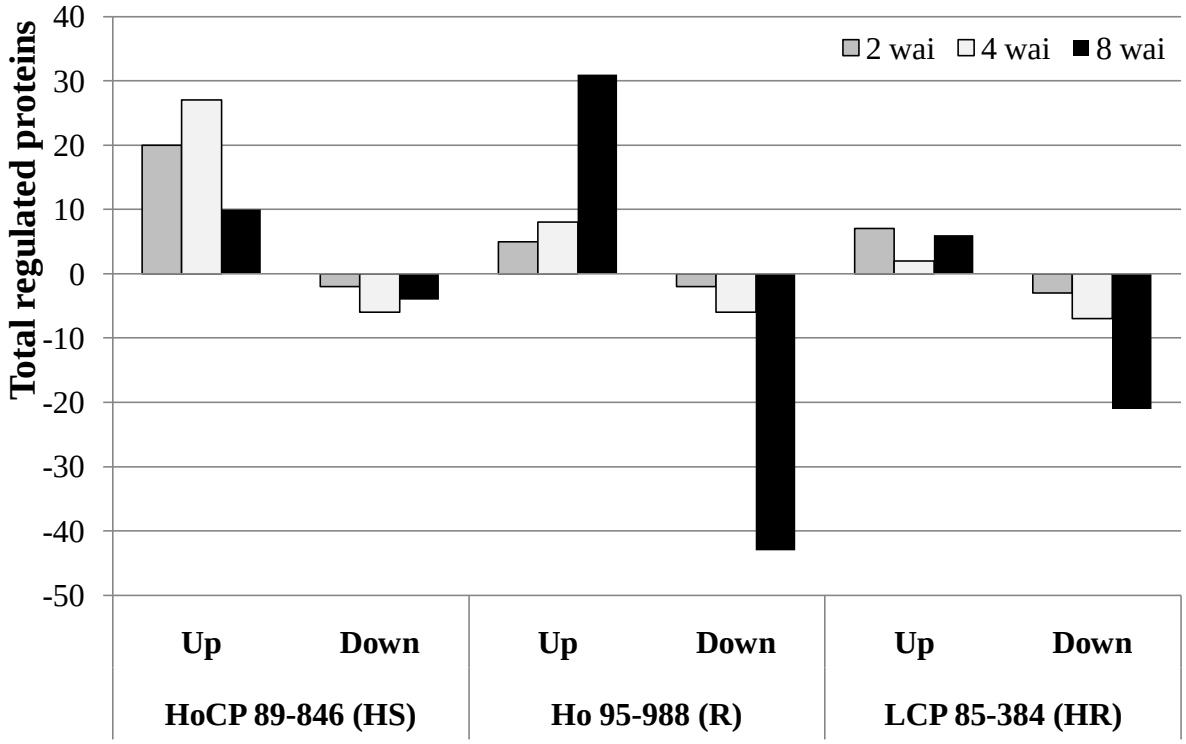


Figure 3.5. Comparison of up- and down-regulated spots in *X. albilineans*-infected compared to mock-inoculated plants of a highly susceptible (HS) cultivar, HoCP 89-846, a resistant (R) cultivar, Ho 95-988, and a highly resistant (HR) cultivar, LCP 85-384, at 2, 4, and 8 weeks after inoculation (wai).

- **Selection and Comparative Analysis of Proteins Differentially Expressed in the Resistant Cultivar Ho 95-988**

A total of 30 spots significantly up- or down-regulated in response to Xa infection during at least one time point of the time course experiment were initially selected for comparative analysis of expression between highly susceptible HoCP 89-846 and resistant Ho 95-988. Eleven of the spots (Figure 3.6) were selected for additional comparative analysis of expression with highly resistant LCP 85-384 and susceptible HoCP 85-845 followed by protein identification.

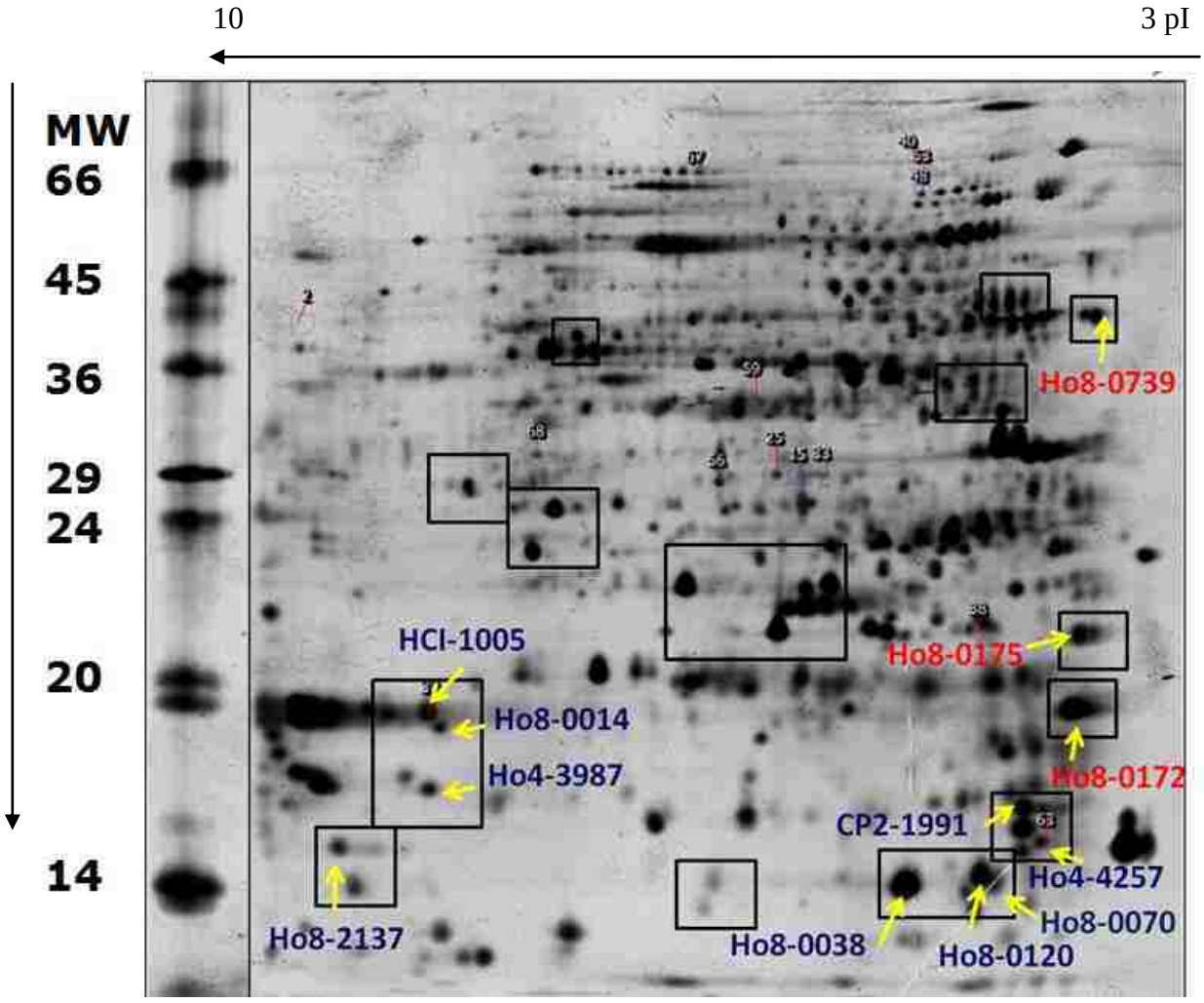


Figure 3.6. Spots differentially expressed in proteome of resistant cultivar, Ho 95-988, in response to infection by *X. albilineans*. Spots were down- regulated (identified in red) or up-regulated (identified in blue). MW = molecular weight; pI = isoelectric point, pH 3-10 (non-linear).

Differential expression of spots in response to Xa infection was similar or contrastive between the resistant and the highly susceptible cultivars. For example, spot Ho8-0038 was up-regulated in Xa-infected plants of both resistant Ho 95-988 and susceptible HoCP 89-846 showing a significant difference 8 wai (Figure 3.7). Spot Ho8-0120 was down-regulated in the susceptible cultivar during initial infection 2 wai but was up-regulated 8 wai in the resistant cultivar (Figure 3.8). In contrast, spot Ho8-0739 was significantly down-regulated in response to Xa infection 8 wai in resistant Ho 95-988 (Figure 3.9).

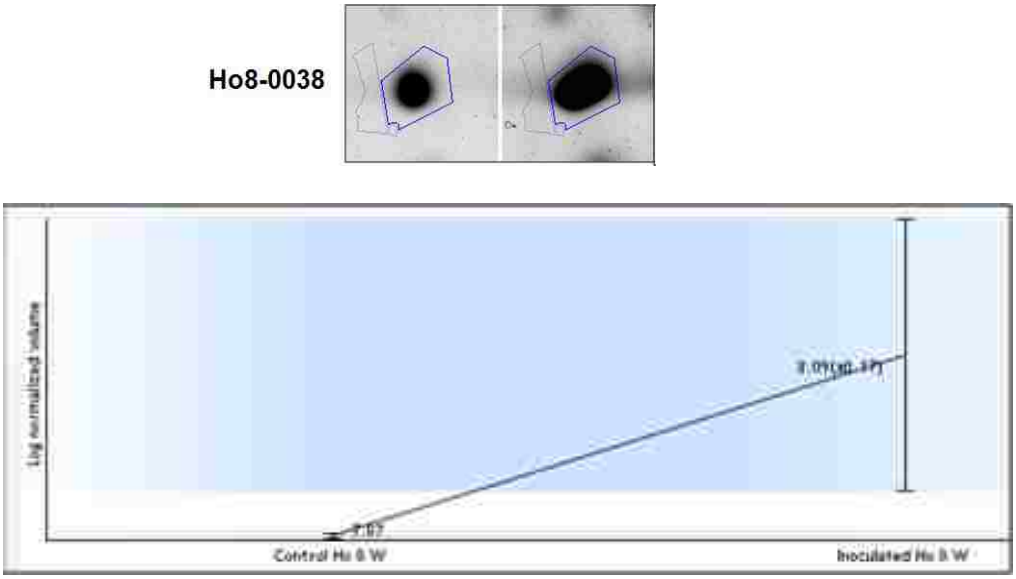


Figure 3.7. Spot Ho8-0038 up-regulated in resistant Ho 95-988 in response to infection by *X. albilineans* (Xa). Above: gel spots from mock-inoculated control (left) and Xa-infected (right) plants. Below: Analysis results illustrating magnitude of difference in regulation between the spots from the control and infected plants with standard deviation for the three biological replicates indicated.

Differential expression of the 11 spots selected in the comparative analysis of resistant Ho 95-988 and highly susceptible HoCP 89-846 was contrasted with expression of the same spots in highly resistant LCP 85-384 during the Xa infection time-course experiment (Figure 3.10).

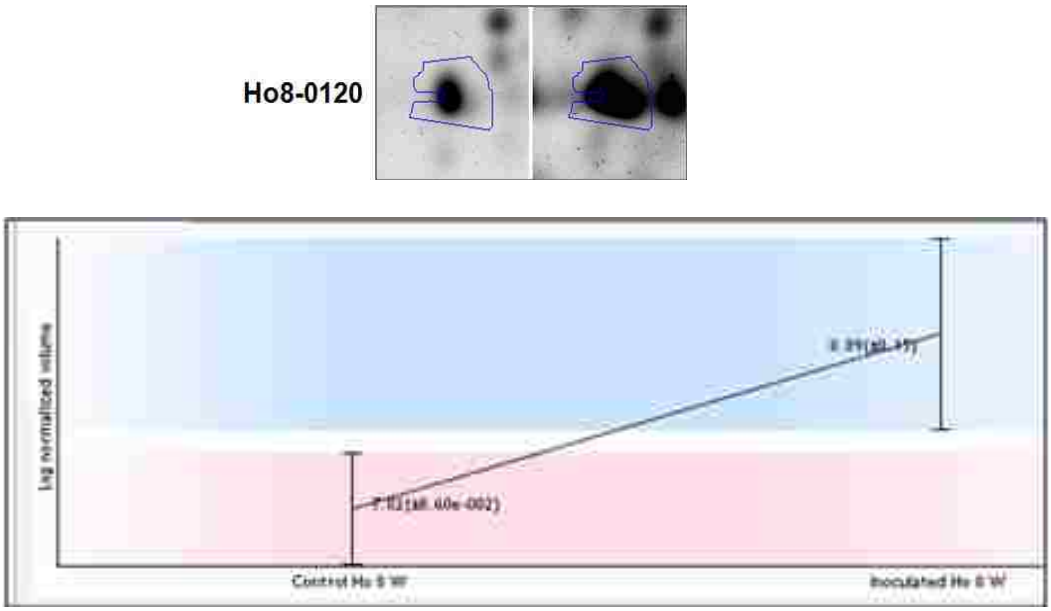


Figure 3.8. Spot Ho8-0120 up-regulated in resistant Ho 95-988 in response to infection by *X. albilineans* (Xa). Above: gel spots from mock-inoculated control (left) and Xa-infected (right) plants. Below: Analysis results illustrating magnitude of difference in regulation between the spots from the control and infected plants with standard deviation for the three biological replicates indicated.

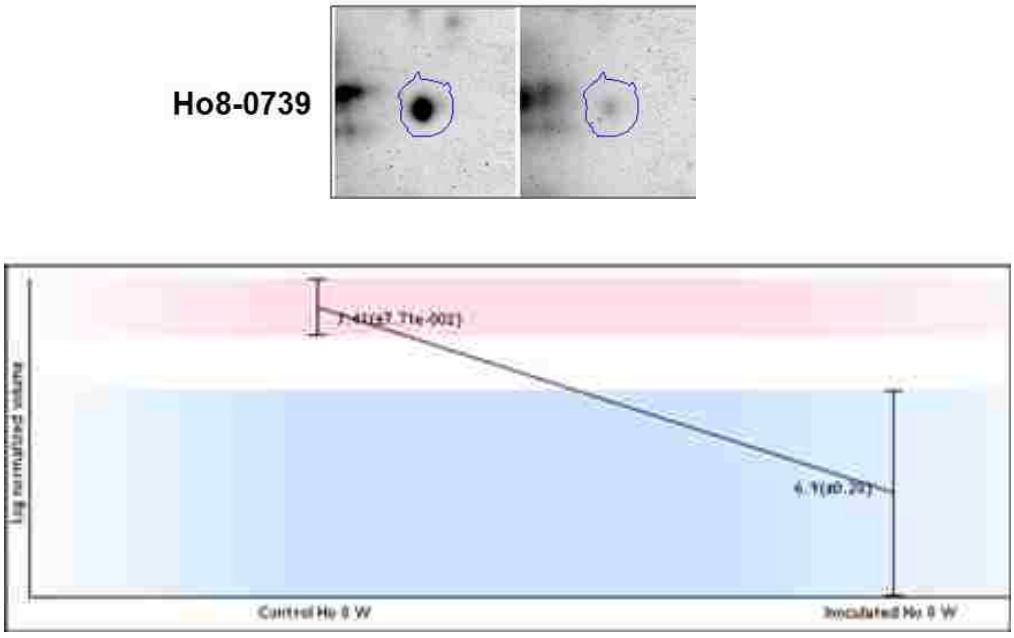
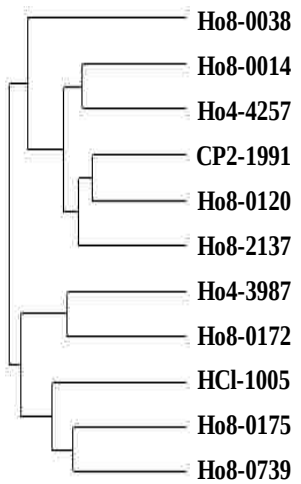


Figure 3.9. Spot Ho8-0739 was down-regulated in resistant Ho 95-988 in response to infection by *X. albilineans* (Xa). Above: gel spots from mock-inoculated control (left) and Xa-infected (right) plants. Below: Analysis results illustrating magnitude of difference in regulation between the spots from the control and infected plants with standard deviation for the three biological replicates indicated.

Differential expression of the 11 spots selected in the comparative analysis of resistant Ho 95-988 and highly susceptible HoCP 89-846 was contrasted with expression of the same spots in highly resistant LCP 85-384 during the Xa infection time-course experiment (Figure 3.10). Cluster analysis resulted in numerous clusters indicating variable expression patterns across cultivars and within cultivars for the different time points (Figure 3.10).



	Spot expression rate								
	Highly susceptible HoCP 89-846			Highly resistant LCP 85-384			Resistant Ho 95-988		
Spot Id	2 wai	4 wai	8 wai	2 wai	4 wai	8 wai	2 wai	4 wai	8 wai
Ho8-0038	-1.0	1.1	1.1	1.3	-1.1	-1.5	1.0	0.0	1.7
Ho8-0014	1.3	1.3	-1.1	1.2	1.1	-1.2	1.3	1.5	1.6
Ho4-4257	1.1	1.5	-1.2	2.4	-1.0	-2.1	1.1	1.9	-1.1
CP2-1991	1.3	1.5	-1.2	1.2	-1.0	-1.3	-1.0	1.4	1.2
Ho8-0120	-1.1	1.4	-1.0	1.3	-1.1	-1.4	0.0	1.6	1.9
Ho8-2137	0.0	0.0	-2.6	1.4	0.0	-1.1	0.0	1.1	2.6
Ho4-3987	-1.2	1.6	-1.1	-1.3	-1.0	1.2	2.2	2.1	1.1
Ho8-0172	-1.1	-1.1	-1.1	-1.7	-1.1	1.1	1.3	1.5	-2.1
HCl-1005	1.2	-1.0	-1.8	1.4	1.0	1.3	1.1	1.4	1.4
Ho8-0175	1.3	0.0	-1.1	-1.3	1.0	1.1	1.5	1.0	-2.4
Ho8-0739	1.0	-1.1	-1.0	0.0	1.2	0.0	1.0	-1.3	-3.1

Figure 3.10. Differential regulation and cluster analysis of the first group of 11 spots selected based on differential regulation in response to infection by *X. albilineans* in different cultivars during the time-course experiment. Magnitude of up (positive values) and down (negative values) regulation indicated for each cultivar at 2, 4, and 8 weeks after inoculation (wai); dark blue = up-regulation, light blue = up-regulation with $P \leq 0.05$, red = down-regulation, and orange = down-regulation with $P \leq 0.05$.

The increased number of differentially expressed spots in the resistant cultivars at 8 wai with more spots down-regulated in LCP 85-384 reflected the results of the overall spot analyses (Figure 3.5). The expression of four of 11 selected spots at 8 wai was significantly up- regulated in the resistant Ho 95-988 but not in the highly resistant LCP 85-384 or susceptible HoCP 89-846. During systemic infection, highly resistant LCP 85-384 showed down-regulation of the spots Ho8-0038 and Ho8-0120 while resistant Ho 95-988 showed up-regulation, and each

resistant cultivar had three down-regulated spots not in common with the other, suggesting possible differences in the expression of resistance. Two additional spots that showed contrastive differences in expression were spot HCl-1005 that was down-regulated in the highly susceptible and up-regulated in the resistant cultivar and spot Ho8-0175 that was significantly up-regulated in the susceptible cultivar 2 wai and down-regulated in the resistant one under systemic infection. The results suggest these spots may play a role in susceptibility or resistance to Xa.

Differences in expression level also were compared separately for only inoculated or mock-inoculated plants across cultivars for the 11 selected spots. Differential expression of the spots was detected between resistant and highly resistant cultivars and either the highly susceptible or the susceptible cultivar in Xa-infected plants at 8 wai (Figure 3.11). Two major clusters were observed, indicating a similar pattern of differential expression under Xa infection for both resistant cultivars compared to each susceptible cultivar. The patterns of up- and down-regulation of spots were similar (91%) in the comparison of the resistant cultivars with susceptible HoCP 85-845 and more variable (63% similarity) in the comparison of the resistant cultivars with highly susceptible HoCP 89-846. A cluster of six spots showed a general pattern of down-regulation for both resistant cultivars compared to highly susceptible HoCP 89-846 and up-regulation compared to susceptible HoCP 85-845. In contrast, spot HC1-1005 exhibited up-regulation in the comparison of the resistant cultivars with the highly susceptible one and down-regulation in the comparison with the susceptible one. The spot expression differences detected in the comparisons of resistant to susceptible cultivars further suggest differences in mechanisms of resistance and susceptibility among cultivars.

Differences in expression level also were detected in comparisons of mock-inoculated, negative controls of different cultivars (Figure 3.12) that need to be taken into account when

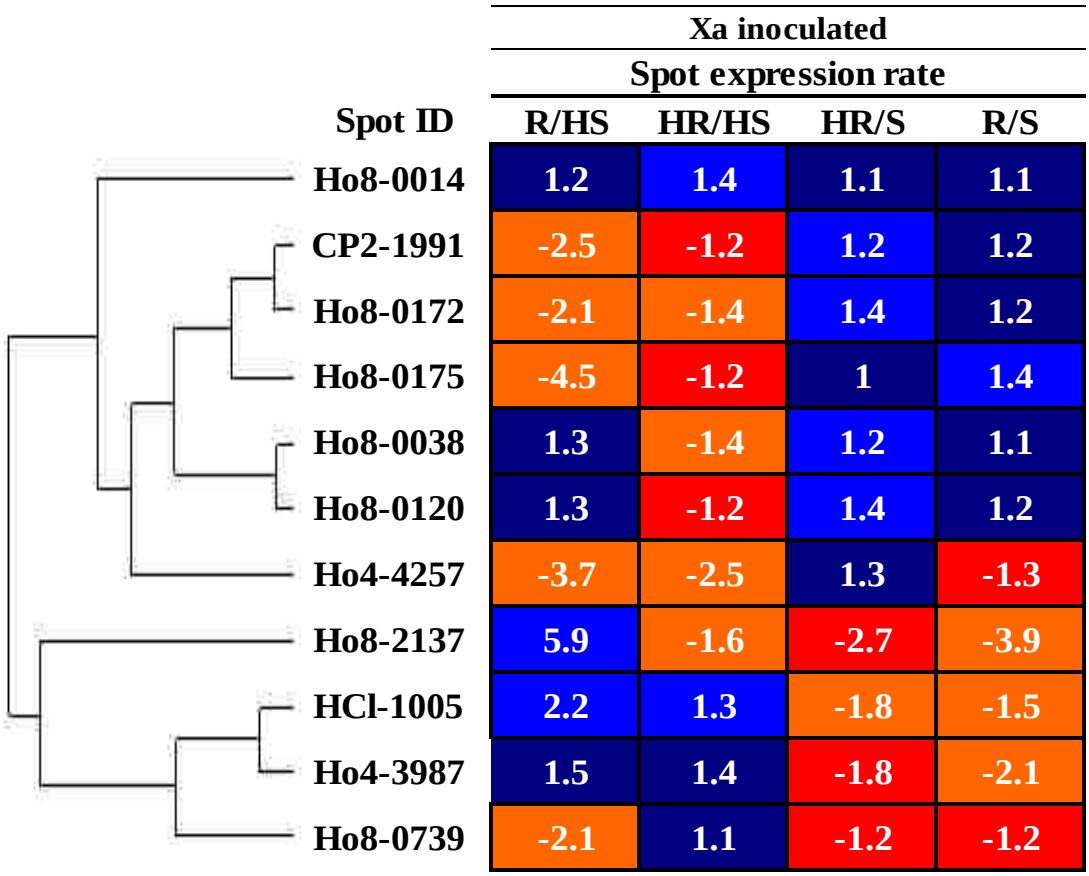


Figure 3.11. Differential regulation and cluster analysis of the first set of 11 selected spots across cultivars between *X. albilineans*-infected leaf scald resistant (R) Ho 95-988 or highly resistant (HR) LCP 85-384 compared to susceptible (S) HoCP 85-845 and highly susceptible (HS) HoCP 89-846. Magnitude of higher (positive values) and lower (negative values) expression indicated for each cultivar comparison; dark blue = higher expression, light blue = higher expression with $P \leq 0.05$, red = lower expression, and orange = lower expression with $P \leq 0.05$.

interpreting the differences in spot expression among cultivars under Xa infection. There was a trend toward lower expression of spots in mock-inoculated plants of the two resistant cultivars compared to highly susceptible HoCP 89-846. Two spots, CP2-1991 and Ho4-4257, were expressed at a lower level in both resistant cultivars. Only one spot, Ho8-2137 was expressed at a higher level in resistant Ho 95-988. Comparing spot expression levels in non-inoculated plants of the two resistant cultivars, one spot, Ho4-4257, also was expressed at a lower level in highly resistant LCP 85-384 than resistant Ho 95-988 while one spot, Ho8-0175 was expressed at a

higher rate. Taking into consideration the constitutive differences, one spot, Ho8-0175, showed greater down-regulation in inoculated plants of resistant Ho 95-988 compared to highly susceptible HoCP 89-846, while two spots, Ho8-2137 and HC1-1005, showed increased up-regulation. Three spots, Ho8-0172, Ho4-4257, and Ho8-2137, showed greater down-regulation in highly resistant LCP 85-384 compared to highly susceptible HoCP 89-846. These differences in expression detected in comparisons accounting for constitutive differences suggest involvement of these spots in the resistance response to Xa infection.

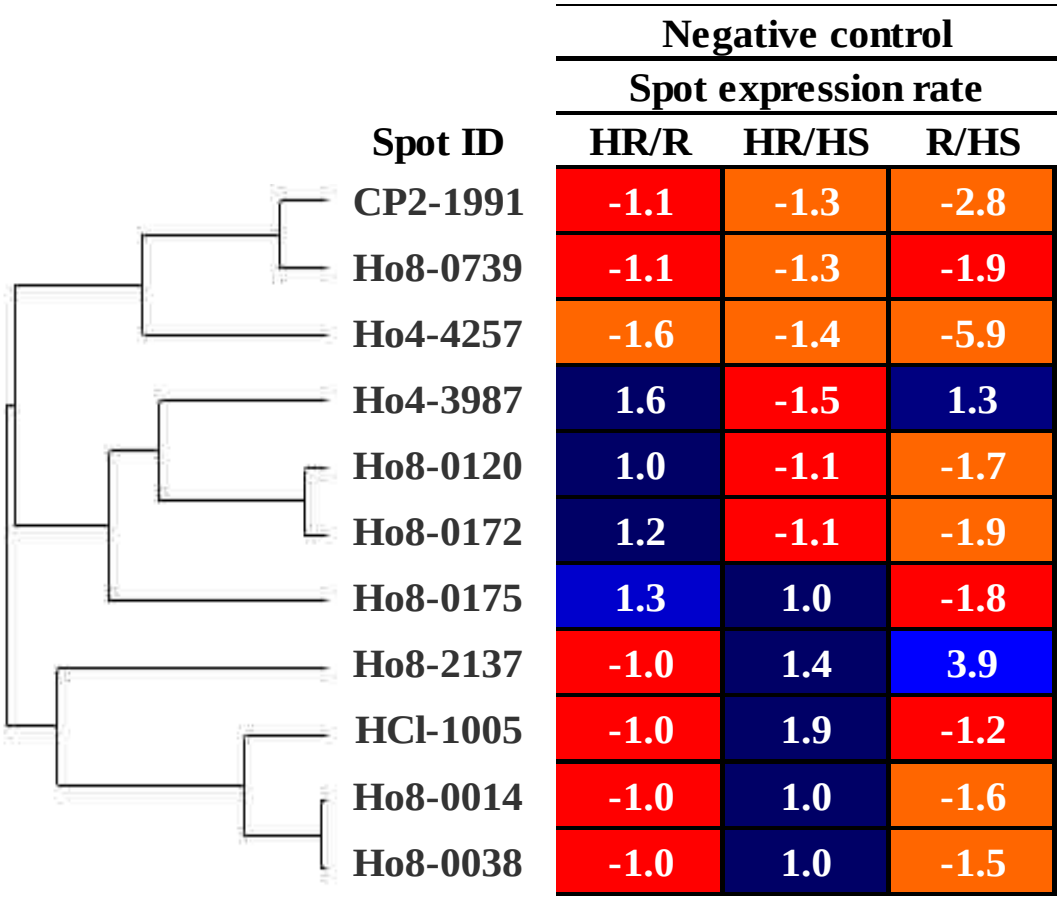


Figure 3.12. Differential regulation and cluster analysis of selected spots across cultivars between mock-inoculated negative controls of highly resistant (HR) LCP 85-384 and resistant Ho 95-988, highly resistant LCP 85-384 and highly susceptible HoCP 89-846, and resistant Ho 95-988 and highly susceptible HoCP 89-846. Magnitude of higher (positive values) and lower (negative values) expression indicated for each cultivar comparison; dark blue = higher expression, light blue = higher expression with $P \leq 0.05$, red = lower expression, and orange = lower expression with $P \leq 0.05$.

- **Selection and Comparative Analysis of Differentially Expressed Proteins from the Highly Resistant Cultivar LCP 85-384**

An additional set of 19 spots exhibiting differential expression for at least one time point in the Xa infection time-course experiment were selected for analysis from highly resistant cultivar, LCP 85-384 (Figure 3.13). Spot expression patterns varied between the inoculated leaves 2 wai and the systemically infected leaves at 8 wai (Figure 3.14). During initial infection (2 wai), three spots were up-regulated and two spots were down-regulated in the inoculated leaves. The cluster analysis reflected an increase in down-regulation of spots at 8 wai. Two spots, L1 and L17, increased in expression over time. However, the results suggest the resistance response in LCP 85-384 involves down-regulation of protein expression by 8 wai.

Differences in expression level of 15 spots selected from LCP 85-384 were detected when spot up- and down-regulation was compared across the two resistant and highly susceptible cultivars at 8 wai (Figure 3.15). The cluster analysis reflected the variability in expression patterns between the three cultivars. LCP 85-384 had the greatest number of differentially expressed spots (10) with seven down-regulated. Five of the down-regulated spots showed a similar but non-significant down-regulation in resistant Ho 95-988. Spot L1 was up-regulated in highly resistant LCP 85-384 and down-regulated in highly susceptible HoCP 89-846. The results suggest these differentially expressed spots could be involved in the resistance response to Xa infection. Spot L6 was the only differentially expressed spot in Ho 95-988 at 8 wai. Interestingly, L6 was up-regulated in LCP 85-384 at 2 wai suggesting possible involvement in the initial expression of resistance to suppress infection in the LCP 85-384 and an induced response during systemic infection in the resistant Ho 95-988.

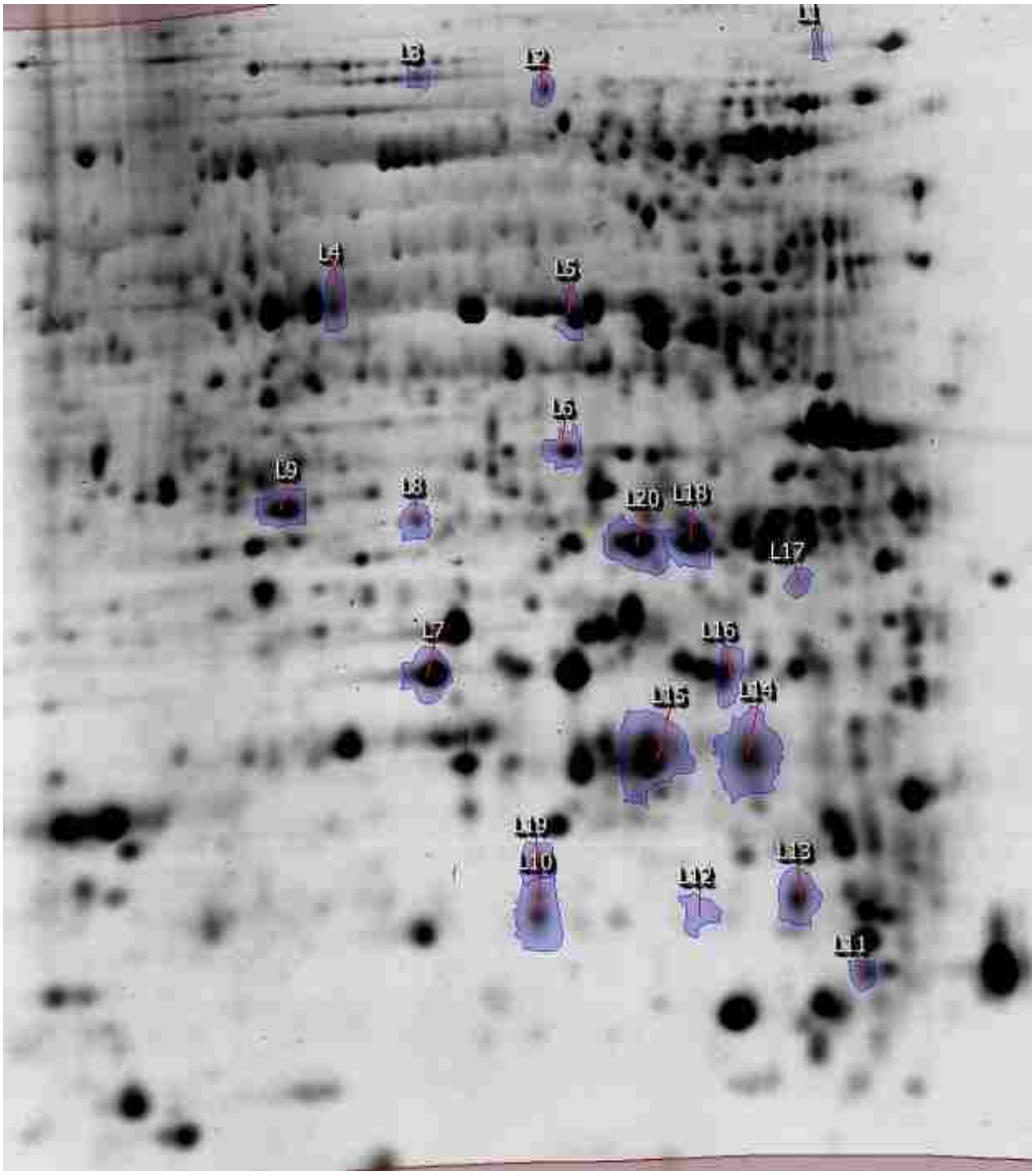


Figure 3.13. Spots differentially expressed in proteome of highly resistant cultivar, LCP 85-384, in response to infection by *X. albilineans*.

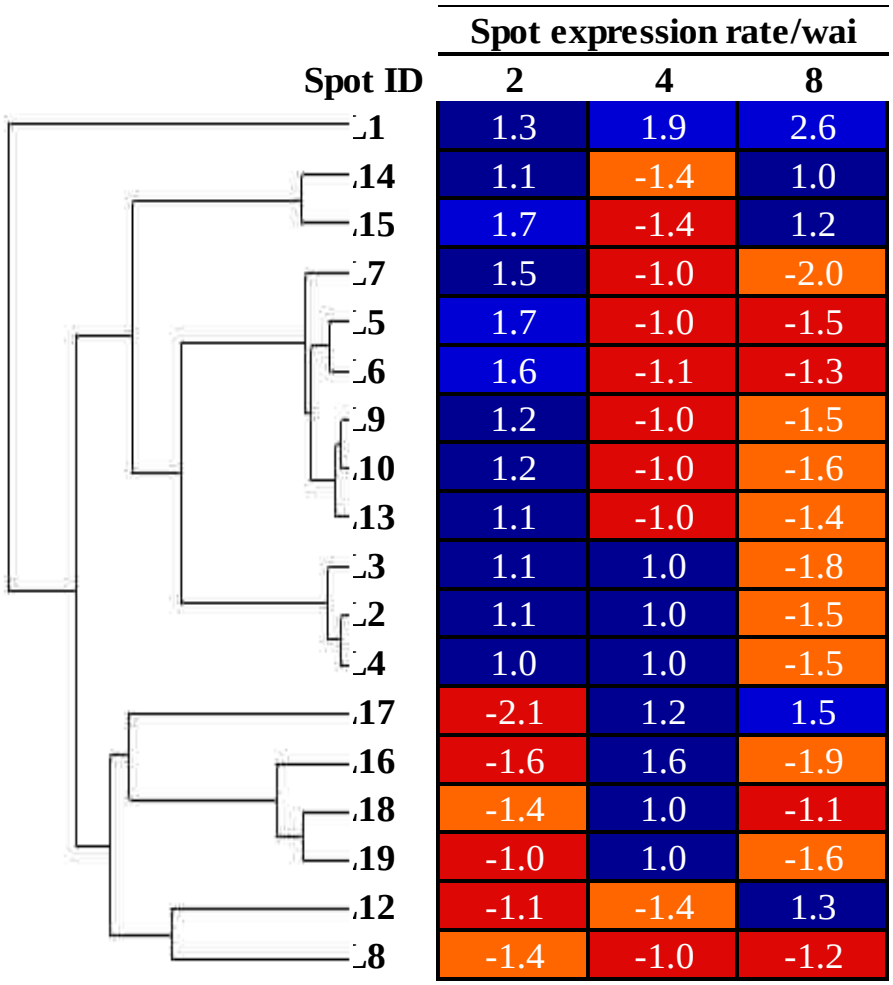
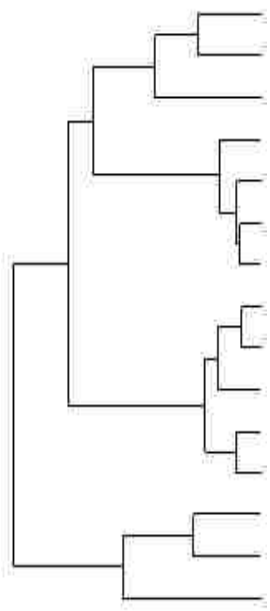


Figure 3.14. Differential regulation and cluster analysis of 18 spots selected based on expression pattern differences in highly resistant LCP 85-384 during the time-course experiment. Magnitude of up- (positive values) and down- (negative values) regulation indicated for 2, 4, and 8 wk after inoculation; dark blue = up-regulation, light blue = up-regulation with $P \leq 0.05$, red = down-regulation, and orange = down-regulation with $P \leq 0.05$.

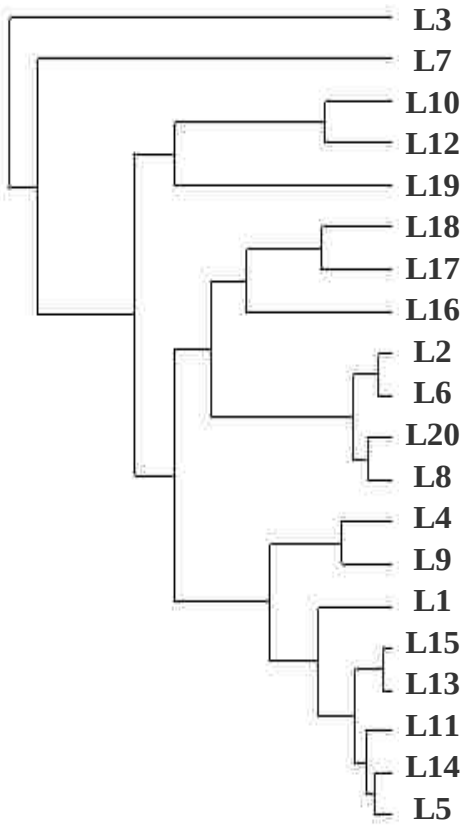
Differences in expression level again were compared separately for only inoculated or mock-inoculated plants across cultivars for the spots selected from LCP 85-384. Differential expression of the spots was detected between the resistant and highly resistant cultivars and either the highly susceptible or the susceptible cultivar in Xa-infected plants at 8 wai (Figure 3.16). As in the comparative analysis of the spots from Ho 95-988, differences in expression level varied among comparisons, and the cluster analysis showed numerous small clusters.

There was a general trend towards lower expression (eight spots) in both resistant cultivars compared to both susceptible cultivars. Five spots exhibited a similar lower expression in both resistant cultivars compared to highly susceptible HoCP 89-846. L7 had lower expression and L10 and L12 had higher experession in both the resistant cultivars compared to susceptible HoCP 85-845. Spot L3 had lower expression in all four comparisons of the resistant with the susceptible cultivars suggesting consistent involvement in the resistance response to Xa infection.



	Spot expression rate		
Spot ID	LCP 85-384	Ho 95-988	HoCP 89-846
L15	1.2	0.0	1.2
L17	1.5	1.1	1.5
L20	2.6	0.0	0.0
L7	-2.0	1.2	1.7
L2	-1.5	1.3	1.5
L6	-1.3	1.4	1.1
L18	-1.1	1.1	1.3
L13	-1.4	-1.9	1.6
L19	-1.6	-1.9	1.3
L3	-1.8	-1.2	1.7
L4	-1.5	-2.8	1.4
L16	-1.9	-2.5	1.4
L1	2.6	1.1	-2.5
L12	1.3	1.1	-2.0
L14	1.0	-1.0	-1.2

Figure 3.15. Differential regulation and cluster analysis of 15 spots in highly resistant LCP 85-384 compared to regulation of the same spots for resistant Ho 95-988 and highly susceptible HoCP 89-846 in response to *X.albilineans* infection at 8 wk after inoculation (wai). Magnitude of up (positive values) and down (negative values) regulation indicated for 2, 4, and 8 wai for LCP 85-384 and 8 wai only for Ho 95-988 and HoCP 89-846; dark blue = up-regulation, light blue = up-regulation with $P \leq 0.05$, red = down-regulation, and orange = down-regulation with $P \leq 0.05$.

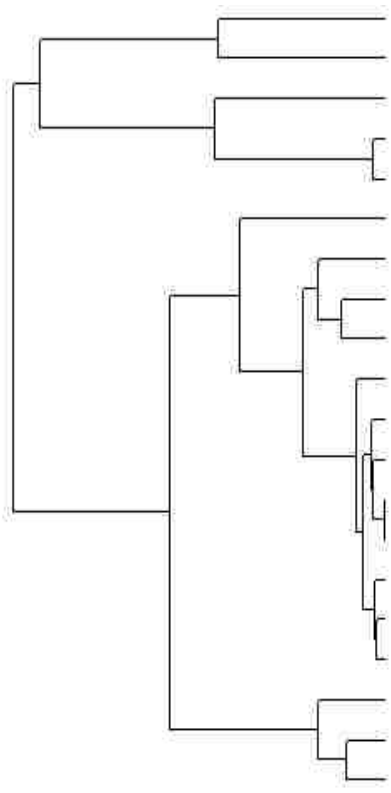


Spot expression rate			
R/HS	HR/HS	HR/S	R/S
-6.2	-4.5	-2.8	-2.0
1.1	-1.3	-3.9	-5.7
-1.3	-1.0	1.4	1.7
-1.9	-1.1	1.8	2.9
1.4	-1.1	1.1	-1.4
-1.0	1.3	-1.3	1.0
-2.0	1.1	-1.0	2.1
1.2	2.6	-1.6	1.4
1.9	1.2	-1.2	-1.8
1.8	1.4	-1.3	-1.8
1.2	1.2	-1.3	-1.3
1.4	1.6	-1.4	-1.2
-2.3	-2.2	-1.2	1.1
-1.6	-1.5	-1.0	1.1
-1.4	-2.3	-1.5	-2.4
-1.2	-1.1	-1.4	-1.3
-1.2	-1.1	-1.3	-1.2
-1.7	-1.9	-1.1	-1.2
-1.7	-1.5	-1.2	-1.1
-1.6	-1.5	-1.4	-1.3

Figure 3.16. Differential regulation and cluster analysis of 20 selected spots from LCP 85-384 across *X. albilineans*-infected cultivars between leaf scald resistant (R) Ho 95-988 or highly resistant (HR) LCP 85-384 and susceptible (S) HoCP 85-845 and highly susceptible (HS) HoCP 89-846. Magnitude of higher (positive values) and lower (negative values) expression indicated for each cultivar comparison; dark blue = higher expression, light blue = higher expression with $P \leq 0.05$, red = lower expression, and orange = lower expression with $P \leq 0.05$.

Differences in expression of the same 20 spots were detected in comparisons of mock-inoculated, negative control plants across cultivars (Figure 3.17). In a comparison of highly resistant LCP 85-384 and resistant Ho 95-988, only spot L1 had a higher expression level in the highly resistant cultivar, while 11 spots formed a large cluster with lower expression. Spot L1 also was expressed at a higher level in susceptible HoCP 85-845 (Figure 3.18). Spot expression levels were very different in comparisons between the two resistant cultivars and the highly

susceptible cultivar. The same cluster of spots exhibiting lower expression in LCP 85-384 compared to Ho 95-988 had generally lower expression also in the comparison to highly susceptible HoCP 89-846. In contrast, a general trend towards higher expression was evident for 12 of the same spots when comparing the resistant to the highly susceptible cultivar. Three spots had significantly lower expression in the highly resistant compared to the highly susceptible cultivar, and four spots had significantly higher expression in the resistant cultivar.



Spot ID	Spot expression rate		
	HR/R	HR/HS	R/HS
L1	2.2	-1.2	-2.7
L13	1.0	-1.1	-1.1
L10	1.2	1.2	-1.0
L2	1.1	1.1	1.0
L3	1.1	1.2	1.0
L7	-2.5	-2.2	1.1
L12	-2.0	-1.5	1.3
L16	-2.0	-1.2	1.7
L6	-2.3	-1.1	2.1
L5	-1.1	-1.1	1.1
L15	-1.4	-1.3	1.1
L14	-1.4	-1.2	1.2
L20	-1.3	-1.2	1.1
L4	-1.3	-1.2	1.1
L17	-1.4	-1.1	1.2
L18	-1.5	-1.1	1.3
L8	-1.4	-1.1	1.3
L11	-1.5	-1.9	-1.2
L19	-1.2	-1.2	-1.0
L9	-1.4	-1.5	-1.1

Figure 3.17. Cluster analysis and differences in expression level of second set of selected spots across cultivars between mock inoculated, leaf scald resistant (R) Ho 95-988 and highly resistant (HR) LCP 85-384 and both resistant cultivars compared to highly susceptible (HS) HoCP 89-846. Magnitude of higher (positive values) and lower (negative values) expression indicated for each cultivar comparison; dark blue = higher expression, light blue = higher expression with $P \leq 0.05$, red = lower expression, and orange = lower expression with $P \leq 0.05$.

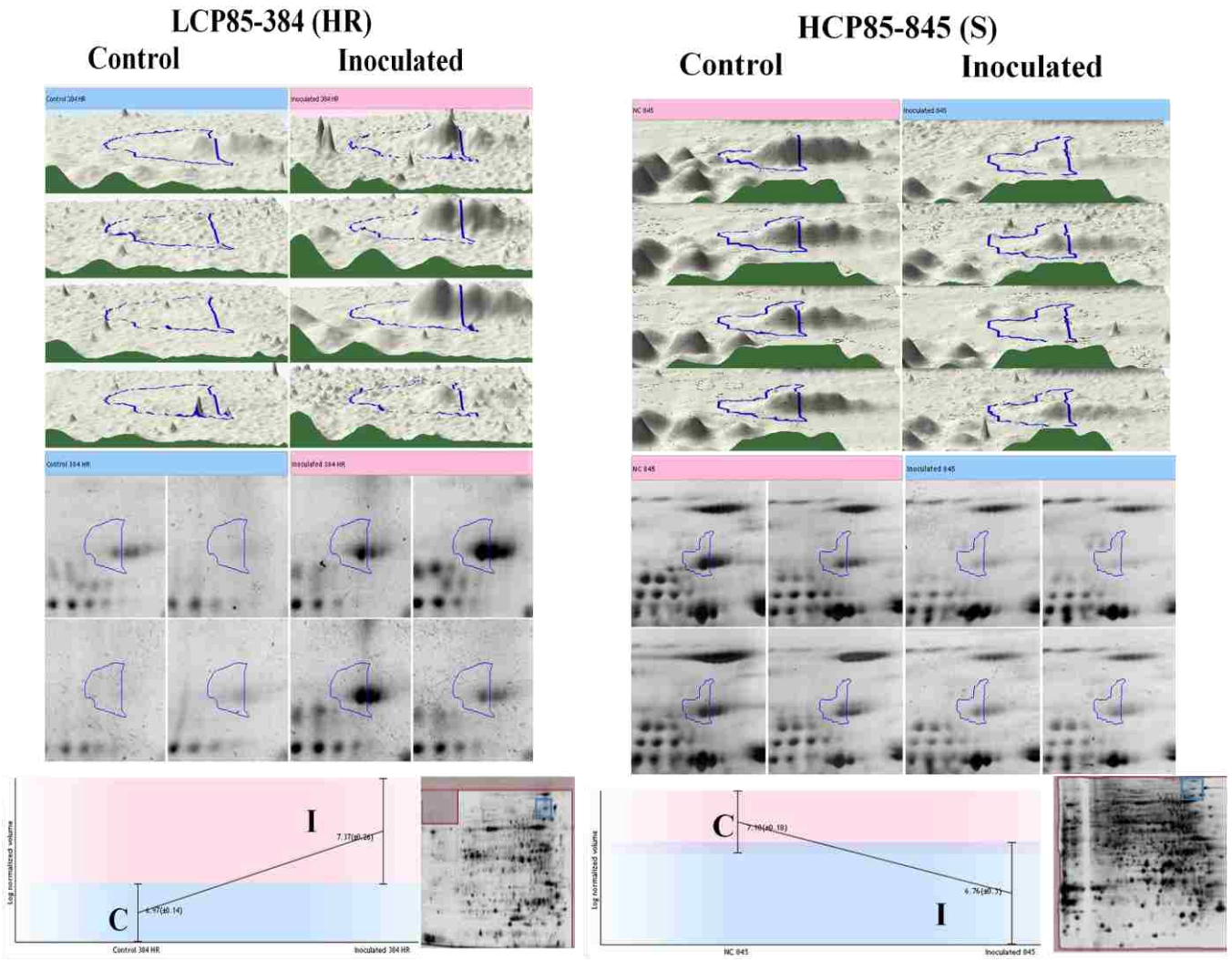


Figure 3.18. Comparison of spot L1 by imaging analysis of three dimension normalized spot volume (top) and the comparative profile map between plants of highly resistant (HR) LCP 85-384 and susceptible (S) HoCP 85-845 infected (I) by *X. albilineans* and mock-inoculated control (C) plants.

Four spots, L3, L4, L9, L11, and L14, had lower expression in the resistant cultivars compared to highly susceptible HoCP 89-846 after taking into consideration the constitutive differences in spot expression evident in the comparisons across mock-inoculated cultivars. L3 had the greatest magnitude of differential expression observed among all spots. Spots L2 and L19 had higher expression in Ho 95-988, and spot L16 had higher expression in LCP 85-384. These differences in expression detected in comparisons accounting for constitutive differences suggest involvement of these spots in the resistance response to Xa infection.

3.3.6 Identification of Differentially Expressed Proteins

The protein identity was determined for spots selected based on differential expression in response to Xa infection from resistant cultivar Ho 95-988 and highly susceptible HoCP 89-846 (Table 3.1) and from highly resistant LCP 85-384 (Table 3.2). After MS/MS spectrometry analysis, some spots did not have enough peptide recovery for protein identification. A total of 21 spots were identified as homologues of proteins from five monocotyledonous plants, rice, maize, sugarcane, wheat and sorghum, and one dicotyledonous plant, potato. Based on the identifications, sub-cellular location and biological function were inferred from protein databases (Tables 3.3 and 3.4). The greatest numbers of identified proteins (67%) are putatively located in the chloroplast, followed by the mitochondrion (14%) and the cytosol (14%) (Figure 3.19). Classification according to biological function determined that the identified proteins are involved in photosynthesis (48%) followed by plant defense (20%), metabolism (12%), and development (12%) (Figure 3.20).

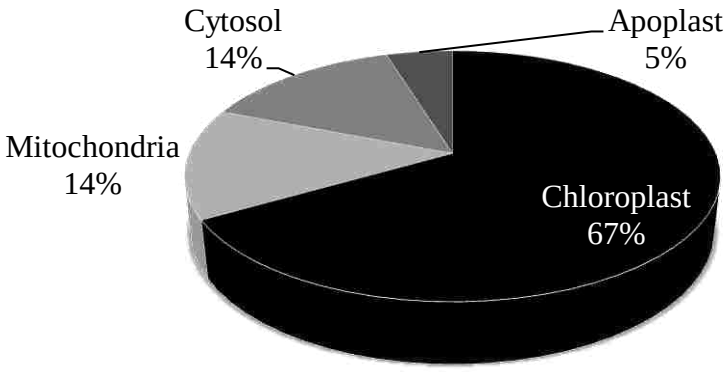


Figure 3.19. Sub-cellular localization of identified proteins in sugarcane differentially expressed in response to infection by *X. albilineans.*

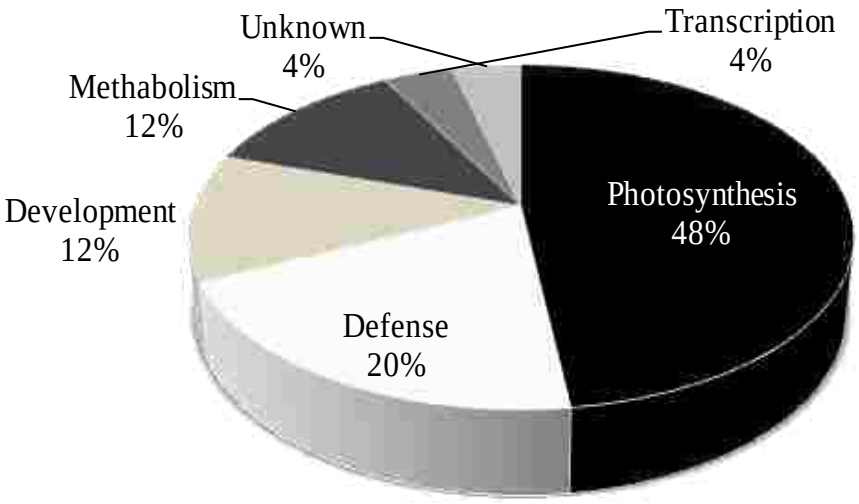


Figure 3.20. Biological function of identified proteins in sugarcane differentially expressed in response to infection by *X. albilineans.*

Table 3.1. Protein indentifications and properties of spots differentially expressed in resistant cultivar Ho 95-988 and highly susceptible HoCP 89-846 in response to infection by *X. albilineans*.

Id[a]	p[b]	Fold[c]	Exp. pI[d]	Exp. MW[e]	The.MW[f]	Homologous protein	Organism	Peptide sequence	Accession No.[g]	Id p (%)	Unique peptides
Ho8-2137	0.05	2.6	3.8	17	14	Ribosomal protein L14	*Saccharum sp.*	IGDVIIAVIK VFGAVAEELR MIQPQTLLNVADNSGAR	YP_054666.1	100.0	3
HCI-1005	0.02	2.2	4.7	19	18	Cyclophilin	*Zea mays*	IVMELYANEVPK VFFDMTVGGAPAGR GNGTGGESIYGEKFPDEK HVVFGQVVEGMDVVK VIPEFMCQGGDFTR VADCGQLS	AAA63403.1	100.0	6
Ho4-3987	0.05	2.1	4.8	17	15	Photosystem I reaction center subunit IV A	*Zea mays*	AEKPPPIGPK ESYWYNGIGNVVTVDQDPNTR ESYWYNGIGNVVTVDQDPNTRYPVVVR RESYWYNGIGNVVTVDQDPNTR	ACG36438.1	100.0	4
Ho8-0120	0.02	1.9	7.8	16	19	Rubisco small subunit	*Miscanthus x Giganteus*	ELQEAIASYPDAYVR	ABY66908.1	100.0	8
Ho8-0070	0.04	3.5	8.0	14				ENSTSPCYYDGR ILGFDNIR KFETLSYLPPLTEEQLLK LPMFGCTDASQVYK MQVWPAYGNK NNWVPCLEFSK QTQCVSFIAYK		99.8	
Ho8-0038	0.05	1.7	6.8	14	19	Hp[h] SORBIDRAFT	*Sorghum bicolor*	ILGFDNIK QTQCVSFIAYKPAGSE MQVWPAYGNK FETLSYLPPLTEEQLLK NNWVPCLEFSK KFETLSYLPPLTEEQLLK ENSTSPCYYDGR ELQEAIASYPDAYVR ILGFDNIK LPMFGCTDASQVYK ELQEAIASYPDAYVR	EES09292.1	100.0	11

a/Identification, b/Significant difference according to p value, c/Fold level of regulation according to normalize volume, d/pI = isoelectric point, e/Molecular weight. Peptide sequences were based on MALDI TOF/TOF analysis.

Table 3.1(continued). Protein indentifications and properties of spots differentially expressed in resistant cultivar Ho 95-988 and highly susceptible HoCP 89-846 in response to infection by *X. albilineans*.

Id[a/]	p[b/]	Fold[c/]	Exp. pI[d]	Exp. MW	The.MW	Homologous protein	Organism	Peptide sequence	Accession #	Id p (%)	Unique peptides
CP2-1991	0.01	1.6	8.3	18	18.1	Thioredoxin M1	*Zea mays*	KESVIGAVPK MIAPVIDELAK NWDGLVMACETPVLVEFWAPWCGPCR STLTTLIDK STLTTLIDKYIGSS	ACG40204.1	100.0	5
Ho8-0739	0.02	-3.1	9.2	43	46	Hp SORBIDRAFT_07g019320	*Sorghum bicolor*	EVQKPLEDITDSLK SLILAGLAEPK LAVGLEELQR EEFDDNSASSQIFWLLK TVPLEIMVDGDKAPVYGETLEELGR YAVFGYVTENEDYLADVK LPFNAFGTMAMAR APVYGETLEELGR IVLDGYNAPVTAGNFIDLVERK ESELTPSNANILDGR VGDVIESIQVVSGLDNLVNPSYK ADGFVVQTGDPEGPAEGFIDPSTGK TVPLEIMVDGDKAPVYGETLEELGR	EES13766.1	100.0	13
Ho8-0175	0.01	-2.4	9.1	21	18	Translationally-controlled tumor protein	*Zea mays*	DEASVVFAYYK DGATNPTFLYFSHGLK ELENGVLWEVEGK LKDLQFFVGESMKDEASVVFAYYK NLTAVLEPEKADEFK	ACG47605.1	100.0	5
Ho8-0172	0.04	-2.1	9.1	20	20794	Hp LOC100273459 electron carrier/ iron ion binding protein	*Zea mays*	DEASVVFAYYK DGATNPTFLYFSHGLK ELENGVLWEVEGK LKDLQFFVGESMKDEASVVFAYYK NLTAVLEPEKADEFK	ACG27776.1	100.0	5

a/Identification, b/Significant difference according to p value, c/Fold level of regulation according to normalize volume, d/pI = isoelectric point, e/Molecular weight. Peptide sequences were based on MALDI TOF/TOF analysis.

Table 3.2. Protein indentifications and properties of spots differentially expressed in the highly resistant cultivar LCP 85-384 in response to infection by *X. albilineans*.

Id[a/]	Fold[b/]	Exp. pI[c]	Exp. Mw[e]	Homologous protein	Organism	Peptide sequence	Accession #	Distinct Summed MS/MS Score	Unique peptides
L1	2.6	5.7	105,2	Pyruvate orthophosphate dikinase	Zea mays	FAYDSFR GEPFPSDPK SDFEGIFR LLPYQR AGLDYVSCSPFR NNGAQGIGLCR SLFEEK	ADC32810	91.1	7
L20	2.6	8.4	39.8	ATP synthase subunit gamma,	Zea mays	GLCGSFNNNVLK ALQESLASELAAR VALVVLTGER FVSLVR	NP_114266	242.7	17
L21	1.8	6	21.8	Germin-like protein 1	Oryza sativa	VTFLDDAQVK	BAA74702	17.7	1
L15	1.7	6.5	36.5	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	Zea mays	VVISAPSK VVDLIR GASYEDIK SSIFDAK AASFNIIPSSTGAAK VPTVDVSVVDLTVR	P08735	86.6	6
L7	-2.0	8.7	18.2	Thioredoxin M-type	Zea mays	SIPTVLIFK ESVIGAVPK STLTTLIDK	AAA92464	46.4	3
L3	-1.8	7.6	47.9	Thylakoid ascorbate peroxidase	Triticum aestivum	VDVTGPEQCPPEGK LPDAGPSSPADHLR YAEDQDAFFR HGANAGLINALK EIVALSGAHTLGR FDVELK FDNSYFK HGANAGLINALK NIEEWPQR VDVTAAEQCPPEGR	AAS80160	41.3	8

a/ Identification, b/Fold level of regulation according to normalize volume, c/pI = isoelectric point, d/Molecular weight. Peptide sequences were based on ESI-MS/MS analysis.

Table 3.2 (continued). Protein indentifications and properties of spots differentially expressed in the highly resistant cultivar LCP 85-384 in response to infection by *X. albilineans*.

Id[a/]	Fold[b/]	Exp. pI[c/]	Exp. MW[d/]	Homologous protein	Organism	Peptide sequence	Accession #	Distinct Summed MS/MS Score	Unique peptides
L19	-1.6	9.5	21.8	FKBP-type peptidyl-prolyl cis-trans isomerase 4	Zea mays	VVGTGAAAQEGQLIR LPPALAYGEK LEDGTVFDSSYK VGVGEVIK LEDGTVFDSSYKR VVGTGAAAQEGQLIR	NP_001148233	97.5	5
L2	-1.5	8.9	20.8	Thioredoxin F-type	Zea mays	DTFWPIVEAAGDK NLDVVFLK	ACG39615	33.8	2
L14	-1.4	5.9	42.0	Chloroplast fructose-bisphosphate aldolase	Triticum aestivum	LASIGLENTEANR IVDILVEQGIVPGIK ALQNTCLK TFEVAQK AAQDALLLR EAAYYQQGAR IVDILVEQGIVPGIK ANSLAQLGK GILAMDESNATCGK TVVSIPNGPSELAVK LASIGLENTEANR GLVPLAGSNNESWCQGLDGLASR	ACM78035	163.3	9
L18	-1.4	8.5	36.1	Putative mitochondrial NAD-dependent malate dehydrogenase	Solanum tuberosum	TQDGGTEVVEAK DDLFNINAGIVK LFGVTTLDVVR	CAD33243	56.8	3

a/Identification, b/Fold level of regulation according to normalize volume, c/pI = isoelectrical point, d/Molecular weight. Peptide sequences were based on ESI-MS/MS analysis.

Table 3.3. Hypothetical function and sub-cellular localization of identified proteins from sugarcane cultivars resistant (Ho 95-988), highly resistant (LCP 85-384), and highly susceptible (HoCP 89-846) to leaf scald.

Spot	Homologous protein	Hypothetical function	Subcellular localization
Accumulation of reactive oxygen species			
L3	Thylakoid ascorbate peroxidase	Detoxification	Chloroplast
L21	Germin-like protein 1 (GLP)	Defense, antioxidant, plant height	Chloroplast
Ethylene biosynthesis and methabolism			
L15	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 1	Metabolism and ethylene biosynthesis.	Cytosol
L14	Chloroplast fructose-bisphosphate aldolase	Fructose-biphosphate aldolase (glycolisis)	Plastid-Chloroplast
L18	Putative mitochondrial NAD-dependent malate dehydrogenase	Citric acid cycle, gluconeogenesis, catalizes the convertion of malate into oxaloacetate	Mitocondrion
Defense regulation			
CP2-1991	Thioredoxin M1 (Chloroplastic)	Thiol-disulfide oxidoreductase (Calvin cycle, and oxidative pentose phosphate pathway. Activates glyceraldehyde-3-phosphate dehydrogenase and the phosphoribulokinase, and inhibits. the glucose-6-phosphate dehydrogenase. Activates NADP-malate dehydrogenase. NPR1 activator. Electron donor of peroxiredoxin.	Chloroplast stroma
L7	Thioredoxin M-type	Similar to above	Chloroplast stroma
L2	Thioredoxin F-type	Similar to above	Chloroplast
HCI-1005	Cyclophilin	Protein folding, detoxification and control levels of reactive oxygen species, mRNA processing, protein degradation, protein chaperon-like, apoptosis. protein trafficking and maturation receptor complex stabilization, receptor signaling , RNA processing spliceosome assembly. May prevent oxidative damage to photosystems.	Stroma thylakoid lumen-Chloroplast
L19	FKBP-type (PPIase)	Similar to above	Stromatal, thylakoid lumen (Chloroplast)
Plant growth regulation			
Ho8-0175	Plant TCTP	Programmed cell death, signaling molecule, auxin homeostacis. Long distance movement of phloem proteins.	Cytosol
Photosynthesys and methabolism			
L1	Pyruvate orthophosphate dikinase	Photosynthesis	Chloroplast
L20	ATP synthase subunit gamma, chloroplastic precursor	Energy transfer	Chloroplast
Ho4-3987	Photosystem I reaction center subunit IV A	Electron transfer, energy transfer	Chloroplast
Ho8-0172	Hp LOC100273459 electron carrier/ iron ion binding protein	Electron carrier (Ferredoxin)	Chloroplast
Ho4-3987	Photosystem I reaction center subunit IV A	Electron transfer, energy transfer	Chloroplast
Ho8-0120	RuBisCO small subunit	Carboxylase - oxygenase reactions	Chloroplast
Ho8-0070	RuBisCO small subunit	Carboxylase - oxygenase reactions	Chloroplast
Transcription			
Ho8-2137	Ribosomal protein L14	Chloroplastic protein transcription	Chloroplast
Unknown			
Ho8-0739	Hp SORBIDRAFT_07g019320	Unknown	Unknown
Ho8-0038	Hp SORBIDRAFT	Unknown	Unknown

3.4 DISCUSSION

The time-course experiment detected differences in protein expression in response to Xa infection between two leaf scald resistant sugarcane cultivars and a susceptible cultivar. An increase in protein expression occurred during the systemic phase of infection in the resistant cultivar Ho 95-988 and in the highly resistant cultivar LCP 85-384. In contrast, protein expression in response to infection was greatest in the highly susceptible cultivar HoCP 89-846 at 2 and 4 wai. This pattern of increasing protein expression in resistant cultivars following infection is consistent with the findings of Rott et al. (1994 and 1997) that the leaf scald resistance response results in lower Xa populations during systemic infection.

Differences in protein expression between the resistant cultivars during systemic infection suggest variability exists in the resistance response to Xa infection. The resistant cultivar Ho 95-988 showed an increase in both up- and down-regulated proteins during systemic infection, whereas the highly resistant cultivar LCP 85-384 exhibited a pattern of down-regulation of proteins. The increased protein regulation in the resistant cultivar Ho95-988 was accompanied by a 30% reduction in height after Xa infection compared to mock-inoculated plants even though there was little or no systemic symptom expression. This suggests a diversion of resources to resistance-associated proteins resulted in some physiological cost during the extended infection response. In contrast, Xa populations were lower in the inoculated leaves of LCP 85-384, and plant growth was not reduced following inoculation. This suggests that resistance expression in this cultivar more rapidly reduced Xa populations with less prolonged fitness cost to the host. Differences in expression of specific identified proteins to be discussed subsequently also suggest the reduction of Xa populations during systemic infection was accomplished by similar but different resistance mechanisms in Ho 95-988 and LCP 85-384.

Homologous identified proteins associated with reactive oxygen species (ROS), ethylene biosynthesis, and defense regulation were differentially expressed in Xa- and mock-inoculated plants and between resistant and susceptible cultivars. The differential expression of proteins homologous to thylakoid ascorbate peroxidase (tAPOD), germin-like protein (GLP), and several thioredoxins may be involved in the regulation of ROS and in particular, the accumulation of H_2O_2 during the response to Xa infection that may reduce bacterial populations, and serve as a signal molecule for an induced systemic mechanism of resistance.

A thylakoid ascorbate peroxidase (tAPOD) homologous protein was down-regulated in highly resistant LCP 85-384 during systemic infection and strongly down-regulated in both resistant cultivars compared to both susceptible cultivars. APOD reduces the accumulation of hydrogen peroxidase ($H_2O_{2)}$ and photo-oxidative damage to plant cells (Azada, 1992). The collective terms active oxygen species or reactive oxygen species (ROS) are used in the literature in reference to oxygen radicals that include superoxide radical (O_2^-), H_2O_2, and hydroxyl radical (^-OH) (Wojtaszek, 1997; Sutherland, 1997). ROS in mammals are released externally to the cell membrane and contribute to control of microbial infection (Sutherland, 1997). O_2^- is and converted to H_2O_2 that is catalyzed by superoxide dismutase (SOD) (Wojtaszek, 1997; Alscher et al., 2002). Under pathogen attack, ROS are generated and serve as a protectant to reduce bacterial (Hyslop et al., 1995; Kumar et al., 2011), fungal (Mellersh et al., 2002) and viral (Andrew et al., 2001; Rossetti et al., 2001) infection. Moreover, ROS induce the expression of different systemic acquired resistance (SAR) genes (Lee et al., 2004), mediating a signal network that leads to local and systemic responses (Alvarez et al., 1998). H_2O_2 is a small, diffusible molecule with direct antimicrobial properties. In addition, apoplastic exogenous H_2O_2 can stimulate the accumulation of phytoalexins (Apostal et al.,

1989) and serves as a late signaling molecule in JA mediated induced resistance (Orozco-Cardenas et al., 2001).

Across sugarcane cultivars, tAPOD showed lower levels of expression in the resistant and highly resistant cultivars compared to the susceptible and highly susceptible cultivars while mock-inoculated plants did not show any differences in expression levels among cultivars indicating an induced response after Xa infection. Lower levels of tAPOD in the tissues of resistant cultivars could cause accumulation of H_2O_2 directly reducing bacterial populations, and/or serving as a signal molecule for ISR.

A homolog of a germin-like protein (GLP) classified as PR16 (Park et al., 2007) was up-regulated in response to Xa infection in highly resistant LCP 85-384. GLP are involved in resistance to bacteria, viruses and fungi and generate H_2O_2 during the oxidation of oxalate by oxalate oxidase (Lane et al., 1993). Germin-like protein GLP1 was accumulated in *Capcicum annuum* after inoculation with *Xanthomonas campestris* pv. *vesicatoria* (Xcv; avrBs2) or *Tobacco mosaic virus* (Park et al., 2007). A group of 12 GLPs were identified and localized in a cluster that overlapped with a QTL that conferred resistance in rice to *Magnaporthe oryzae* and *Rhizoctonia solani* in rice (Manosalva et al., 2008). GLPs are common in cereal genomes and might be used to develop non-race specific disease resistant crops (Breen and Bellgard, 2010). Exogenous application of methyl jasmonate induced extracellular accumulation of GLP in *Capsicum chinense* suspension cells (Sabater-Jara et al., 2010).

The accumulation of GLP in the cell wall (Lane et al., 1992) and extra-cellularly in the apoplast (Christensen et al., 2004), its role in the generation of antimicrobial H_2O_2 that also is a signal for ISR, and the association of GLPs with QTLs providing resistance to different diseases suggest that this differentially expressed protein is involved in resistance to Xa. After the

characterization of the GLP homologous protein found in sugarcane and confirmation of its involvement in the defense reaction, this could be a candidate to analyzed as a functional marker for resistance to leaf scald.

The down-regulation of APOD and up-regulation of GLP after Xa infection in resistant cultivars, particularly LCP 85-384, suggest a role for H_2O_2 in an induced defense response to Xa infection. Apoplastic H_2O_2 could reach xylem cells and reduce bacteria colonization or serve as a signaling intermediate to trigger systemic resistance and thereby reduce Xa colonization. Further research is needed to confirm the role of H_2O_2 and those enzymes involved in its production and scavenging, that may include the analysis of H_2O_2 concentration in inoculated and newly emerging leaves of Xa-infected plants, enzymatic activity testing for proteins, such as peroxidases and superoxide dismutase, and gene expression analysis for GLP and APOD upon Xa infection.

Several different thioredoxin homologs were down-regulated in the resistant cultivars in response to Xa infection. Spot CP2-1991 (a thioredoxin M1 homolog) was up-regulated in susceptible HoCP 85-845 during initial infection, while it was down-regulated in highly resistant LCP 85-384 during systemic infection. The comparison between cultivars infected by Xa showed lower levels of expression in resistant Ho 95-988 versus highly susceptible HoCP 89-846. In mock-inoculated plants, there were lower levels in the highly resistant and resistant cultivars in contrast to the highly susceptible one. Similarly, spots L2 (a thioredoxin F homolog) and L7 (a thioredoxin M homolog) were down-regulated in the highly resistant and up-regulated in the highly susceptible cultivar during systemic infection. Across inoculated cultivars, thioredoxin M had the highest levels of regulation among all the different identified spots, being down-regulated in the highly resistant and resistant cultivars compared to the susceptible one. Thioredoxin F was down-regulated

in the highly resistant and resistant cultivars versus the susceptible one. Constitutive expression of both thioredoxins was significantly lower in the highly resistant cultivar than the resistant one.

Thioredoxins are important regulatory proteins in plant resistance responses to pathogen infection. A tomato thioredoxin interacted with the resistance protein Cf-9 and was negatively expressed in the interaction between Cf-9 and the Avr9 protein that leads to cell death and defense responses (Rivas et al., 2004). Silencing of the thioredoxin caused an increase of H_2O_2 and a stronger and accelerated hypersensitive response (HR) with the activation of several defense related genes against *Cladosporium fulvum* (Rivas et al., 2004). Similarly, after silencing the gene for ferredoxin:thioredoxin reductase (FTR), cell death, necrosis, and accumulation of ROS were observed in tomato leaves (Lim et al., 2010). Additionally, resistance to *Pseudomonas syringae* pv. tomato DC3000 was increased with the accumulation of pathogenesis-related proteins. Conformational changes in NPR1, a regulator of salicylic acid (SA) mediated defense genes were observed, suggesting that thioredoxin H is the enzyme that catalyzes the NPR1 oligomer to monomer reduction during plant defense (Tada et al., 2008).

Down-regulation of the thioredoxin-type proteins in the resistant sugarcane cultivars could be a mechanism to increase the accumulation of ROS and limit systemic infection by Xa. The identification of three spots associated with thioredoxins and the levels of regulation observed in different cultivars under Xa infection and across cultivars suggest that these proteins could play an important role in the mechanism of resistance to leaf scald.

Three enzymes involved in metabolism and in pathways important for ethylene hormone biosynthesis showed variable expression patterns under Xa infection. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was up-regulated in the highly resistant cultivar in the early response to Xa infection, while chloroplast fructose-biphosphate aldolase, and NAD-dependent malate

dehydrogenase (NAD-MDH) were down-regulated. Ethylene (ET) biosynthesis enzyme, S-adenosylmethionin decarboxylase, was reported to be repressed in a susceptible sugarcane cultivar after Xa infection (Dabbas et al., 2006). The level of Xa was reduced in sugarcane previously inoculated with *Gluconacetobacter diazotrophicus* (Gd), and genes involved in transcription factors related to ET response were expressed based on cDNA-AFLP sequencing analysis (Arencivia et al., 1999). ET and jasmonic acid (JA) acted synergistically in defense responses to the biotrophic and obligate powdery mildew pathogens *Erysiphe cichoracearum, E. orontii and Oidium lycopersicum* (Ellis and Turner, 2001). Exposure of bean leaves to ET triggered formation of a gel occluding the vascular system and thereby obstructing the vascular pathogen *Fusarium oxysporum* f.sp. *cubense* (VanderMolen et al., 1983). However, ET biosynthesis also can be associated with susceptibility (Van Loon et al., 2006). The inhibition of ET biosynthesis or its removal from environment reduced disease severity caused by *Botrytis cinerea* (Elad, 1993). On the other hand, the rhizobacterium *Bacillus subtilis* and its volatile compounds also induced basal plant immunity or induced systemic resistance (ISR) against bacterial pathogens inducing up-regulation of the enzymes involved in ET biosynthesis (Kwon et al., 2010). S-adenosilmetionin descarboxilase, glyceraldehyde-3-phosphate dehydrogenase, chloroplast fructose-biphosphate aldolase, NAD-dependent malate deshydrogenase, and other ET biosynthesis involved enzymes were up-regulated following exposure to plant growth-promoting rhizobacteria (PGPR) or their volatile compounds (Kwon et al., 2010).

The glyceraldehyde-3-phosphate dehydrogenase (G3PDH) homologous protein was up-regulated in the cytosol of the inoculated leaves of highly resistant LCP 85-384, without any significant change of expression in the systemically infected leaves of any cultivar. In contrast, the putative mitochondrial NAD-dependent malate dehydrogenase (NAD-MDH) homologous enzyme showed early down-regulation in inoculated leaves of the same cultivar. The chloroplast fructose-

biphosphate aldolase homologous protein was down-regulated in Xa-infected LCP 85-384 at 4 wai, and the analysis of systemically infected plants across cultivars showed lower expression in both resistant cultivars compared to the highly susceptible cultivar.

The results indicate variability in the pattern of expression of enzymes associated with ET biosynthesis during the time-course of the resistance response. Although the evidence is not conclusive, the differential expression of multiple enzymes suggests that ET biosynthesis may be involved in the early resistance response to leaf scald in LCP 85-384 that is down-regulated later during systemic infection after reduction of the pathogen infection.

A cyclophilin homolog selected from the proteome of resistant Ho 95-988 was differentially up-regulated in the resistant cultivar and down-regulated in the highly susceptible cultivar during systemic infection. In contrast, a cyclophilin homolog selected from highly resistant LCP 85-384 was down-regulated in that cultivar during systemic infection. Taking into account constitutive differences, the homolog selected from LCP 85-384 was expressed at a higher level in resistant Ho 95-988 than in highly susceptible HoCP 89-846.

Cyclophilins (CyP) are members of the inmunofilins that share similar enzymatic activities with the FK506 binding protein FKBP that has peptidyl-prolyl cis-trans isomerase PPIase activity. Meza et al. (1989) and later Godoy et al. (1999) found that a CyP-type protein was accumulated after wounding damage and was differentially expressed with methyl jasmonate treatment and concluded that CyP may be involved in the unfolding of induced defense response proteins. A differentially expressed sequence tag (EST) study found that a CyP was up-regulated in the resistance response of mandarin orange to infection by *Xylella fastidiosa* (de Sousa et al., 2007). In humans, these proteins regulate T cell activation and other metabolic activities (Harding et al., 1989). Cyclophilin A up-regulates the expression of genes involved in drug transport and

metabolism, drug resistance and cytokine related genes (Chen et al., 2007). Plant cyclophilins are located in the chloroplast, mitochondria, and the cytosol (Lippuner et al. 1994; Romano et al., 2004) in both monocotyledoneous and dicotyledoneous plants (Godoy et al., 1999) and are induced by biotic and abiotic stress (Marivet et al., 1992; Godoy et al., 1999). They have antifungal activity (Lee et al., 2007), are associated with salt tolerance in rice, prevent oxidative damage to photosynthesis, regulate circadian rhythm, and act as a general regulator of environmental stress (Ruan et al., 2011).

CyP was down-regulated in highly susceptible HoCP 89-846 and up-regulated in resistant Ho 95-988 but not highly resistant LCP 85-384 during systemic infection. This change in expression coincides with the differential changes in bacterial population in the highly susceptible and resistant cultivars. The analysis of constitutive expression in mock-inoculated plants did not detect any differences in expression level between cultivars, suggesting an induced role in the expression of resistance in Ho 95-988 against systemic infection by Xa.

The reduced growth observed in resistant cultivar Ho 95-988 during response to Xa infection that could indicate a plant fitness cost was associated with differential expression of a protein related to growth regulation and several proteins involved in primary metabolism. Translationally controlled tumor protein (TCTP) is a positive regulator of cell division in *Arabidopsis thaliana* controlling the duration of mitosis (Brioudes et al., 2010) that was down-regulated in systemically infected leaves of Ho 95-988. The *Arabidopsis* TCTP is highly expressed in physiologically active and proliferating meristematic tissues of the root or embryo, and a tTCTP gene knock-out showed reduced leaf expansion and vegetative growth (Berkowitz et al., 2008). A TCTP knock-out in cabbage reduced vegetative growth because of smaller cell size, and tolerance to low or high temperature and salt stress was impaired (Cao et al., 2010).

The sugarcane TCTP homolog was down-regulated in resistant Ho 95-988 during systemic infection. In non-inoculated plants, this protein was expressed at a higher level in highly resistant LCP 85-384 and a lower level in highly susceptible HoCP 89-846. The reduction in plant height observed in Xa-infected Ho 95-988 plants without any symptom expression could be related to the reduced expression of TCTP and to a change in plant fitness altering primary metabolism for a late induced mechanism of defense against Xa. Dabbas et al. (2006) found a repressed EST for plant cellular expansion in a leaf scald resistant cultivar. The induction of defense mechanisms after pathogen multiplication is detected requires a massive redistribution of energy to be used in an active defense response (Bolton, 2009). The increased number of differentially expressed proteins over time, the reduction in bacteria over time following infection, and the involvement of a TCTP homologous protein suggest that the mechanism of resistance to leaf scald in Ho 95-988 involves an induced defense response during systemic infection by the pathogen that has a plant fitness cost.

Several homologues of proteins involved in primary metabolism pathways were differentially expressed during the sugarcane-Xa interaction that may be involved in providing carbon and energy transfer to enable the defense response. Glyceraldehyde-3-phosphate, a key enzyme in glycolysis that converts glucose to pyruvate, resulting in a small net gain of ATP, and an ATPase synthase homologue were up-regulated in highly resistant LCP 85-384. ATPase synthase had higher expression in the highly resistant cultivar than the resistant one. In addition, a protein homologous to pyruvate orthophosphate dikinase, a key enzyme in gluconeogenesis and photosynthesis, was up-regulated in the highly resistant cultivar 4 and 8 wai. Similarly, there were two enzymes involved in photosynthesis that were up-regulated in response to Xa infection in the resistant cultivar Ho 95-988. A protein homologous to photosystem I subunit IV A was

significantly up-regulated 4 wai, and a rubisco small subunit protein was up-regulated during systemic infection.

These primary metabolism homologous proteins identified are involved in pathways that have been linked to important mechanisms of disease resistance (Bolton, 2009). Plant respiration, which includes glycolysis, was increased 80% early in a resistant barley cultivar in contrast to a susceptible one after powdery mildew (*E. graminis* f.sp. *hordei*) inoculation (Smedegaard-Petersen and Stolen, 1981). Rooney and Hood (1989) found an increase in photosynthesis in healthy areas of infected leaves inoculated with *Septoria nodorum* possibly as a mechanism to compensate for the energy cost of resistance in winter wheat. Proteomic analyses of the responses of plants to pathogenic bacteria have demonstrated up-regulation of photosynthesis and metabolism proteins, including *Arabidopsis* infected by *Pseudmonas syringae* pv. tomato DC3000 (Jones et al., 2006), tomato infected by *Clavibacter michiganense* subsp. *michiganense* (Coaker et al., 2004), and rice infected by *Xanthomonas oryzae* pv. o*ryzae* (Mahmood et al., 2006).

The increased regulation of this group of proteins could represent part of a mechanism to provide the carbon and energy to support the induced, non-specific disease resistance response in sugarcane against Xa. However, there is a need to determine whether changes in photosynthetic proteins are associated with changes in final products of photosynthesis.

Most plant pathogenic bacteria colonize intercellular space and come in direct contact with living host cells, most possessing a type III secretion system and often inducing a hypersensitive response (Pieretti et al., 2009). However, *Xanthomonas albilineans* colonizes the dead, lignified cells of the xylem, does not possess a type III secretion system or induce hypersensitive responses, but produces a potent host toxin, albicidin, that affects chloroplast development in surrounding mesophyll cells (Birch and Patil, 1985 a and b; Birch and Patil, 1987 a and b). It also possesses

pathogenicity factors in addition to albicidin of which a group of membrane associated proteins may be most important (Rott, et al., 2011). The mechanisms of resistance to leaf scald must respond to this atypical set of pathogen characteristics. Resistance does not prevent infection but limits systemic colonization of the xylem by the pathogen and prevents severe symptom expression (Rott et al., 1997).

Albicidin associated damage and exposure to other molecules from the bacterium may serve as pathogen associated molecular patterns (PAMPs) (Medzhitov and Janeway, 1996) or damage associated molecular patterns (DAMPs) (Matzinger, 1994) to provide signals that trigger the regulation and expression of proteins associated with resistance in photosynthetic tissues of stomatal guard cells and the bundle sheath cells of the vascular bundles. The findings of Rott et al. (1998) that resistance to leaf scald is associated with the extent of Xa colonization in the shoot apex and the demonstration by qPCR that lower levels of Xa occur in newly emergent leaves of resistant host genotypes (Chapter 2) indicate that resistance to Xa is expressed during the establishment of systemic infection. After protein extraction from systemically infected leaves, the comparative proteomic analysis showed that more proteins were differentially expressed during systemic infection. Furthermore, 67% of the identified differentially expressed proteins were located in the chloroplast and 48% of the differentially expressed proteins were involved in photosynthesis. Considered altogether, the evidence suggests that the mechanisms of resistance could be associated with cells involved in photosynthetic activity either located in the stomatal guard cells and the bundle sheath cells (Martin et al., 1961) or the mesophyll. Dabbas et al. (2006) found up-regulation of a DNA helicase EST involved in plastid differentiation that was suggested to be an important factor conferring resistance to Xa. The injury of photosynthetic cells or the detection of DAMPs or PAMPs by pattern recognition receptors (PRR) (Numbergert et al., 2009), such as the

transmembrane LRR protein detected in the macro-array study (Dabbas, 2006), in sugarcane cells may induce the synthesis of ROS with the consequent down-regulation of thioredoxins and ascorbate peroxidase (APOD) resulting in increased accumulation of H_2O_2. Similarly, Dabbas et al. (2006) found an EST homologous to glutathione synthetase that was down-regulated in the resistant cultivar and hypothesised that low levels of this enzyme may increase the accumulation of H_2O_2. This ROS molecule may act as a signal to induce the activation of enzymes involved in biosynthesis of JA and ET. The JA messenger may induce the expression of cyclophilin and the PR16 germin-like protein (GLP) and other defense proteins. The location of GLP in the cell membrane and translocation to the apoplast could increase levels of H_2O_2 by oxalate oxidase (OXO) activity with subsequent diffusion to the neighboring xylem cells. The increased levels of symplastic and apoplastic H_2O_2 could have a direct antimicrobial effect or serve as a signal to trigger a generation of secondary reactions to reduce the extent of Xa colonization in the apex and newly emerging tissues. The proteomic analysis found homologous proteins involved in ET biosynthesis that agree with the findings of Arencivia et al. (1999) where 26% of differentially expressed transcripts were involved in the ethylene signalling pathway. JA and ET signaling may induce the expression of proteins involved in the synthesis of phenolic compounds, as described by Santiago et al. (2009) and phytoalexin biosynthesis as hypothesized by Dabbas et al. (2006), who found an EST homologous to isoprenoid biosynthesis induced in the resistant cultivar that may be associated with the final biosynthesis of microbial agents like phytoalexins. Free phenolic compounds and phytoalexins might also be mobilized from the symplast of living cells to the apoplast and from there to the neighboring xylem cells to directly reduce the Xa population and the extent of new emergent xylem colonization.

Additionally, mechanisms that involve growth and fitness costs may include down-regulation of proteins like the TCTP homologous protein that provide or compensate for energy required for defense responses, especially in physiologically active cells of emergent tissues. Likewise, sources of energy production, such as glycolysis and the induction of proteins like ATPase synthase that are up-regulated in resistant host genotypes may support the energy cost of the induced mechanisms of defense to Xa infection.

In summary, the induced defense reaction in sugarcane cultivars resistant to Xa may result from cumulative effects of gene regulation that alter H_2O_2, jasmonic acid, and GLP likely causing the xylem environment to become toxic to the bacteria. This may in turn lead to induction of a systemic defense response that, depending on the cultivar, may include different carbon and energy compensations to support defense mechanisms and reduce the extent of Xa colonization. Kinetic studies on H_2O_2 and enzymatic activities of proteins like peroxidases in inoculated and newly emerged tissue, gene expression analysis of proteins like GLP, cyclophilin, thioredoxin M1, and gene regulation markers associated with induced resistance are needed to support the proteomic findings and hypothesized resistance mechanisms. Identification of key genes in defense mechanisms of resistant sugarcane genotypes could be beneficially exploited during breeding and selection for leaf scald resistance supporting development of molecular markers for the different mechanisms of resistance.

CHAPTER 4:
GENERAL CONCLUSIONS AND PROSPECTS FOR FURTHER RESEARCH

4.1 GENERAL CONCLUSIONS

- Initial experiments evaluating vascular infection by *Xanthomonas albilineans* (Xa) confirmed that resistance is associated with low vascular colonization at the shoot apex in local cultivars.
- The qPCR assays developed provide a highly sensitive method for the detection of Xa.
- Xa population quantification by qPCR in newly emerged, systemically infected leaves differentiated resistant and susceptible cultivars, suggesting it could serve as an alternative method for leaf scald resistance screening.
- Symptom expression is not essential for bacterial population comparisons among cultivars. However, a successful inoculation is critical.
- Differential expression of proteins was detected in susceptible and resistant cultivars during both early and systemic infection. More proteins were differentially expressed during systemic infection in resistant cultivars.
- The regulation of proteins homologous to thioredoxins, germin-like protein, and ascorbate peroxidise suggest that the accumulation of reactive oxygen species, particularly H_2O_2, may have an important role in a non-specific, induced defence response, creating a toxic xylem environment and/or triggering induced systemic resistance (ISR).
- The up-regulation of cyclophilin and ethylene biosynthesis proteins provides additional evidence for the involvement of ISR in leaf scald resistance.
- The elevated expression of proteins involved in photosynthesis and metabolism could represent a mechanism to provide the energy to support ISR in sugarcane against Xa.

- The low Xa populations detected by qPCR in resistant cultivars and the comparative proteomic analysis results suggest that resistance to leaf scald in sugarcane involves ISR that limits colonization of the xylem in newly emergent tissue and prevents disease symptom development.

4.2 PROSPECTS FOR FURTHER RESEARCH

- The utility of qPCR as an alternative screening method for resistance to leaf scald in the sugarcane breeding program needs to be further evaluated under variable greenhouse and field conditions using a larger number of cultivars.
- The concentration of H_2O_2 in inoculated and new, emerging leaves of Xa-infected plants of resistant and susceptible cultivars needs to be determined.
- Enzyme activity levels of proteins like peroxidises and superoxide dismutase need to be determined locally in the inoculated and systemically infected leaves.
- Gene expression analysis for proteins like GLP, cyclophilin, and thioredoxins during a time-course after infection with Xa is needed to confirm their induction over time during the leaf scald resistance response.
- The expression of marker genes associated with JA/Ethylene and SA defense pathways is needed to confirm the existence of an induced mechanism of resistance after Xa infection.
- Attempt to develop multiple functional molecular markers for marker-assisted selection of leaf scald resistance based on the proteomics results and gene expression analysis that may include proteins like GLP, cyclophilin, thioredoxins and ascorbate peroxidase.

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APPENDIX 1: PROTOCOLS

XANTHOMONAS ALBILINEANS DNA EXTRACTION FROM LEAF EXUDATE (Jacobs et al., 2008 modified method)

1. Vortex the microcentrifuge tube containing 20 leaf discs in 1 ml sterile dH_2O briefly. Flick/tap the microcentrifuge tube to settle the leaf discs to the bottom of the tube.
2. Incubate the tube for 2 h at room temperature or overnight at 4 °C for bacteria to diffuse from the leaf discs in to the sterile dH_20.
3. Transfer the bacterial suspension to a new sterile 1.5 ml microcentifuge tube.
4. Centrifuge at 9000 rpm for 5 min to precipitate bacterial cells.
5. Discard the supernatant and add 100 µl of lysis buffer (0.05 M KCl, 0.01 M Tris-HCl, 0.2% Tween 20, pH 8.3) to the tube.
6. Vortex the tube briefly and incubate at 95 °C for 10 min in water bath.
7. Cool on ice and store at -20 °C.
8. DNA is ready for PCR or qPCR.

XANTHOMONAS ALBILINEANS ISOLATION AND INOCULUM PREPARATION

1. Collect leaves containing pencil-line symptoms and wash the surface thoroughly with abundant tap water.
2. Cut leaf pieces approximately 7 cm long along the pencil-line symptom.
3. Surface-sterilize the leaf pieces in NaOCl solution (10% commercial bleach) for 30 sec and rinse twice in sterile dH_2O.
4. Dry the leaf pieces on sterile paper towels inside laminar flow hood.
5. Divide the 7 cm long piece into small pieces and place these in a sterile 1.5 ml microcentrifuge tube, and add 1 ml of sterile dH_2O.
6. Briefly vortex the microcentifuge tube containing small pieces and flick/tap the tube to settle the pieces to the bottom.
7. Incubate the microcentrifuge tubes at room temperature for 2 h or overnight at 4 ^oC.
8. Transfer the bacterial suspension to a new sterile 1.5 ml microcentrifuge tube.
9. Using a sterile loop, streak the bacterial suspension from the microcentifuge tube onto XAS modified Wilbrinks medium (Davis et al., 1994) (Appendix 2).
10. Incubate the plates at 28 ^oC for 7 days or until single colonies are observed, and transfer single colonies to new plates of XAS medium without antibiotics.
11. Incubate the single colony culture plates at 28 ^oC for 2 days. Prepare 5 ml of bacterial suspension containing 10^8 CFU with distilled water using a spectrophotometer (0.18 absorbance at 590 wavelength). Do not use as inoculum colonies that have been sub-cultured more than once.
12. Do not expose bacterial suspension to direct sunlight and keep it on ice until use.

SUGARCANE LEAF PROTEIN EXTRACTION PROTOCOL
(Wan, 2006; U. of Missouri, 2002) (Modified)

1. Day one (takes 3-5 h). Grind sample into a fine powder in a mortar with pestle under liquid nitrogen. Keep samples, tools, mortar and pestle in liquid nitrogen. For each sample, you need 250 ml of liquid nitrogen (LN).

2. Transfer the powder (2 g) into a 50 ml phenol resistant tube. To wash the sample, add 10 ml of 10% tricholoroacetic acid (TCA)/acetone. Mix well by vortexing for 1 min. Place on ice 1 min and then vortex for 1 min (no more). Centrifuge at 5,500 x g for 10 min at 4 ºC. Carefully remove the supernatant by decanting.

3. Wash the TCA and other contaminants (phenols) with 15 ml of 80% methanol plus 0.1 M ammonium acetate. Mix well by vortexing for 3 min then placing on ice for 1 min. Repeat twice. Centrifuge at 5,500 x g for 10 min at 4 ºC. Discard the supernatant.

4. Wash the pellet with 15 ml of 80% acetone. Vortex for 3 min until the pellet is fully dispersed. Centrifuge at 5,500 x g for 10 min at 4 ºC. Discard the supernatant.

5. Air-dry at room temperature or incubate at 35 ºC in an airflow incubator for 10 min to remove residual acetone.

6. Add 2.5 ml/ g starting plant material of Tris-satured phenol (pH 8.0) and 2.5 ml of SDS extraction buffer. Mix and disperse the sample for 1 min at 12,500 rpm using a bench-top Polytron PT 3100 homogenizer (Kynematica, Lucerne-Switzerland). Mix thoroughly by vortexing for 1 min then place on ice 1 min. Repeat 14 times. Separate the phenol phase by centrifugation at 5,500 x g for 10 min at 4 ºC.

7. Transfer the upper phenol phase (3-4 ml) into a new 50 ml tube, being careful not to disturb the SDS interphase (white color) with a 1 ml pipette. Add 20 ml of 80% methanol containing 0.1 M ammonium acetate and store at -20 ºC for 2 h to overnight (add at least 5-7 volumes more than the phenol phase). Note: If no phase separation occurs, add more phenol (1.5 ml) to the mixture, mix well, and centrifuge again.

8. Day two (takes 3 h). Precipitate the protein pellet at 5,500 x g for 30 min at 4 ºC. Carefully discard the supernatant. A white pellet should be visible.

9. Wash the pellet once with 15 ml of 100% methanol. First, disturb the pellet with a spatula and then vortex for 3 min. Centrifuge the sample at 5,500 x g for 20 min at 4 ºC.

10. Finally, wash once with 5 ml of 80% acetone with vortexing for 3 min. Disturb and precipitate the sample again at 5,500 g at 4 ºC for 20 min, and allow the proteins to air dry during 10-15 min or in an air flow chamber. Store the dried pellet at -20 ºC, or dissolve the proteins in a buffer of choice (e.g. SDS buffer or IEF rehydration buffer). Note: All wash solutions should be pre-cooled at -20 ºC for at least 1 h. Keep the 100% methanol at -80 ºC.

Solution and Buffers Recipes:

1. **10%TCA/acetone**. (Keep at -20 ºC)
    a. Recipe for 100 ml
    b. 10 g trichloroacetic acid in 100 ml acetone

2. **0.1 M ammonium acetate in 80% methanol** (Keep at -20 ºC)
    a. Recipe for 1000 ml
    b. 200 ml of dH_2O and 800 ml of methanol (keep at -80ºC).
    c. Ammonium acetate 1 M = 77.08 g/l. 0.1 M = 7.708 g/l.

3. **SDS Buffer** (Keep at 4º C and take out 1 h before use.)
    a. Recipe for 100 ml
    b. 0.1 M Tris-HCl pH 8.0, (Add 1.21 g of Tris into 80 ml of dH_2O, adjust pH with HCl until pH is 8.0, Q.S. to 100 ml)
    c. 2% SDS, 2 g
    d. 0.5% 2-B-mercaptoethanol, 500 ul
    e. 30% sucrose, 30 g

4. **Tris satured Phenol Buffer** (Keep at 4 ºC)
    a. Recipe for 200 ml
    b. 0.1 M Tris-HCl pH 8.0 (Add 1.21 g of Tris into 80 ml of dH2O, adjust pH with HCl until pH is 8.0, Q.S. to 100 ml).
    c. Add 100 ml of phenol and mix. When the two phases could be see storage under refrigeration.

5. **80% acetone**. (Keep at -20 ºC)
    a. Recipe for 1000 ml
    b. 200 ml of dH_2O and 800 ml of acetone

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XAS MEDIUM FOR *XANTHOMONAS ALBILINEANS* ISOLATION
(Davis et al., 1994; LSU-Agcenter Sugarcane Disease Detection Lab)

1. Recipe for 1000 ml

a. Mix the following reagents while stirring

Bacto-peptone	5.00 g
sucrose	10.00 g
$K_2HPO_4 \cdot 3H_2O$	0.50 g
$MgSO_4 \cdot 7H_2O$	0.25 g
Na_2SO_3	0.25 g
Kbr	5.00 g
Benomyl (2 mg active ingredient/ml of stock solution)	2.00 ml

b. Adjust pH to 6.8

c. Add 15 g of Bacto-agar and autoclave

d. Let the media to cool down in water bath at 50 °C

e. Under laminar flow hood, add 1 ml/l of the following stock solutions:

	Stock solutions:
Benomyl	100 mg Benlate 50DF in 25 ml of acetone.
Cyclohexamide	Mix 1 g in 10 ml of 95% ethanol and adjust volume to 100 with sterile ddH_20.
Cephalexin	Mix 0.25 g in 10 ml of ethanol and adjust volume to 100 with sterile ddH_20.
Novobiocin	Mix 0.30 g in 29.7 ml of sterile ddH_20 and adjust volume to 100 with sterile ddH_20.
Kasugamycin	Mix 0.50 g in 49.5 ml of sterile ddH_20 and adjust volume to 100 with sterile ddH_20.
	Filter all the stock solutions using syringe filters, make 1 ml aliquots in 1.5 ml centrifuge tubes and store at -20 °C.

Reference

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APPENDIX 2:
MASTER MIX AND CONDITIONS OF AMPLIFICATION

TAQMAN DOUBLE-QUENCHED™ ZEN qPCR

MASTER MIX

Reagents	**Volume/reaction**
TaqMan Universal master mix (Roche®)	10.0 µl
Nuclease free water	5.0 µl
Probe XaQ (2 µM)	1.0 µl
Primer XaQf (10 µM)	1.0 µl
Primer XaQr (10 µM)	1.0 µl
	18.0 µl
DNA sample	2.0 µl
Final vol.	**20.0** µl

AMPLIFICATION PROTOCOL

Cycle/step	**Temperature**	**Time (min.)**	**Optics**
Cycle 1: (1X)			
Step 1:	50 °C	10:00	off
Cycle 2: (1X)			
Step 1:	95 °C	2:00	off
Cycle 3: (40X)			
Step 1: Denaturation	94 °C	0:15	off
Step 2: Annealing-polimerization	60 °C	1:00	on
Data collection and real-time analysis enabled.			
Cycle 4: (1X) Step 1:	4 °C	Hold	

SYBR GREEN qPCR

MASTER MIX

Reagents	**Volume/reaction**
Power SYBR Green PCR (Applied Biosystems®)	7.5 µl
Nuclease free water	5.3 µl
Primer XaAlbIQf (100 µM)	0.1 µl
Primer XaAlbIQr (100 µM)	0.1 µl
	13.0 µl
DNA sample	2.0 µl
Final vol.	**15.0** µl

AMPLIFICATION PROTOCOL

Cycle/step	**Temperature**	**Time (min.)**	**Optics**
Cycle 1: (1X)			
Step 1: Denaturation	95 °C	10:00	off
Cycle 2: (40X)			
Step 1: Denaturation	94 °C	0:10	off
Step 2: Annealing-polymerization	60 °C	0:30	on
Data collection and real-time analysis enabled.			
Cycle 3: (81X)			
Step 1:	55-95 °C	0:30	on
Increase set point temperature after cycle 2 by 0.5 °C			
Melt-curve data collection and analysis enabled.			
Cycle 4: (1X) Step 1:	4 °C	Hold	

VITA

Freddy Fernando Garces Obando, son of Roberto Garces Correa and Arbelia Obando Arce and brother of Mauricio, was born in 1972 in an always traditional coffee production town of the Colombian Mountains "Quinchia". He started his elementary school in "Escuela Salvador Duque" followed by high school in the Instituto San Andres, where he discovered his passion for music. Later, he obtained his degree as Agronomy Engineer from Universidad Nacional de Colombia-sede Medellin in 1997, where he started to focus his attention in biotechnology, phytogenetic resources and a little bit in plant pathology. Afterwards, he had the opportunity to work as one of the first young scientists supported by the Colombian Administrative Department of Science, Technology and Innovation COLCIENCIAS in the Sugarcane Research Station of Colombia "Centro de Investigacion de la cana de Azucar del Colombia-CENICANA" under the mentoring of Dr. Jorge Victoria, that opened a great opportunity to work in sugarcane pathology. Later, in 1998, he joined the Foundation for the Sugar Research of Ecuador "Fundacion para la Investigacion Azucarera del Ecuador- FIADE" to develop in conjunction with a group of Ecuadorian researchers the Sugarcane Research Center of Ecuador CINCAE. After 13 years, more than 20% of the sugarcane mills area is planted with cultivars resistant to the main diseases of Ecuador. During 2001-2003, he pursued Master degree studies in Molecular Biology and Genetic Engineer at the Guayaquil State University of Ecuador. In June 2007, he joined Dr. Jeff Hoy's lab and started to work on the sugarcane reaction to leaf scald caused by *Xanthomonas albilineans* using a qPCR and proteomic approach. During his time in graduate school he was involved in student organizations, leading the Plant Pathology and Crop Physiology Graduate Student Association in 2009-2010, and at the same time re-activated the Latinoamerican Student Association at LSU. During his time as a graduate student, he attended different international and

national meetings to present the findings of his work. In 2010, he received from the Plant Pathology and Crop Physiology Department at LSU the C.W. Edgerton Honor Award in recognition of outstanting performance as a graduate student in Plant Pathology. After his experience at LSU, he is going back to Ecuador to rejoin CINCAE and work for the Ecuadorian Sugarcane Industry.