2013

Sweetpotato storage root rots: flooding-associated bacterial soft rot caused by Clostridium spp. and infection by fungal end rot pathogens prior to harvest

Washington Luis da Silva
Louisiana State University and Agricultural and Mechanical College, wdasilva@agcenter.lsu.edu

SWEETPOTATO STORAGE ROOT ROTS: FLOODING-ASSOCIATED BACTERIAL SOFT ROT CAUSED BY *Clostridium* spp. AND INFECTION BY FUNGAL END ROT PATHOGENS PRIOR TO HARVEST

A Thesis

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

in

The Department of Plant Pathology & Crop Physiology

by
Washington Luis da Silva
B. S., Universidade Federal de Viçosa, 2010
May 2013

DEDICATION

Dedicated to my wife Rachel and daughter Aurelia

ACKNOWLEDGEMENTS

This manuscript would not have been possible without the assistance and the help of those who in one way or another donated their valuable assistance in the conduct of this study, to only some of whom it is possible to give individual mention herein.

First and foremost, I would like to thank my wife Rachel for being the support and the reason of my accomplishments all through this journey. She makes me see and pursue my dreams beyond my capability. Our relationship taught me to give value to the most important things in life, the simple ones. She gave me the most precious treasure, our daughter Aurelia, whom, consciously and un-consciously, gives me the essence of life and shows me how good it is to breathe every morning.

My utmost gratitude to Dr. Christopher A. Clark, the best mentor ever, whose sincerity and encouragement I will never forget. Dr. Clark taught me the pleasure of doing good research. He was there for me, academically and as a friend, giving me the support and inspiration as I hurdled all the obstacles in the completion of this important period of my life. It has been an honor to be his student.

My deepest gratitude to Mrs. Mary Hoy for sharing her vital knowledge and experiences in and beyond the laboratory's environment. Her energetic and positive attitude is a source of inspiration. I will never forget the Hoy family as they made me feel at home far from home, and will be forever friends. I will come back for the warm thanksgiving dinners.

I would like to thank my committee members Dr. Jong Hyun Ham and Dr. Lawrence Datnoff for their time and helpful comments. I would like to thank my ex-committee members Dr. Catherine Aime and Dr. Donald Ferrin for their support and initial push on this study.

Unfortunately, Dr. Ferrin is no longer among us, but his dedication and legacy to sciences will never be forgotten.

I am very grateful to have had the opportunity to be part of the Louisiana State University Department of Plant Pathology and Crop Physiology's family. It was a prolific and exciting experience that I will carry along forever. I am indebted and grateful to the students, staff and faculty members of this department for the precious help and support during my masters studies.

I would like to acknowledge the academic and technical support of Louisiana State University and the Louisiana State University Agricultural Center. My special thanks to the United States Department of Agriculture (USDA) Specialty Crop Research Initiative (SCRI) for the financial support, which made my time at LSU possible.

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ABSTRACT

Sweetpotato production in the southern United States is being threatened by a soft rot that develops in storage roots when fields are flooded and by an important post-harvest disease caused mainly by the fungi *Fusarium solani* and *Macrophomina phaseolina*.

To identify the pathogens responsible for development of the soft rot, samples were collected from storage roots with soft rot from intentionally flooded fields and decayed tissue was streaked on plates of nutrient dextrose agar plus 0.05% cysteine and incubated anaerobically. Two distinct groups of Gram positive strict anaerobic bacteria were re-isolated from rotting storage roots. Endospores were observed in all isolates by differential staining. Genomic DNA was extracted from representative isolates of each group, LSU-B1 and LSU-B7, and the 16s ribosomal RNA region was amplified and sequenced. BLASTn analysis of the 1425 bp sequence of LSU-B1 resulted in 99% homology with *Clostridium puniceum* strain BL 70/20 from rotting Irish potatoes. Isolate LSU-B7 generated a sequence 1376 bp long, which resulted in 99% homology with *C. saccharobutylicum* strain P262.

To determine how and when end rot pathogens enter sweetpotato storage roots, two greenhouse experiments were designed using tissue culture-derived plants free of *F. solani* and *M. phaseolina*. In one experiment, plants were grown in autoclaved soil and one month after transplanting, plants were inoculated at the soil line with either non-infested toothpicks or with toothpicks infested with each fungus alone or combined. In the other experiment, plants were grown in non-infested soil or in soil infested with each fungus alone or combined. Isolations were attempted from different parts of the plants. *F. solani* and *M. phaseolina* were recovered from roots, storage roots, and plant stems below the soil line, at the soil line, and

five centimeters above the soil line in both experiments. This suggests that these fungi are capable of invading the sweetpotato plants and storage roots from infested soil, and systemically colonize the plant from infected plant propagation material, eventually reaching the storage roots. These findings indicate that infection with *F. solani* and *M. phaseolina* can occur prior to harvest adding crucial information to end rot disease control.

CHAPTER 1: INTRODUCTION

Sweetpotato (*Ipomoea batatas,* (L) Lam.) is an important crop in the United States and internationally. Because of its high nutrient values, especially in vitamins and carbohydrates, it plays an important role in the human diet, principally in developing countries, where sweetpotato is an essential source of food for humans (Bovell-Benjamin, 2007) and animals alike (Martin and Woolfe, 1993). In the United States, sweetpotato storage roots are traditionally used in preparation of holiday dishes. Canned sweetpotato products have been widely available in the commercial markets for years. However, demand has increased rapidly in recent years due to the popularity of newer sweetpotato products such as fries, chips, and easy-to-prepare frozen products as Americans are becoming increasingly more health conscious, and aware of the high nutrient value of this crop. Sweetpotato is cultivated in several states in the United States, with Louisiana being one of the top five states in production, generating around $85 million annually (www.LSUAgCenter.com/agsummary).

Frequent flooding events caused by storms and hurricanes, have disrupted sweetpotato production in Louisiana, especially since 2005. In many cases, storage roots of sweetpotato growing in flooded fields develop a soft rot, which is distinguished by a strong odor and by the rapid decay of the roots within a few days. Sweetpotato storage root soft rot diseases are recognized to be caused by *Dickeya dadantii* (*Erwinia chrysathemi*) (Martin and Dukes, 1997; Samson *et al.*, 2005; Schaad and Brenner, 1977), *Rhizopus stolonifer* (Clark and Moyer, 1988; Clark and Hoy, 1994; Harter and Weimer, 1921), and most recently *Geotrichum candidum* (Holmes and Clark, 2002). Under anaerobic conditions created by flooding, soft rot in sweetpotato storage roots seems to be triggered by an association of pathogens (Duarte, 1990),

which was found to include pectolytic anaerobes, possibly clostridia (Duarte and Clark, 1990). These authors isolated putative clostridia from soft-rotted storage roots, and when inoculated into sweetpotato storage roots that were kept in an anaerobic environment, soft rot symptoms were reproduced and pectolytic anaerobic bacteria were re-isolated from the rotten roots. However, they were not able to fully characterize the strains, and the species that are involved in this sweetpotato storage root soft rot were still unidentified.

Increasingly, sweetpotato growers must store sweetpotatoes for up to a year to be able to sufficiently supply and maintain their markets. Thus, losses that occur after harvest and after the full cost of production have been paid out are especially costly. Rotting sweetpotatoes may also produce the phytoalexins ipomeamarone and ipomeanols that have been implicated in deaths of cattle that were fed sweetpotatoes culled from storage (Hansen, 1928; Hiura, 1943; Woolfe, 1992).

Among the important postharvest disease complexes in sweetpotato are end rots, which are characterized by a visible dry decay at either or both ends of the storage roots. In Mississippi, these diseases have increased significantly in the last few years, causing loss in storage facilities, and drastic reductions in production. Little is known about the etiology of this disease complex, which makes it hard to integrate methods of management and disease control. Although curing has long been established as an efficient practice to reduce water loss and infections by several pathogens during storage (Artschwager and Starrett, 1931; Weimer and Harter, 1921) , many growers have not invested in facilities to provide humidification and heating necessary to properly cure sweetpotatoes. At the LSU AgCenter Burden Center in Baton Rouge in 2010, almost 60% of uncured sweetpotato storage roots stored for 6 months were partially decayed by end rots, but only 10-20% of cured roots developed end rots (Clark,

personal communication). Weather conditions, such as intermittent flooding and/or droughts, may also influence end rot occurrence.

In Louisiana, it is common to have periods of flooding during the sweetpotato growing season, and many farmers associate the increase of the incidence of end rots with flooding events. Among the many effects of flooding on sweetpotato plants is the induction of ethylene synthesis (Paterson *et al.*, 1979). Ethylene can also be induced by ethephon, which is a product used as a defoliant on potato (*Solanum tuberosum* L.) and pre-harvest application of this product also has been shown to reduce skinning in sweetpotato (Schultheis *et al.*, 2000). However, preliminary data suggest that ethephon may in fact trigger greater incidence of end rots and/or internal necroses. The results from an experiment designed to investigate these findings showed that *Fusarium* sp., *Macrophomina phaseolina*, and other fungal species were present inside of symptomless storage roots, apparently growing as endophytes (Experiment A1).

Fungal endophytes are fungi that live internally in plants without inducing symptoms for at least a part of their life cycle (Wilson, 1995). Fungal endophytes consist of several types of fungi, including latent pathogens (Saikkonen *et al.*, 1998) and dormant saprophytes (Yuan *et al.*, 2010). The role of these fungi is not well established, and the little information known is based on their symbioses with a few agronomic grasses, mainly *Festuca arundinacea* (tall fescue) and *Lolium perenne* (perennial ryegrass); these interactions can extend from strong antagonism to obligate mutualism (Saikkonen *et al.*, 1998). Endophytic fungi are able to grow in the plant inter- or intracellularly (Boyle *et al.*, 2001; Schulz and Boyle, 2005). The plant-endophyte interaction is driven by: the plant's physiology and genotype (Donoso *et al.*, 2008), the fungal genotype (Freeman and Rodriguez, 1993), and the environmental circumstance (Redman *et al.*,

2001; Rodriguez *et al.*, 2009). In addition, the endophyte-plant interaction seems to be quite variable (Yuan *et al.*, 2010). More stressful environments, such as summer drought (Malinowski *et al.*, 2008) or flooding (Stanosz *et al.*, 2001), drive the selection toward higher infection frequencies of endophytes (Jensen and Roulund, 2004). However, endophytes can also play an important role in plant resistance to abiotic stresses (Cheplick, 2004; Donoso *et al.*, 2008; Yuan *et al.*, 2010), to certain herbivores (Cheplick and Clay, 1988), and to pathogen infections (Schulz and Boyle, 2005; Schulz *et al.*, 1999; Wilson, 1995). Fungal endophyte interactions in sweetpotato storage roots have not yet been studied and thus their effects on the plant are unknown.

CHAPTER 2: FLOODING-ASSOCIATED SOFT ROT OF SWEETPOTATO STORAGE ROOTS CAUSED BY *Clostridium* SPECIES

2.1. LITERATURE REVIEW:

Pectolytic Clostridia

Pectolytic clostridia have been reported to cause soft rot in potato, *Solanum tuberosum* (Campos *et al.*, 1982; Lund, 1972; Perombelon *et al.*, 1979) and yam, *Dioscorea rotundata* (Obi, 1981), are associated with wetwood diseases (Schink *et al.*, 1981), and are known to cause cavity spot in carrots (Perry and Harrison, 1977). An important common factor among these diseases is that their occurrence was associated with wet seasons in poorly drained soils with restricted aeration.

Clostridium puniceum has been well described as a cause of potato soft rot. *C. puniceum* is characterized as: anaerobic, spore-forming, rod-shaped, pink-pigmented on potato infusion agar (PIA), and forming pits (craters) on double-layer pectate medium (DLPM) (Lund *et al.*, 1981). This bacterium is different from the putative pectolytic clostridia strains isolated from decayed sweetpotato storage roots by the localization of the endospore in the cells and by pigment production (Duarte, 1990). Strains isolated from decayed sweetpotato storage roots tend to have vegetative cells with endospores located in the end of the cells and no pigment production was observed from colonies grown on PIA (Duarte, 1990). Whereas, *C. puniceum* strains isolated from Irish potato produce sub-terminal endospores and pink pigment on PIA (Lund *et al.*, 1981).

Although pectolytic clostridia have been reported to be associated with soft rot in several plants, the species *C. puniceum* is the only *Clostridium* species described as a plant

pathogen that has been thoroughly characterized and published as a valid name in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (Bull *et al.*, 2010). Identification of the strains of pectolytic clostridia responsible for causing soft rot in sweetpotato storage roots growing in flooded fields is an important step to manage the disease and reduce losses associated with flooding. The objective of this study was to identify the strictly anaerobic, pectolytic bacterium involved in the souring of sweetpotato storage roots.

2.2. MATERIAL AND METHODS:

2.2.1. Media Used

All media used in this study were reduced by incubating the plates for four days in an anaerobic jar (BD GasPakTM 100, Anaerobic Jars, Becton Dickinson Microbiology Systems, Franklin Lakes, NJ, USA) in an atmosphere containing H_2/CO_2 (90:10 by volume) at 32^{o}C, prior to being used (Figure 1). Streaked plates were also incubated in this anaerobic jar to maintain anaerobiosis. Nutrient dextrose agar plus 0.05% cysteine hydrochloride (NDAC) (Difco nutrient agar, 23.0 g; dextrose, 2.5 g; cysteine HCl, 0.5 g; distilled water, 1000.0 ml) was used for isolations, bacterial growth, and morphological characterization of the bacterial isolates (Lund, 1972). Potato infusion agar (PIA) [white potatoes, 200.0 g; glucose, 5.0 g; $(NH_4)_2SO_4$, 1.0 g; $CaCO_3$. 3.0 g; cysteine HCl, 0.5 g; Davis agar, 15.0 g; distilled water, 1000.0 ml] was used for morphological characterization and pigment production (Lund *et al.*, 1981). Potato infusion medium (PIM) [white potatoes, 200.0 g; glucose, 5.0 g; $(NH_4)_2SO_4$, 1.0 g; $CaCO_3$. 3.0 g; cysteine HCl, 0.5 g; Davis agar, 0.5 g; distilled water, 1000.0 ml] was used for preservation of the isolates (Holdeman *et al.*, 1977). Pectinase activity was evaluated using a

double-layer pectate medium (DLPM) [medium for basal layer – Tryptone, 10.0 g; Lab-lemco powder, 2.4 g; yeast extract, 5.0 g; $CaCl_2.6H_2O$, 5.2 g; cysteine HCl, 0.4 g; Bacto agar, 19.0 g; polymyxin B sulphate solution (24,000 units/ml), 10.0 ml; distilled water, 1000.0 ml. Medium for upper layer – sodium polypectate, 20.0 g; ethanol (absolute), 60.0 ml; EDTA (di-sodium salt), 1.0 g; distilled water, 1000.0 ml] (Lund, 1969).

Figure 1 – Anaerobic jar, plates incubated in an atmosphere containing H_2/CO_2 (90:10 by volume).

2.2.2. Bacterial Isolations

Isolations were conducted from sweetpotato storage roots with soft rot symptoms obtained from plants growing in a field that was intentionally flooded two weeks before harvest. In total, there were 10 samples from 10 different rotting storage roots, and one storage root was collected per plant. Eight of the roots were of the cultivar Evangeline, one of Bonita, and one of 07-146. Portions of the decayed tissue were suspended in sterile distilled water (SDW) and streaked on NDAC. Streaked plates were then inverted and fumigated with 1 mL of chloroform for 10 minutes to eliminate non spore-forming bacteria (Bauernfeind and

Burrows, 1978). Finally, the plates were incubated in an anaerobic jar for seven days at 32°C. Isolated bacterial colonies from these plates were re-streaked on NDAC and incubated in aerobic and anaerobic atmospheres. Colonies from isolates that grew in the anaerobic environment but not in the aerobic environment were collected and preserved in screw-cap tubes (13 x 100 mm) containing PIM for further evaluations.

2.2.3. Differentiation of Isolates

Isolates of pectolytic clostridia were separated based on size and morphology of colonies growing on PIA and NDAC (Lund, 1972; Lund *et al.*, 1981), on size and morphology of vegetative cells and endospores from colonies growing on PIM, and on pectolytic activity of colonies growing on DLPM (Lund, 1969).

Pectolytic ability was evaluated by streaking bacterial cells kept in PIM onto plates of DLPM. Streaked plates were incubated for seven days at 32°C in an anaerobic jar. After incubation, the plates were inspected for pit formation, which is an indication of pectin degradation. Colonies that formed pits were classified as pectolytic while colonies that did not form pits were considered negative for pectolytic activity.

Bacterial endospores were observed by using a modified differential bacterial staining protocol Reynolds *et al.* (2005). A loop of bacterial cells grown on PIM was spread on a microscope slide, and completely air dried. The smear was heat-fixed by moving the slide quickly over a Bunsen burner flame 3 or 4 times. Then, the slide was covered with a small piece of paper towel, and flooded with malachite green dye solution (0.5 g of malachite green [Sigma Chemicals] in 100 ml of distilled water). The slide was left for five minutes on a wire screen on top of a beaker containing boiling water; which was previously placed on a hot plate

to boil. The slide was then washed thoroughly with water and flooded with safranin dye solution (2.5 g of safranin O [Sigma Chemicals] in 100 ml of 95% [v/v] ethanol), and left for one minute. Finally, the slide was washed thoroughly with tap water, blotted dry with paper towels, and observed with the aid of a light microscope under a 100 X oil immersion objective. Pictures were taken with an Olympus DP72 microscope digital camera (OLYMPUS CORPORATION).

Bacterial cell measurements, length and thickness, were taken using differential interference contrast (DIC) microscopy. 10 µl of bacterial suspension grown on PIM were placed on a microscope slide, covered with slide cover and directly observed with the aid of a Leica TCS SP2 Spectral Confocal microscope in the Socolofsky Microscopy Center, Department of Biological Sciences, Louisiana State University.

2.2.4. Pathogenicity Test

Inoculum was prepared by suspending bacterial cells that had been grown for 7 days on NDAC in sterile distilled water amended with 0.05% cysteine and diluting to an optical density (OD_{620}) of 0.1 (approximately 1 x 10^8 CFU/ml). For each isolate, 10 storage roots were inoculated and 10 control storage roots were mock inoculated with sterile distilled water (SDW) plus 0.05% cysteine. Whole asymptomatic sweetpotato storage roots were washed with tap water, surface disinfected with 1% NaOCl for 10 minutes, and inoculated by inserting and leaving a pipette tip containing 50 µl of inoculum suspension in each root. The storage roots were then individually wrapped with moist paper towels, placed in a plastic bag that was then tied shut, and incubated at 30°C for 7 days. To gain information on host range, the same approach was used for inoculation of potato tubers, onion bulbs, and carrot roots purchased

from a local grocery store. Re-isolations were performed from inoculated decaying tissue on NDAC as described above.

2.2.5. Genomic DNA Extraction and 16S Ribosomal DNA Gene Amplification

Genomic DNA of isolates of pectolytic clostridia were extracted by using a method modified from (Pospiech and Neumann, 1995):

1. Bacterial isolates were grown on NDAC under anaerobic conditions at 28^{o}C for 5 days;
2. Cells were harvested using a sterile toothpick and re-suspended in 500 µl of SET (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5).
3. 0.5 mg of lysozyme (1 mg ml^{-1} in sterile water) was added and incubated at 37^{o}C for 30 min.
4- 50 µl (1/10 volumes) of 10% SDS and 14 µl of proteinase K (0.5 mg ml^{-1}) were added and incubated at 55^{o}C with inversion every 20 min for 2 h.
5- 190 µl (1/3 volume) of 5 M NaCl and 750 µl (1 volume) of chloroform were added, and incubated at room temperature for 30 min with frequent inversion.
6- After centrifuging at 4500 g for 15 min, the aqueous phase was transferred to a new tube using a blunt-ended pipette tip.
7- DNA was precipitated by adding ≈ 750 µl (1 volume) of isopropanol and gently inverting the tube.
8- After centrifugation at 14,000 RPM for 10 min, the supernatant was discarded.
9- 500 µl of 70% ethanol was added, the tube was inverted several times and centrifuged at 14,000 RPM for 2-5 min.
10- The supernatant was discarded. For a second wash, 500 µl of 70% ethanol was added, inverted several times and centrifuged at 14,000 RPM for 5 min.
11- The supernatant was discarded and the tube centrifuged again to remove the excess ethanol.

12- The extract was air dried for 15-20 min, then the pellet resuspended in 50-100 µl of TE for ≈ 20 minutes on ice.

13- The DNA was stored at -70°C until ready to use.

The 16S rDNA gene was amplified using the set of universal bacterial primers FD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and RD1 (5′-AAG GAG GTG ATC CAG CC-3′) (Weisburg *et al.*, 1991). PCR reactions were done in a reaction mixture containing 5 µl of 5 X thermophilic DNA buffer (Promega, Madison, WI - USA), 0.5 µl of 10 mM deoxyribonucleoside triphosphates (dNTPSs), 0.25 µl of Taq polymerase (0.5 U/ µl), 3.33 µl of 25 mM $MgCl_2$, 1.0 µl each of 10 mM stock of each primer (RD1 and FD1), 1.0 µl of 100 ng/µl of DNA sample, and 12.92 µl of SDW. The PCR program included an initial denaturation period of 98°C for 2 min, 30 cycles of 98°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and a final extension period of 72°C for 5 min.

Amplicons were purified with QIAGEN's QIAquick® PCR Purification Kit and sequenced by Eton Bioscience (Eton Bioscience Inc., Durham, NC - USA). DNA sample concentration was adjusted to 60 ng in 6 µl of deionized water per reaction. Then, 10 pmols of primer, 6 µl of DNA sample, 8 µl of big dye v. 3.1, and 6 µl of deionized water was added in each well of a 96-well plate and placed on a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA – USA) . The cycle sequencing included an initial denaturation period of 98°C for 1 min; 30 cycles of rapid thermal ramp to 98°C, 98°C for 10 sec, rapid thermal ramp to 50°C, 50°C for 5 sec, rapid thermal ramp to 60°C, 60°C for 4 min, rapid thermal ramp to 4°C, and 4°C for 5 min. Finally, the samples were purified, vortexed, and loaded on ABI automated 3730xl sequencers (Applied Biosystems, Foster City, CA – USA), which translated the fluorescent signals into their corresponding base pair sequence.

The two 16S rDNA query sequences, LSU-B1 and LSU-B7, were edited in BioEdit v 7.1.3 (Hall, 1999), and compared with the NCBI data base using BLASTn (Altschul *et al.*, 1990) at the National Center for Biotechnology Information website (http://ncbi.nlm.nih.gov/blast).

2.2.6. Phylogenetic analyses

16S rDNA bacterial sequences used for comparative analyses were obtained from the NCBI GenBank (Table 1). The sequences from 18 strains of *Clostridium* spp. and one sequence of *Dickeya dadantii,* as an outgroup, were aligned with Clustal W (Higgins *et al.*, 1994). A maximum likelihood (ML) search and a maximum parsimony (MP) analysis of taxa were performed in MEGA5 (Tamura *et al.*, 2011). Analyses were run with 1000 bootstrap replicates and a bootstrap consensus tree was inferred to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree was drawn to scale, with branch lengths calculated using the average pathway method (Nei and Kumar, 2000) and are in the units of the number of changes over the whole sequence. All positions containing indels and missing data were eliminated. There were a total of 1213 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

Alternative topological hypotheses were tested by using the ML method based on the Data specific model (Nei and Kumar, 2000). The tree with the highest log likelihood (-6446.0885) was selected and the bootstrap support values of 1000 runs were included on the phylogenetic tree.

Table 1. Bacterial sequences used in the phylogenetic analysis.

Species Designation	**Strain Designation**	**Geographical Origin**	**GenBank Accession Number**	**Accession Number Description**
Clostridium acetobutylicum	NCIMB8052	Weizmann, Israel	U16165	Partial 16S rRNA
C. baratii	IP 2227	Inst. Pasteur, Paris	NR_029229	Complete 16S rRNA
C. beijerinckii	JCM 8023	Riken, Japan	AB647331	Partial 16S rRNA
C. botulinum	E134	Lake Erie, USA	JN617090	Partial 16S rRNA
C. butyricum	AB33	India	JQ993878	Partial 16S rRNA
C. corinoforum	DSM 5906	DSM, Germany	X76742	Partial 16S rRNA
C. difficile	DSM 11209	DSM, Germany	X73450	Partial 16S rRNA
C. diolis	SH1	DSM, Germany	NR_025542	Partial 16S rRNA
C. favososporum	DSM 5907	DSM, Germany	X76749	Partial 16S rRNA
C. perfringens	JCM 3817	Riken, Japan	AB588015	Partial 16S rRNA
C. puniceum	BL 70/20	Wisconsin, USA	NR_026105	Partial 16S rRNA
C. roseum	N36	Italy	AB601091	Partial 16S rRNA
C. saccharobutylicum	P262	New Zealand	U16147	Partial 16S rRNA
C. saccharoperbutylacetonicum	N1-4	New Zealand	NR_036950	Partial 16S rRNA
C. sordellii	HT3	India	DQ978213	Partial 16S rRNA
C. tetani	NCTC 279	NCTC, UK	NR_029260	Partial 16S rRNA
Dickeya dadantii	CFBP 1269	Comores	NR_041921	Partial 16S rRNA
LSU_B1	LSU_B1	Louisiana, USA	JX258847	Partial 16S rRNA
LSU_B7	LSU_B7	Louisiana, USA	JX258848	Partial 16S rRNA

2.3. RESULTS:

2.3.1. Isolate morphology

Two distinct groups of bacteria were consistently isolated from decaying tissue of soft rotted Evangeline storage roots grown in an intentionally flooded field. One representative isolate of bacteria from each group, LSU-B1 and LSU-B7, were selected for further investigation. Both isolates had a Gram positive reaction, grew on media incubated in an anaerobic environment but not on media exposed to air, and formed sub-terminal endospores and were thus preliminarily identified as *Clostridium* sp. (Lund and Kelman, 2001). In other respects, the morphology of the isolates differed considerably (Table 2). Only isolate LSU-B1 formed pits on DLPM (Figure 2). LSU-B1 produced smaller colonies and vegetative cells and appeared to produce fewer endospores on PIM than LSU-B7 isolate (Figures 3, 4, and 5).

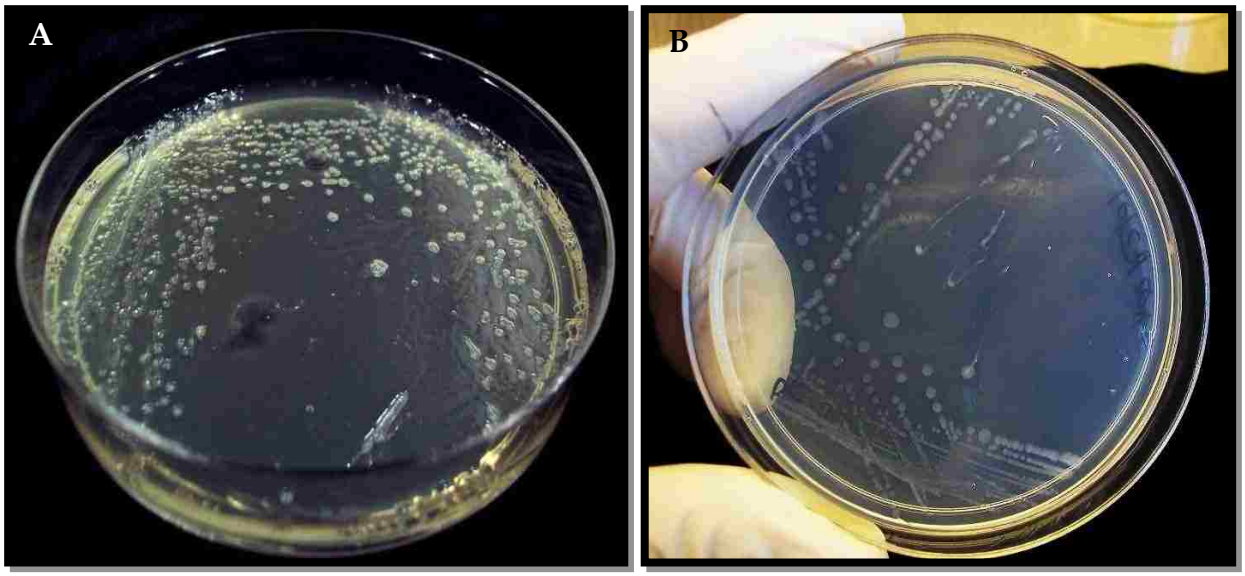

Figure 2 - Pectolytic activity of the isolates was determined by pit formation on DLPM after seven days of anaerobic incubation at 32°C. **A**, pits formed by LSU-B1 colonies. **B,** colonies of LSU-B7 in which no pit formation was observed.

Table 2. Morphological characteristics of the bacterial isolates, LSU-B1 and LSU-B7, following growth in an anaerobic environment at 32°C. Colony characteristics were evaluated after seven days growth on NDAC. Vegetative cell characteristics were evaluated after seven days on PIM.

	Colonies						Vegetative Cells	
Groups	**Color**	**Appearance**	**Diameter (mm)**	**Form**	**Elevation**	**Margin**	**Shape**	**Size (μm)**
LSU-B1	Cream	Butyrous	1-2	Rhizoid to Irregular	Umbonate	Undulate to Lobate	Straight Rods	3-5
LSU-B7	Light Cream	Butyrous	4-7	Circular	Raised to Convex	Entire	Straight Rods	5-11

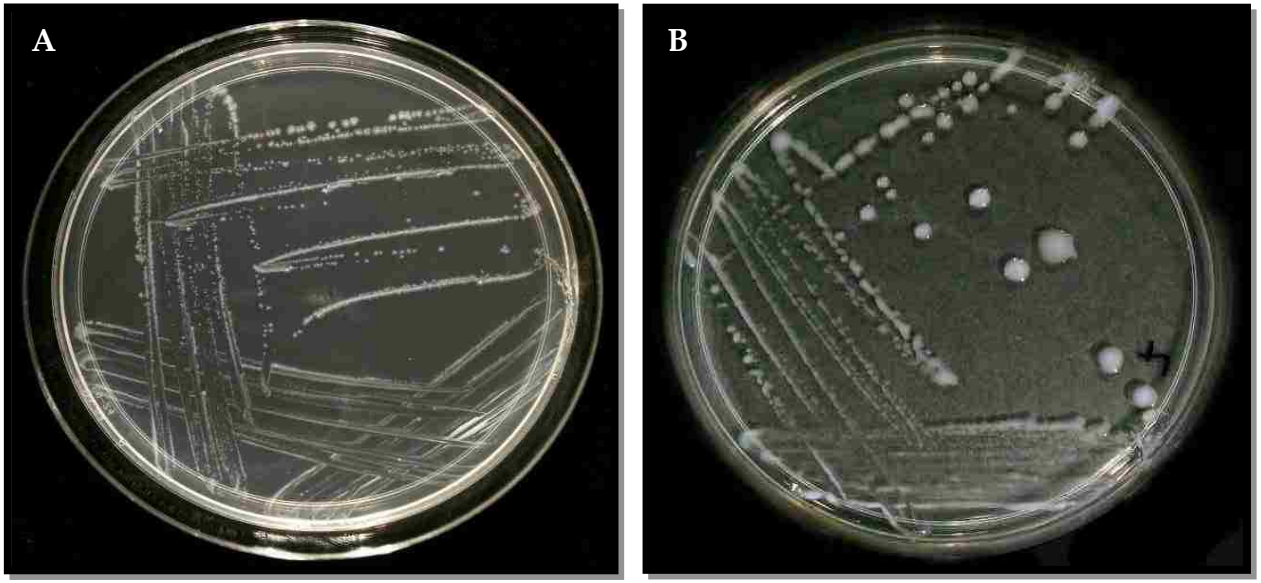

Figure 3 - Bacterial colonies growing on NDAC after seven days of anaerobic incubation at 30°C. **A,** LSU-B1 colonies. **B,** LSU-B7 colonies.

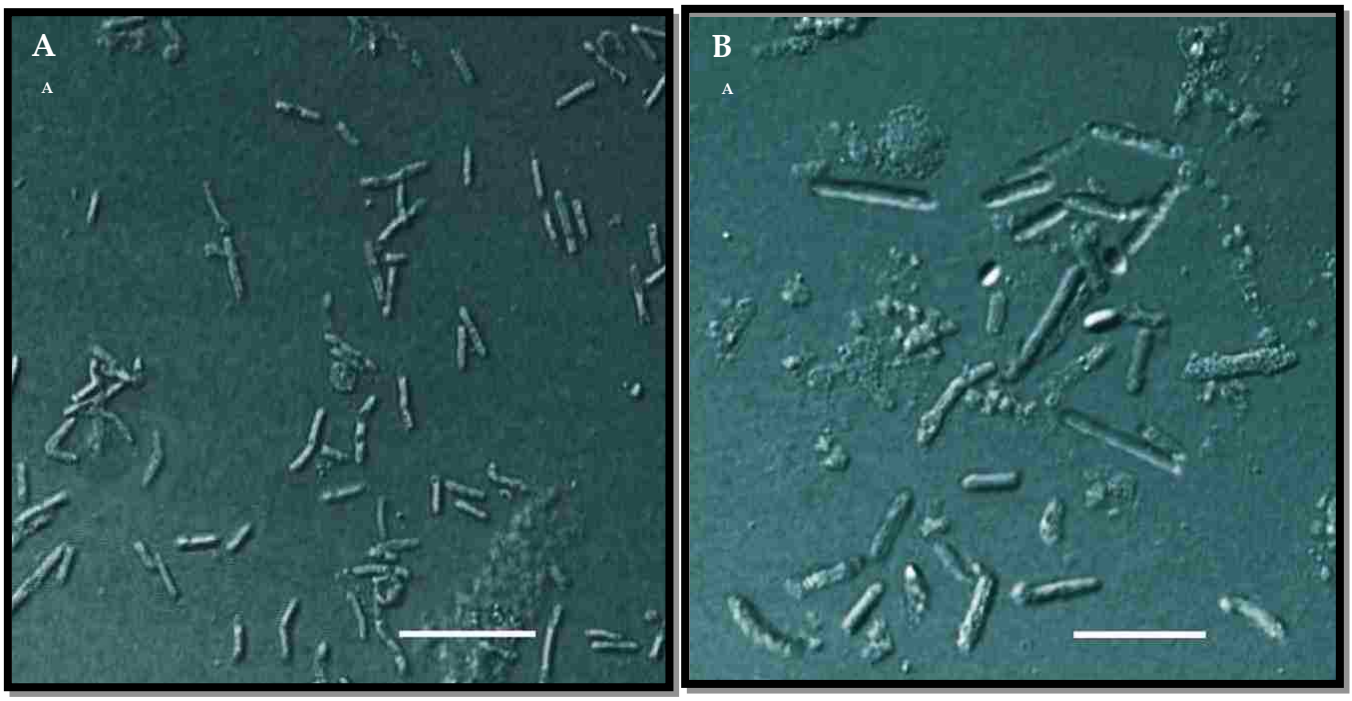

Figure 4 - DIC micrographs of bacterial cells grown on PIM. **A,** LSU-B1 bacterial cells (3-5 µm long). **B,** LSU-B7 bacterial cells (5-11 µm long). Scale bars = 10 µm.

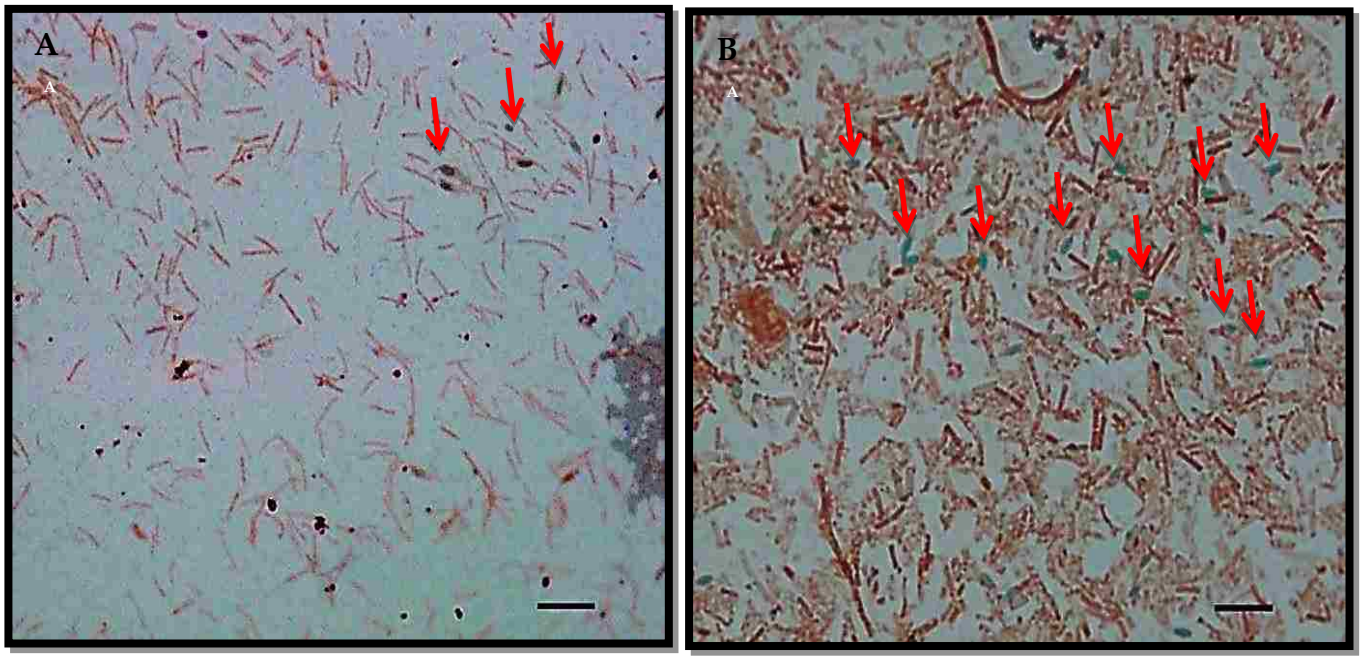

Figure 5 - Light micrographs of differential staining of bacterial cells grown on PIM. **A,** LSU-B1 bacterial cells. **B,** LSU-B7 bacterial cells. Note that there are fewer endospores (red arrows) in LSU-B1 than LSU-B7. Scale bars = 10 µm.

2.3.2. Pathogenicity test

All sweetpotato storage roots inoculated with either of the bacterial isolates developed severe soft rot after seven days of incubation in a hypoxic environment (Figure. 6). Potato tubers, onion bulbs, and carrot roots were completely rotted after five days of incubation, when inoculated with either of the bacterial isolates (Figures 7 and 8). None of the controls developed soft rot after the incubation period (Figures. 9 and 10). However, three onion bulbs and four carrot roots developed fungal infections after the incubation period. In all cases, the inoculated isolates were re-isolated from decaying tissue (Figure 11).

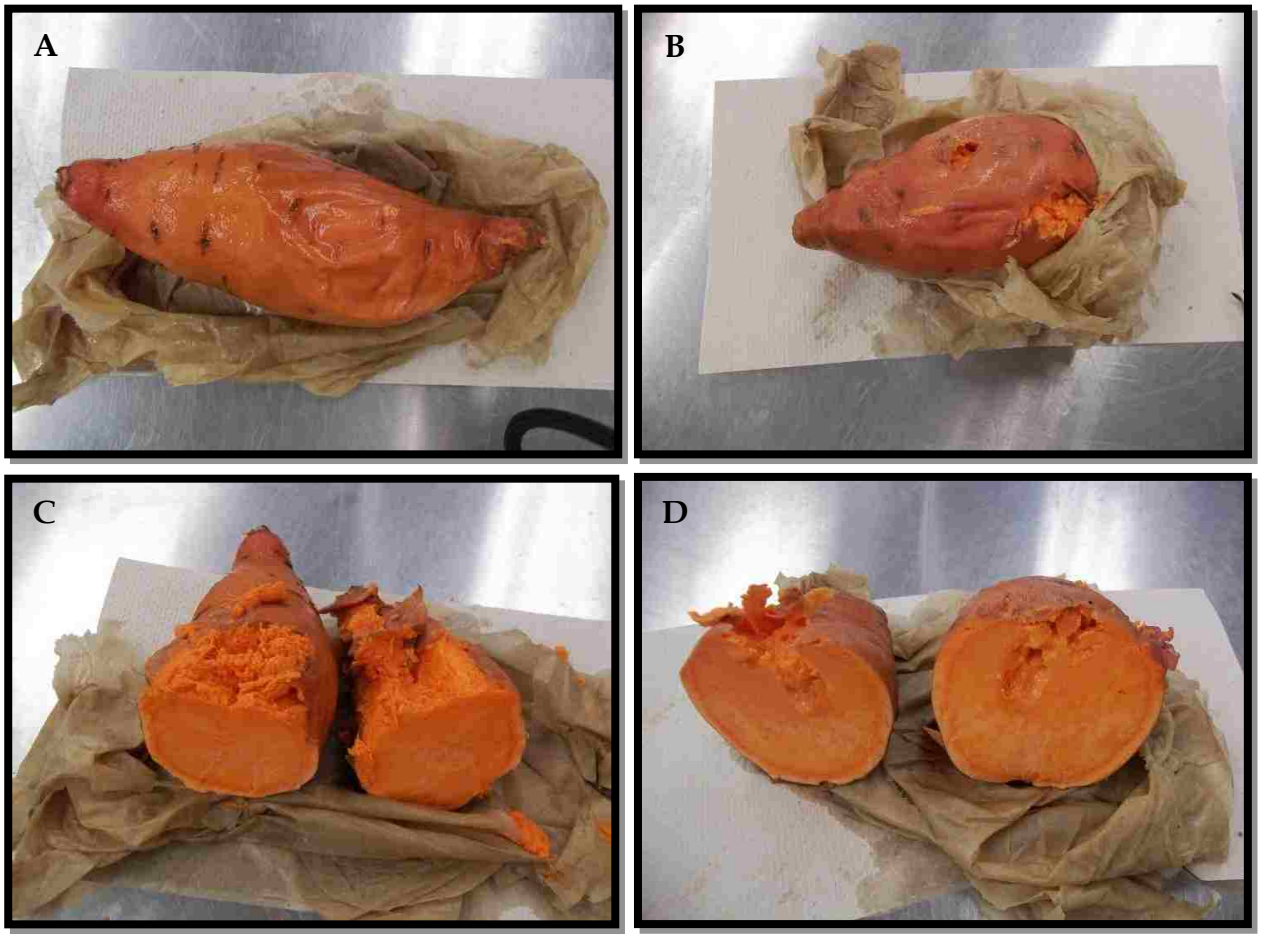

Figure 6 - Soft rot symptoms on Evangeline sweetpotato storage roots inoculated with LSU-B1 isolate **(A)** and **(C),** and LSU-B7 isolate **(B)** and **(D).**

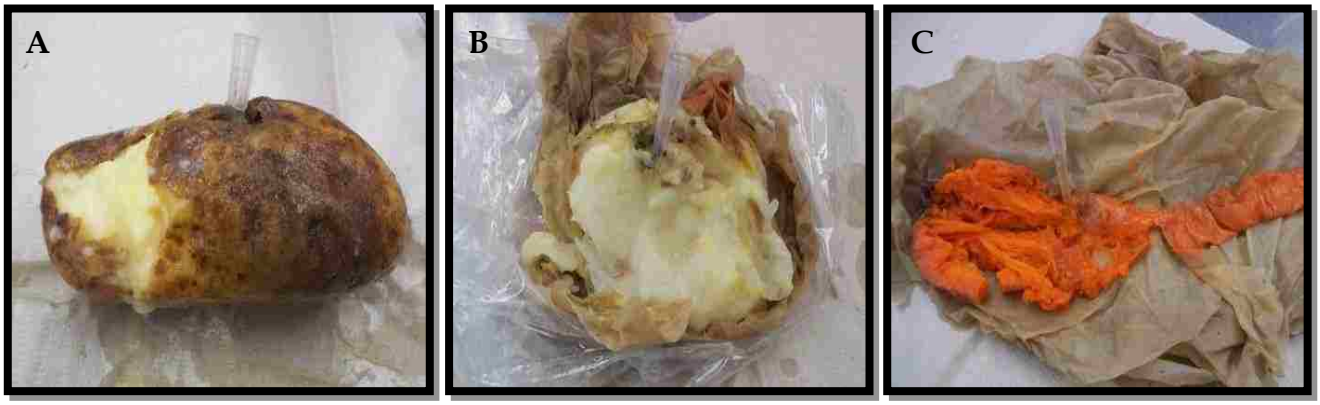

Figure 7 - Soft rot symptoms on potato tuber **(A),** onion bulb **(B),** and carrot root **(C)** inoculated with LSU-B7 isolate.

Figure 8 - Soft rot symptoms on potato tuber **(A),** onion bulb **(B),** and carrot root **(C)** inoculated with isolate LSU-B1.

Figure 9 - Evangeline sweetpotato storage root mock inoculated with 50 µl of sterile distilled water plus 0.05% cysteine as the experiment control.

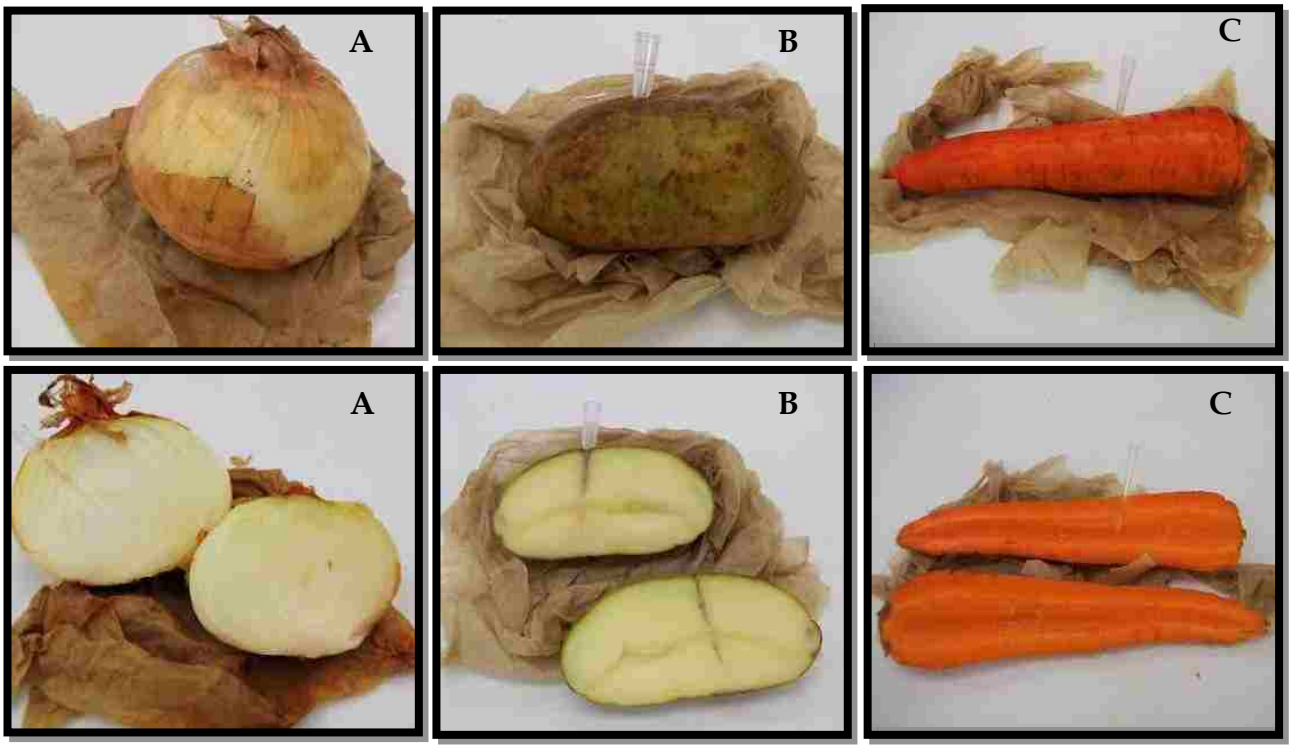

Figure 10 Onion bulb **(A),** Irish potato tuber **(B),** and carrot root **(C)** mock inoculated with 50 µl of sterile distilled water plus 0.05% cysteine as the experiment control.

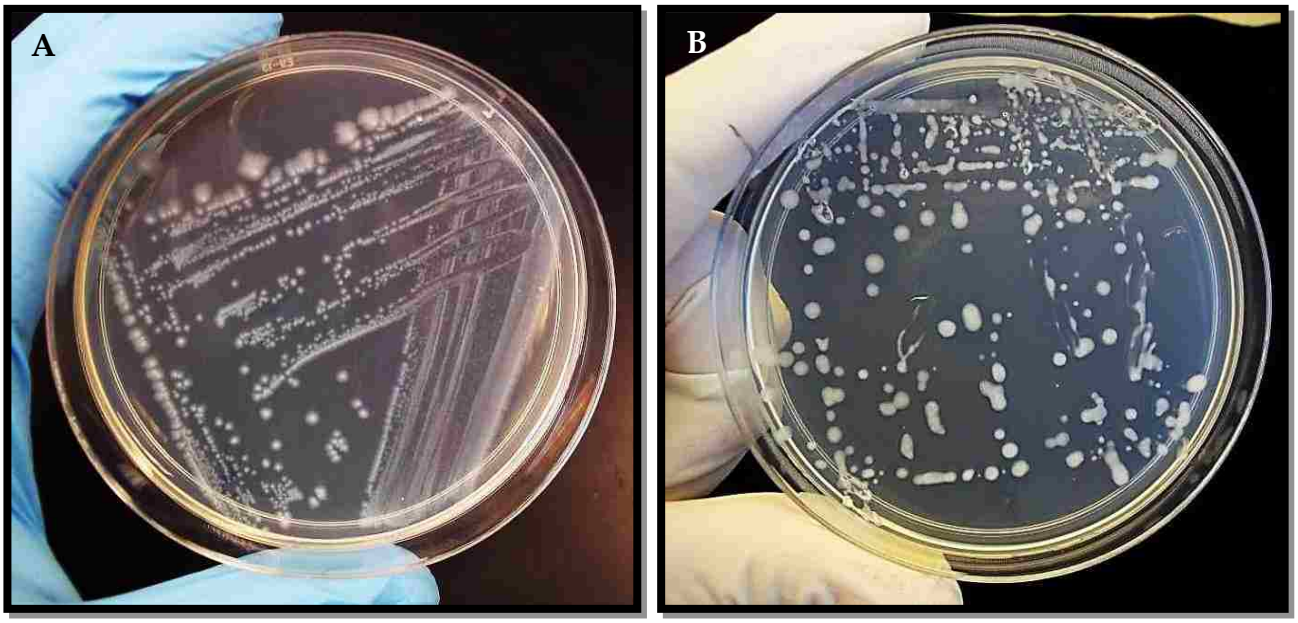

Figure 11 - Bacterial colonies of isolate LSU-B1 **(A)** and isolate LSU-B7 **(B)** recovered from decaying sweetpotato storage root previously inoculated with the respective isolate.

2.3.3. 16S ribosomal DNA gene sequence analyses

Amplification and sequencing of the 16S rRNA region from isolate LSU-B1 generated a sequence 1425 bp long (GenBank database Accession No. JX258847). BLASTn analysis against the NCBI database of this nucleotide sequence resulted in 99% homology with *C. puniceum* strain BL 70/20 (GenBank Accession No. NR_026105.1). However, it resulted in high homology (98%) with other *Clostridium* species as well (Figure 12).

Isolate LSU-B7 generated a sequence 1376 bp long (GenBank database Accession No. JX258848). This nucleotide sequence resulted in 99% homology with *C. saccharobutylicum* strain P262 (GenBank Accession No. U16147), after a BLASTn analysis on the NCBI database website. This analysis also showed that LSU-B7 is highly similar (98%) to other *Clostridium* species in regard to the 16S rDNA gene (Figure 13).

A sequence comparison analysis between the LSU-B1 and LSU-B7 16S rRNA sequences revealed that although they were 97% similar, there are many mismatching and indels (insertion and/or deletion of nucleobases) among the sequences (Figure 14).

2.3.4. Phylogenetic analysis

Three distinct clades were recovered from maximum parsimony (MP) and maximum likelihood (ML) analyses using the 16S rRNA sequence dataset (Figure 15).

Clade I, which was well supported by the ML and MP bootstrap values of 60 and 88, respectively, included the LSU-B7 isolate from this study and the species *C. saccharobutylicum.* Clade II, included the LSU-B1 isolate from this study and the species, *C. puniceum, C. corinoforum,* and *C. favosporum*.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NR_026105.1	Clostridium puniceum strain BL 70/20 16S ribosomal RNA, part	2516	2516	100%	0.0	99%
X71857.1	C.puniceum gene for 16S ribosomal RNA	2516	2516	100%	0.0	99%
FN667340.1	Uncultured compost bacterium partial 16S rRNA gene, clone PS	2483	2483	99%	0.0	99%
AB594682.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2473	2473	100%	0.0	98%
X76742.1	Clostridium corinoforum partial 16S rRNA gene, strain DSM 59	2471	2471	100%	0.0	98%
X76749.1	Clostridium favososporum partial 16S rRNA gene, strain DSM 5	2470	2470	99%	0.0	98%
HQ397040.1	Uncultured Clostridium sp. clone HAHS13.33 16S ribosomal RN	2466	2466	100%	0.0	98%
U16122.1	Clostridium saccharoperbutylacetonicum N1-4 16S ribosomal R	2462	2462	100%	0.0	98%
AB594694.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2460	2460	100%	0.0	98%
EU828377.1	Uncultured bacterium clone T4_2_55 16S ribosomal RNA gene,	2460	2460	100%	0.0	98%
FJ535169.1	Uncultured Clostridia bacterium clone ATB-LH-5969 16S riboso	2459	2459	100%	0.0	98%
JN038600.1	Uncultured Firmicutes bacterium clone MA-R17 16S ribosomal F	2453	2453	100%	0.0	98%
JN038619.1	Uncultured Firmicutes bacterium clone MA-R45 16S ribosomal F	2453	2453	100%	0.0	98%
EU037333.2	Uncultured bacterium clone G3DCM-67 16S ribosomal RNA gen	2440	2440	100%	0.0	98%
AY168741.2	Uncultured bacterium clone Hot Creek 31 16S ribosomal RNA g	2440	2440	100%	0.0	98%
AB640693.1	Clostridium beijerinckii gene for 16S ribosomal RNA, partial seq	2438	2438	100%	0.0	98%
FR872745.1	Clostridium saccharoperbutylacetonicum partial 16S rRNA gene	2435	2435	98%	0.0	98%
AB678391.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2433	2433	100%	0.0	98%
AB678390.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2433	2433	100%	0.0	98%
AB678386.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2433	2433	100%	0.0	98%
AB647330.1	Clostridium butyricum subsp. convexa gene for 16S ribosomal	2433	2433	100%	0.0	98%
AB286478.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2431	2431	98%	0.0	98%
DQ296468.1	Uncultured bacterium clone AR-23 16S ribosomal RNA gene, pa	2429	2429	100%	0.0	98%
AB678395.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2427	2427	100%	0.0	98%
AB678394.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2427	2427	100%	0.0	98%

Figure 12 - BLASTn analysis of the 16S rRNA sequence of LSU-B1 representative isolate showing 99% homology with *C. puniceum* and 98% homology with other *Clostridium* species.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
U16147.1	Clostridium saccharobutylicum P262 16S ribosomal RNA (rrn) g	2460	2460	100%	0.0	99%
AM998793.1	Clostridium saccharobutylicum partial 16S rRNA gene, strain O	2455	2455	100%	0.0	99%
NR_036951.1	Clostridium saccharobutylicum strain P262 16S ribosomal RNA,	2453	2453	99%	0.0	99%
AY188845.1	Clostridium sp. L1/8 16S ribosomal RNA gene, partial sequence	2446	2446	99%	0.0	99%
AY188843.1	Clostridium sp. F7/7 16S ribosomal RNA gene, partial sequence	2442	2442	99%	0.0	99%
AY188842.1	Clostridium sp. F7/4 16S ribosomal RNA gene, partial sequence	2431	2431	99%	0.0	98%
DQ196629.2	Clostridium sp. BL-8 16S ribosomal RNA gene, partial sequence	2422	2422	100%	0.0	98%
AB594687.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2414	2414	100%	0.0	98%
FN667423.1	Uncultured compost bacterium partial 16S rRNA gene, clone PS	2411	2411	100%	0.0	98%
AB594682.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2394	2394	100%	0.0	98%
AB240281.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2394	2394	100%	0.0	98%
AB114250.1	Clostridium sp. B908-1 gene for 16S rRNA, partial sequence	2392	2392	98%	0.0	98%
DQ296468.1	Uncultured bacterium clone AR-23 16S ribosomal RNA gene, pa	2388	2388	98%	0.0	98%
FR872745.1	Clostridium saccharoperbutylacetonicum partial 16S rRNA gene	2385	2385	100%	0.0	98%
CP000721.1	Clostridium beijerinckii NCIMB 8052, complete genome	2383	3.318e+04	100%	0.0	98%
AY188844.1	Clostridium sp. C1T/10 16S ribosomal RNA gene, partial seque	2383	2383	97%	0.0	99%
U16122.1	Clostridium saccharoperbutylacetonicum N1-4 16S ribosomal R	2383	2383	100%	0.0	98%
AB594694.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clo	2381	2381	100%	0.0	98%
AB610548.1	Clostridium diolis gene for 16S rRNA, partial sequence	2377	2377	100%	0.0	98%
AB647333.1	Clostridium beijerinckii gene for 16S ribosomal RNA, partial seq	2377	2377	100%	0.0	98%
AB647331.1	Clostridium beijerinckii gene for 16S ribosomal RNA, partial seq	2377	2377	100%	0.0	98%
AB601074.1	Clostridium beijerinckii gene for 16S rRNA, partial sequence, st	2377	2377	100%	0.0	98%

Figure 13 - BLASTn analysis of the 16S rRNA sequence of LSU-B7 representative isolate showing 99% homology with *C. saccharobutylicum* and 98% homology with other *Clostridium* species.

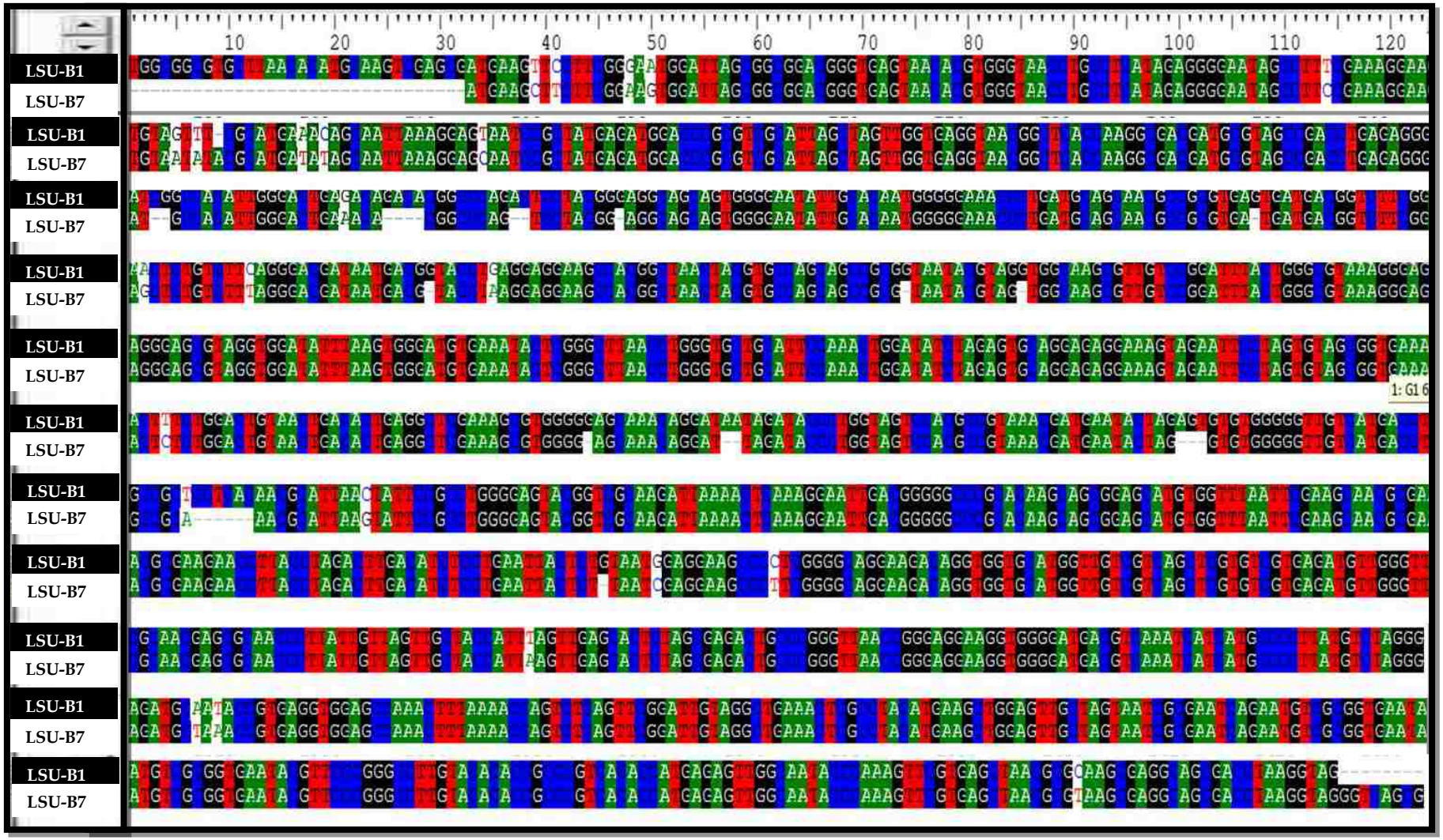

Figure 14 - Sequence comparison between the 16S rRNA sequences of LSU-B1 and LSU-B7 representative isolates showing 97% homology. The color codes of the bases (A= green, T= red, C= blue, and G= black) represent the homology between the two sequences. Bases with white color code characterize the mismatching and indels between the sequences.

Additionally, a strong phylogenetic relationship was indicated between the LSU-B1 isolate and the species *C. puniceum*, which was well supported by the ML and MP bootstrap values (96 and 97). Clade III, included *Clostridium* species that have industrial uses (*C. saccharoperbutylacetonicum, C. butyricum, C. beijerinckii, and C. acetobutylicum*), and soil inhabitant species (*C. roseum and C. diolis*).

The human toxin producing *Clostridium* species (*C. baratii, C. perfringens, C. tetani, C. difficile, C. sordellii,* and *C. botulinum*), included in the phylogenetic study and the outgroup species *Dickeya dadantii,* did not form any distinct group and were far removed phylogenetically from isolates LSU-B1 and LSU-B7.

2.4. DISCUSSION:

Pectolytic anaerobic bacteria resembling clostridia were found to cause soft rot in sweetpotato storage roots submerged in sterile distilled water (Duarte and Clark, 1990). However, until now, no study had been conducted under field conditions to characterize the occurrence of these pathogens in natural infections. In this study, we were able to isolate two distinct isolates of clostridia involved in the occurrence of this disease complex from rotting storage roots in flooded fields. Both isolates caused soft rot in sweetpotato storage roots and in other hosts following artificial inoculation and were re-isolated to fulfill Koch's Postulates.

Clostridium species are considered to be difficult microorganisms to control and work with (David and Stefanie, 2005; Kelly and LaMont, 2008). They produce resistant structures, endospores, which are hard to kill and can result in cross contamination among cultures when working with the isolates. They are also strictly anaerobic, requiring special conditions for growth in culture, which are expensive and time consuming. I also encountered unique

challenges in amplifying and sequencing DNA of these bacteria. The G+C contents of isolates LSU-B1 and LSU-B7 were over 50% in the 16S rDNA gene (Table 3) and the G+C content was not uniformly distributed along the gene (Figure 15). Some portions of the gene had G+C content much greater than 50% while for others the incidence was very low. This seemed to induce the formation of secondary structures such as hairpin loops, which created problems in the amplification and sequencing of the gene. However, these complications were resolved by increasing the melting temperature in the PCR and sequencer protocols from the standard 95°C to 98°C and adding 5% DMSO to the mix reactions.

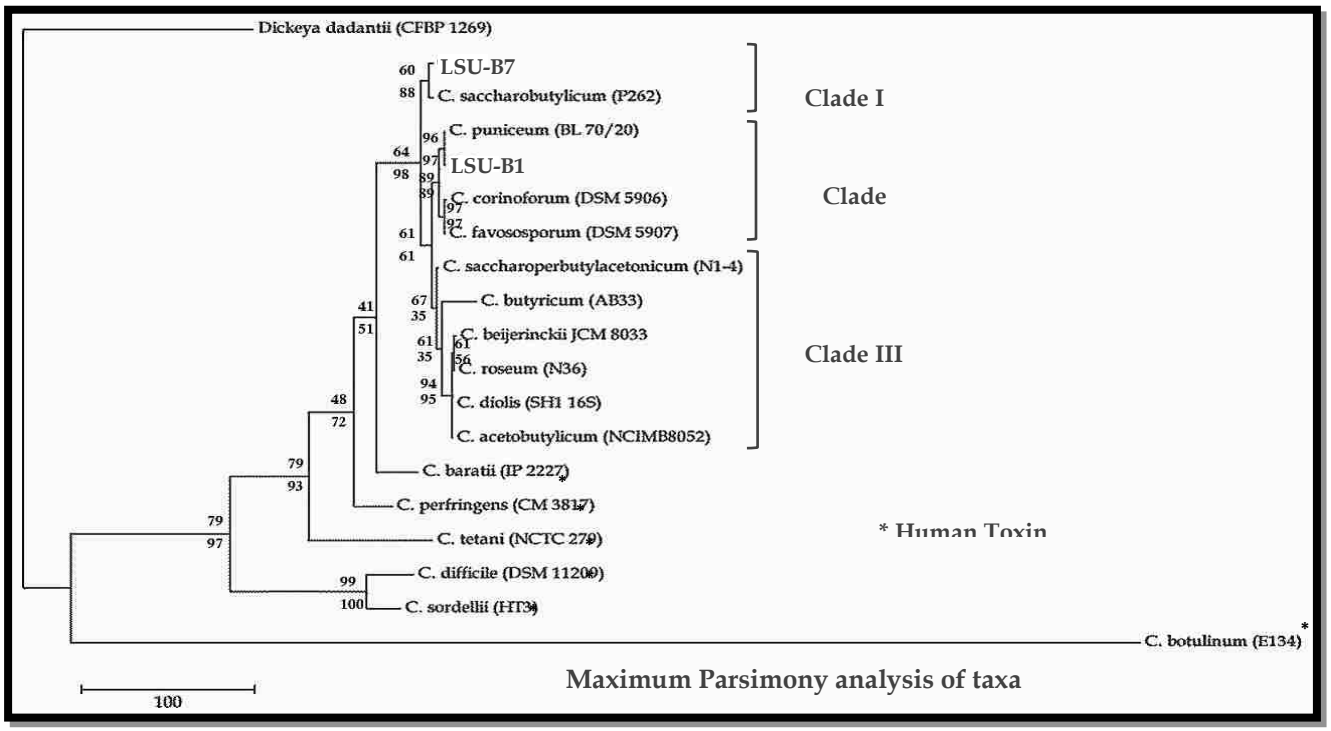

Figure 15 - Phylogram of the 16S rRNA dataset for 18 species of *Clostridium* and one species of *Dickeya* included as an outgroup. Branch tip labels include the species name with the strain designation in parentheses. Sequences from all species were obtained from the National Center for Biotechnology Information GenBank. The phylogram was produced by the maximum parsimony (MP) method. Numbers on nodes represent bootstrap support values for maximum likelihood (top) and maximum parsimony (bottom). The scale bar indicates number of substitutions per site.

Table 3. Distribution of nitrogen-containing bases along the 16S rRNA sequences of LSU-B1 and LSU-B7 isolates.

Isolate	Base Count					
	Adenine (A)	**Thymine (T)**	**Guanine (G)**	**Cytosine (C)**	**%G~C content**	**Total**
LSU-B1	380	302	432	311	52.1	1425
LSU-B7	372	294	413	297	51.6	1376

The identification of isolates LSU-B1 and LSU-B7 to species level was not attained due to the complexity of the genus and also due to the limited number of relevant gene sequences available on the NCBI database. In fact, for the species *C. puniceum,* the only known plant pathogen in the genus *Clostridium*, only the 16S rDNA gene had been previously sequenced and deposited in the GenBank database. Sequencing other genes for identification of the isolates was not within the scope of this study, as it would require obtaining numerous reference species and also sequencing them to have sufficient data for comparison. Even though the 16S rDNA gene is widely used to identify bacterial species, this gene alone was insufficient for a reliable identification of the pectolytic clostridia isolated from sweetpotato storage roots. The sequences were highly similar with numerous sequences of different *Clostridium* species within group 1 of the genus (Collins *et al.*, 1994; Wiegel *et al.*, 2006). Inglett *et al.* (2011) encountered similar difficulties with this group of *Clostridium* and found that DNA-DNA hybridization analyses and numerous phenotypic characterizations were necessary to support their description of a new species, *Clostridium chromiireducens*.

The phylogenetic analysis of the 16S rRNA sequence dataset corroborated the BLAST search results, showing isolate LSU-B7 to be very closely related with *C. saccharobutylicum* and isolate LSU-B1 with *C. puniceum* (Figure 15). Furthermore, this analysis showed a low relatedness among human toxin producer *Clostridium* species (*C. baratii, C. perfringens, C. tetani, C. difficile, C. sordellii,* and *C. botulinum*) and the isolates from this study, which reduces the fear of these isolates being able to cause disease in humans. However, more genes should be analyzed for a comprehensive conclusion.

For final species identification of LSU-B1 and LSU-B7, it will be necessary to perform more sequence and phenotypic analyses. Whole genome sequencing and comparison would provide the most thorough information. However, this approach is still expensive, and since many of the genomes of other species in this group have not been completely sequenced yet, there is little available for comparison. DNA finger print analyses and sequencing of other genes might also provide a suitable identification of these isolates. Likewise, morphological studies looking for the presence and type of flagella, pili, s-layer; and studies to determine the cellular fatty acid compositions would be indispensable approaches to help to properly identify those isolates.

Furthermore, this experiment was conducted in a single location, analyzing a small sampling of isolates. Possibly other species are involved in this disease complex and a more detailed study covering multiple diverse locations, and involving more sampling will be needed to reveal the full complex etiology of this disease. Duarte (1990) isolated two distinct isolates of *Clostridium* from decaying tissue of sweetpotato, one of his isolates produced small pits on DLPM while the other produced larger pits. In our study, only the isolate LSU-B1 produced pits on DLPM, while LSU-B7 grew on this medium, but no pit formation was

observed. Additionally, Duarte (1990) also detected a higher severity of the disease when the clostridia isolates were inoculated together with the soft rotter, *D. dadantii,* into sweetpotato storage roots. These facts suggest that more species within and out of the genus may also be triggering disease development, which reinforces the need for more studies.

In potato, pectolytic clostridia have been found primarily infecting lenticels (Perombelon *et al.*, 1979) and when those lenticels open, under wet conditions, these and other microorganisms are able to penetrate into the tuber and initiate soft rot (Pérombelon, 2002). Substantial lenticel proliferations were observed on sweetpotato storage roots when those roots were submerged in water. Possibly in flooded fields, the storage root lenticels proliferate and these are the sites to where *Clostridium* cells enter the root and start the infection. This indicates that finding an efficient way to control lenticel proliferation in sweetpotato storage roots would play a pivotal role in controlling the initiation of this soft rot disease.

The harsh Louisiana weather with constant severe thunderstorms and hurricanes, causing floods in the fields, seems to be the perfect combination for the development of this soft rot disease complex. In this study, important information was determined about this disease etiology, which can be used as a foundation for developing strategies in the disease management and control programs, providing better support to sweetpotato growers in dealing with this problem.

CHAPTER 3: INFECTION OF SWEETPOTATO BY FUNGAL END ROT PATHOGENS PRIOR TO HARVEST

3.1. LITERATURE REVIEW:

End Rots Fungal Pathogens

End rots of sweetpotato storage roots are caused by *Fusarium solani* (Clark, 1980), *Macrophomina phaseolina* (Jenk, 1981; Ray and Edison, 2005; Taubenhaus, 1913), *Lasiodiplodia theobromae* (Clark and Moyer, 1988; Clendenin, 1896) and/or *Diaporthe batatatis* (Harter and Field, 1913). In addition, some end rots were observed in 2010 from which *Sclerotium rolfsii* was isolated (daSilva, unpublished). These fungi are normally isolated alone from roots with end rot symptoms; however, isolations done in our laboratory from roots with end rot revealed that *F. solani* and *M. phaseolina* are often isolated from the same lesion (unpublished data) (Figure 16). *F. solani* and *M. phaseolina* were also isolated from healthy storage roots that did not present any end rot symptoms (data not shown). These findings suggested that these fungi are present inside of symptomless sweetpotato storage roots, suggesting that they are probably fungal endophytes in these roots, and become pathogens when conditions are favorable.

It is well known that *Fusarium* (Dill-Macky and Jones, 2000; Fernando *et al.*, 2000; Shaner, 2003; Sutton, 1982) and *M. phaseolina* (Baird *et al.*, 2003; Bhattacharya and Samaddar, 1976; Meyer *et al.*, 1974; Short *et al.*, 1980) can survive in crop residues and in the soil, from one season to another. However, how and when these fungi enter the sweetpotato storage root are important factors yet to be determined.

In commercial sweetpotato production, storage roots are planted in beds to sprout, and then the sprouts are used as propagative material to establish field plantings. From transplanting, sweetpotato plants take around four months to produce storage roots ready for harvesting. Hence, during their entire life cycle, sweetpotato plants are kept in contact with soil, therefore creating different options for end rot causal agents to penetrate the plant.

The traditional belief regarding end rot diseases is that pathogens infect storage roots through wounds, such those created by the harvesting process (Clark and *et al.*, 2013; Lo and Clark, 1988; Nelsen and Moyer, 1979). However, the discovery that *F. solani* and *M. phaseolina* are found inside of symptomless storage roots suggests other possibilities. These fungi may enter the storage roots even before harvesting, during plant growth. Understanding how and when those fungi invade sweetpotato storage roots are crucial steps to developing an effective end rot disease management program. This study was carried out to investigate how and when sweetpotato storage roots are invaded by the fungi, *F. solani* and *M. phaseolina.*

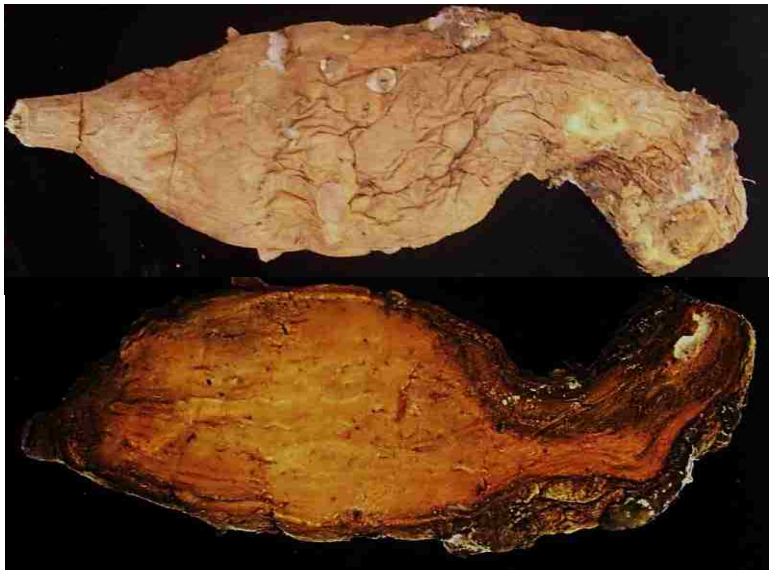

Figure 16 – Sweetpotato storage root infected with *M. phaseolina* and *F. solani.* Surface view **(A)** and longitudinal section view **(B).**

3.2. MATERIAL AND METHODS

3.2.1. Inoculum

Isolate CK-7 of *M. phaseolina* was isolated from an Evangeline sweetpotato storage root with tip rot at the Burden Center in Baton Rouge, LA in 2011. Isolate M-10 of *Fusarium solani* was isolated from a sweetpotato with *Fusarium* root rot in North Carolina and provided by J. W. Moyer (Dept. Plant Pathology, North Carolina State University, Raleigh). Isolates were revived on potato dextrose agar (PDA) and the hyphal tip technique was performed to eliminate any possible contaminants.

3.2.2. Toothpick Inoculation

In order to investigate if the pathogens, *F. solani* and *M. phaseolina,* move from infected mother plants to storage roots during growth and development of the plant, a greenhouse experiment was designed using vines from tissue culture plants (screened for the absence of *M. phaseolina* and *F. solani*) planted in autoclaved soil. After one month from the planting day, vines were mock inoculated with a sterile toothpick or inoculated with the specific fungus at the soil line, by a method modified from Pratt *et al.* (1998). Tips (1.0 cm long) of 100 wooden toothpicks were autoclaved for 20 min in 250 ml of distilled water. They were then blotted and re-autoclaved in additional water to remove inhibitory substances. The toothpick tips were autoclaved for a third time in 250 ml of 20% V-8 juice. Finally, they were cooled in sterile Petri dishes and transferred individually to colonies of the fungi growing on PDA Petri dishes or to Petri dishes with just PDA. There were 30 toothpick tips on colonies of *M. phaseolina*, 30 on colonies of *F. solani*, and 30 on plates with just PDA. After incubation for seven days at

28°C, toothpick tips were removed, inserted in vines of sweetpotato plants at the soil line, and the inoculation site was wrapped with a piece of Parafilm® to avoid desiccation of the wounded region. There were four treatments; plants mock inoculated with toothpick tips left on just PDA as control (Control), plants inoculated with toothpick tips infested with *M. phaseolina* (Mp) plants inoculated with toothpick tips infested with *F. solani* (Fso), and plants inoculated with two toothpick tips, one infested with *M. phaseolina* and another with *F. solani* (Fso+Mp). Each treatment had 10 replicate plants and each plant was cultivated in a 15-cm-diameter clay pot.

After 120 days, storage roots were harvested and washed with tap water to remove excess soil. Pencils roots, stems, and one storage root of each plant were collected for isolations as described below. The remaining storage roots were kept in paper bags, stored at 15°C, and analyzed after three months for end rot symptom development. Percent of storage roots infected with each of the pathogens was determined. This experiment was performed in 2011 and repeated in 2012.

3.2.3. Soil Infestation

This assay was performed to verify if the pathogens, *F. solani* and *M. phaseolina*, move from infested soil into the growing plant and/or into storage roots. Vines from tissue culture (screened for the absence of *M. phaseolina* and *F. solani)* were planted in infested and non-infested soil. The soil was infested by a modified procedure (Miles and Wilcoxson, 1984) in which inoculum was produced in three metal pans (7 X 15 X 21 cm) lined with a double layer of aluminum foil. 150 g of cornmeal and 300 ml of warm 1% PDA were added to each pan. The pans were allowed to stand for 10-15 min, and 75 g of sand was thoroughly mixed with

the cornmeal. Then, the pans were covered with two layers of aluminum foil and autoclaved for 1.5 hours at 1.27 kg/cm^2 (18 psi) and 121^{o}C. After cooling, the pans were inoculated with the agar from one-week-old fungal cultures, one pan with *M. phaseolina* and one with *F. solani*, grown on PDA and one pan was inoculated with sterile PDA. Agar from three petri dishes of each fungus was mixed in the sand-cornmeal of the corresponding pan with a sterile knife, and 50 ml of sterile distilled water was added. The pans were closed with aluminum foil, and incubated for four weeks at 28^{o}C. After the incubation period, the fungal inocula were spread on a tabletop for two days to air-dry. Dried inocula were stored individually in plastic bags at 4^{o}C until needed. Cornmeal-sand inoculum of each fungus was mixed with sterile soil in a 15-cm-diameter clay pot to a concentration of approximately 10^3 CFU/g (determined by serial dilution) of dry soil and approximately 10 g of non-inoculated cornmeal-sand was mixed in each control treatment pot. There were four treatments; pots with cornmeal-sand soil infested with *M. phaseolina* (Mp), pots with cornmeal- sand soil infested with *F. solani* (Fso), pots with cornmeal-sand soil infested with both fungi (Fso+Mp), and pots with non-infested cornmeal-sand soil (Control). One vine cutting of sweetpotato cultivar Beauregard was transplanted into each pot.

Sweetpotato plants and/or storage roots were sampled four times during the first year of this experiment (2011), at three developmental stages prior to harvest and at harvest. According to Villordon and his colleagues (2009), sweetpotato plants have three developmental stages (SR1, SR2, and SR3) (Figure 17). SR1 is when there is a minimum of one adventitious root (minimum length = 0.5 cm) in each of at least 50% of transplants. SR2 is when anomalous cambium is observed in a minimum of one adventitious root in each of at least 50% of transplants. And SR3 is the presence of a minimum of one visible storage root

(adventitious root with visible localized thickening, minimum of 0.5 cm at its widest section) in each of at least 50% of plants.

Modified from A. Villordon *et al*. 2009

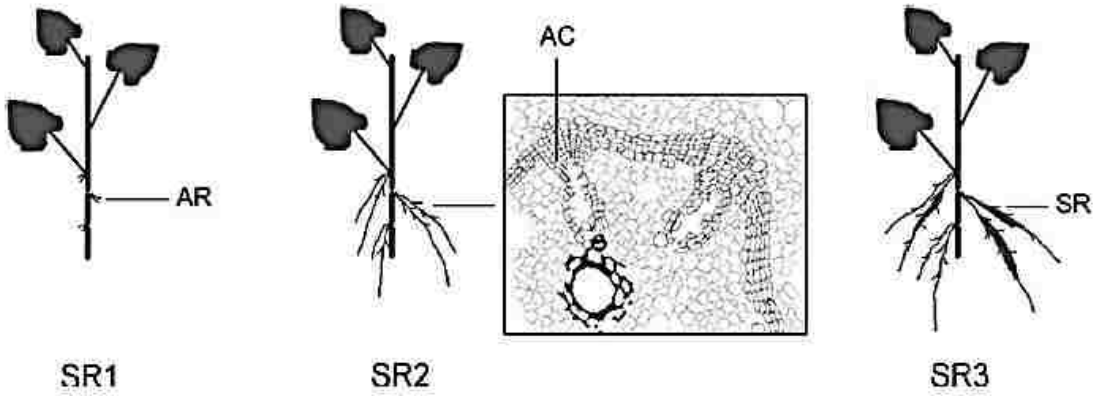

Figure 17 - Phenology scheme for describing morpho-anatomical features related to storage root initiation. Development stages SR1, SR2, and SR3. AC= anomalous cambium, AR= adventitious root, SR= storage root.

The first sampling was done at SR1, 5-10 days after transplanting (DAT) to determine if the pathogens are able to penetrate the plant in the first week after transplanting. It is possible that the wounds on the transplants created during cutting might provide an avenue for ingress of these microorganisms. The plants were sampled above and below the soil line, and 5 cm long segments were used for isolations as described below. The second isolations were sampled at 15-20 DAT, at SR2 when cracks formed on the adventitious roots by the emergence of lateral roots during this developmental stage might provide a natural entrance for the fungi. In addition to the isolations done as in the SR1, isolations were also attempted from the adventitious roots. The third sampling was attempted on plants at SR3, at 30-35 DAT to investigate the possibility that the fungi may enter storage roots through lateral adventitious roots. The isolation procedure was attempted in the same manner as for the SR2 plus isolations of one of the SR formed per plant. The fourth sampling was attempted at harvest

and isolations were done as for the third sampling. In the second year (2012), samples were only collected at one week after transplanting and at harvesting. The stems, pencil roots, and the storage roots were sampled as described below. In total, there were 10 plants sampled per each treatment in each sampling time and the remaining storage roots from the four samplings were kept in paper bags, stored at 15^{o}C, and analyzed after three months for end rot symptom development. Percent of storage roots infected with each of the pathogens was determined.

3.2.4. Isolation Procedures

Isolations were attempted from different areas of the sweetpotato vines including the pencil roots, storage roots, the portion of the vine located below the soil line, and the portion of the vine from the soil line up to five cm above the soil line. The pencil roots from each plant were chopped into small pieces and placed on each of the selective media. One storage root was selected from each plant, split in half longitudinally, and transverse strips were taken from both ends (distal and proximal) and from the center of the storage root and placed on each of the selective media. Segments from the vine of each plant were split in half, and the two parts were placed on each of selective medium with the split side facing the media. All plant materials for isolations were surface disinfested in 1% sodium hypochlorite for 10 minutes and placed on two different media: Peptone PCNB Agar (PPA) medium selective for *Fusarium* species first described by Snyder and Hansen (1941) with modifications by Nelson and colleagues (1983) and RB medium (Cloud and Rupe, 1991) selective medium for *M. phaseolina*. After growing on the specific selective medium, fungal mycelia were transferred to PDA, hyphal tips were transferred to acquire single isolates, and the isolates were kept on PDA for further evaluations.

3.2.5. DNA Extraction, Amplification, and Sequencing

In preparation for DNA extraction, isolates were grown first on PDA plates for seven days at 28°C in the dark. Approximately 100 mg of fresh mycelia was scraped from plates, placed in 1.5 ml microfuge tube, macerated thoroughly with a micro-pestle, and allowed to stand for 6-10 min. DNA extraction and purification was performed using the GenElute™ Plant Genomic DNA Miniprep Kit following the manufacturer's protocol (Sigma-Aldrich Co. St. Louis, MO, USA). Extracted DNA was quantified using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE-USA) and samples in the range 1.8-2.0 ratio of absorbance at 260 and 280 nm were used as a template in the PCR reactions.

PCR reactions were done in a reaction mixture containing 5 µl of 5 X thermophilic DNA buffer (Promega, Madison, WI-USA), 0.5 µl of 10 mM deoxyribonucleoside triphosphates (dNTPSs), 0.25 µl of Taq polymerase (0.5 U/µl), 3.33 µl of 25 mM $MgCl_2$, 1.25 µl of 10 mM of each primer, 1.0 µl of the 100 ng/µl of DNA template, and 12.42 µl of SDW. The PCR program included an initial denaturation period of 95°C for 2 min, 35 cycles of 95°C for 45 sec, 53°C for 1 min, 72°C for 30 sec, and a final extension period of 72°C for 5 min.

Amplicons were purified with QIAGEN's QIAquick® PCR Purification Kit following the manufacturer's protocol (Qiagen Inc., Valencia, CA) and sequenced by Eton Bioscience (Eton Bioscience Inc., Durham, NC - USA). The Eton Bioscience procedure involved adjusting DNA sample concentration to 60 ng in 6 µl of deionized water per reaction. Then, 10 pmols of primer, 6 µl of DNA sample, 8 µl of big dye v. 3.1, and 6 µl of deionized water were added in each well of a 96-well plate and placed on a Veriti™ Thermal Cycler (Applied Biosystems,

Foster City, CA – USA). The cycle sequencing included an initial denaturation period of 95°C for 1 min, 30 cycles of rapid thermal ramp to 95°C, 95°C for 10 sec, rapid thermal ramp to 50°C, 50°C for 5 sec, rapid thermal ramp to 60°C, 60°C for 4 min, rapid thermal ramp to 4°C, and 4°C for 5 min. Finally, the samples were purified, vortexed, and loaded on ABI automated 3730xl sequencers (Applied Biosystems, Foster City, CA – USA), which translate the fluorescent signals into their corresponding base pair sequence.

3.2.6. Isolate Identification

Fusarium isolates from the experimental samples were identified based on morphological characters on PDA and on carnation leaf agar (CLA) following identification keys (Leslie and Summerell, 2006; Nelson *et al.*, 1983). The identification was confirmed by comparing partial translation elongation factor 1-alpha (TEF) sequences with the FUSARIUM-ID database (Geiser *et al.*, 2004). The set of primers ef1 (5'-ATG GGT AAG GA(A/G) GAC AAG AC-3') and ef2 (5'-GGA (G/A)GT ACC AGT (G/C)AT CAT GTT-3') (O'Donnell *et al.*, 1998) were used for the partial gene amplification and sequencing. The partial TEF sequence of isolate M-10, used in the inoculations, was deposited in the GenBank database (Accession number JX945169).

Isolates of *M. phaseolina* were identified by morphological characters on PDA (Barnett and Hunter, 1998) and confirmed by comparing the partial internal transcribed spacer region (ITS) sequences with the GenBank database using BLASTn (Altschul *et al.*, 1990) at the National Center for Biotechnology Information website (http://ncbi.nlm.nih.gov/blast). The amplification and sequencing of the partial ITS region were performed using the primer set ITS1-(5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS4-(5´TCC TCC GCT TAT TGA TAT GC

3´) (White *et al.*, 1990). The partial ITS sequences of the isolate CK-7, used in the inoculations, was deposited in the GenBank database (Accession number JX945170).

3.2.7. Pathogenicity Test

All fungal isolates obtained from parts of the plants and storage roots were tested for pathogenicity in slices of sweetpotato storage roots. Whole asymptomatic sweetpotato storage roots were washed with tap water, peeled, and surface disinfested with 1% NaOCl for 10 minutes. 1 cm thick slices were aseptically cut from those roots and individually placed on top of moistened filter paper that was placed individually in sterile glass Petri plates. Then, in each slice, a 0.5 cm diameter agar plug from a 24-hr-old fungal colony growing on PDA was placed on the center of the sweetpotato slice, incubated for two weeks in dark at 28°C, and evaluated for necrosis development on the slice.

3.2.8. Statistical Analyses

All statistical analyses were performed using PROC GLIMMIX for generalized linear mixed models in SAS (version 9.3; Copyright(c) 2002-2010 by SAS Institute Inc., Cary, NC, USA). The data was arranged in binary distribution. Pairwise treatment comparisons were conducted on the independent variables using LSMEANS comparison, and adjustments were performed using Tukey's honest significant difference (Tukey, 1949).

In both experiments, data for each experimental unit consisted of binary values from each isolation site. Data was analyzed as a complete randomized design, with treatments consisting of toothpick inoculation type or soil infestation type. The independent variables were treatment, isolation sites, and time for the infested soil experiment, and isolation sites for

the toothpick inoculation experiment. The response variables were the fungi isolated: *F. solani, M. phaseolina,* and others (other *Fusarium* species).

To evaluate the incidence of storage roots with end rot infected by each fungus, each storage root was used as an experimental unit and the presence or absence of each fungus was determined in a binary distribution. The independent variables were the treatments and response variables were the fungi isolated from rotting storage roots (*F. solani, M. phaseolina,* and other fungi).

3.3. RESULTS:

3.3.1. Toothpick Inoculation

Both *F. solani* and *M. phaseolina* were recovered from inoculated plants but not from those not inoculated. The percentage of recovery of each fungus did not differ from treatments where they were inoculated alone or where both fungi were inoculated together (Fso+Mp). However, *F. solani* was recovered over 70% of the time while the recovery of *M. phaseolina* was below 53% in both years (Table 4). Interestingly, other *Fusarium* species (Others) were found in all treatments (Table 5), except in plants inoculated with *F. solani* alone in 2012 (Table 4). Also, the incidence of the other *Fusarium* species was lower in plants inoculated with *F. solani*, either alone or with *M. phaseolina*, and higher in plants inoculated with *M. phaseolina* alone (Table 4).

TABLE 4. Toothpick inoculation experiment. Percentage of fungi recovered from sweetpotato plants in each treatment.

Treatment[z]	2011			2012		
	Fungi Isolated[y]			Fungi Isolated[y]		
	F. solani	*M. phaseolina*	Others	*F. solani*	*M. phaseolina*	Others
Control	0 b*	0 b	28 b	0 b	0 b	30 b
Fso	96 a	0 b	20 b	86 a	0 b	0 c
Mp	0 b	28 a	64 a	0 b	52 a	60 a
Fso+Mp	98 a	16 a	20 b	74 a	36 a	2 c
F Value	1060.09	10.81	11.50	135.18	28.09	33.22
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

[z] Plants inoculated with *Fusarium solani* (Fso), plants inoculated with *Macrophomina phaseolina* (Mp), plants inoculated with *Fusarium solani* and *Macrophomina phaseolina* (Fso+Mp), and non-inoculated plants (Control).
[y] Fso (*Fusarium solani*), Mp (*Macrophomina phaseolina*), and Others (other *Fusarium* species).
* Conservative Tukey-Kramer Grouping for Treatment Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE 5. Number of isolates of other *Fusarium* species recovered from different isolation sites[y] in each experiment.

Fusarium species	Toothpick Inoculation Experiment					Soil Infestation Experiment				
	Top	Soil Line	Bottom	Roots	Storage Roots	Top	Soil Line	Bottom	Roots	Storage Roots
F. commune	0	0	0	0	0	3	2	3	0	0
F. concentricum	0	0	0	0	0	0	0	0	0	1
F. lateritium	0	1	1	1	0	0	0	0	0	0
F. oxysporum	2	3	2	2	9	5	7	9	12	1
F. pallidoroseum	8	10	8	13	21	6	6	5	8	0
F. proliferatum	0	0	0	0	0	18	21	22	16	13
*F. solani**	5	7	5	11	13	21	18	24	20	12

[y]Top (stem 5 cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.
*Did not cause any significant rotting in slices of storage roots.

F. solani was recovered from all parts of the plants of the plant tested (isolation sites) in both years, and the rate of recovery did not differ among the isolation sites within year (Table 6). On the other hand, *M. phaseolina* was recovered mainly from the stem near the soil line in

both years. With the exception of the bottom region in year the 2012, *M. phaseolina* was not recovered or recovered at a low rate in the isolation sites above and below the soil line (Table 6). Other *Fusarium* species were isolated from all isolation sites in both years, and they were found in a higher percentage in the storage roots in 2011 (Table 6).

TABLE 6. Toothpick inoculation experiment. Percentage of fungi isolated from different isolation sites in sweetpotato plants.

Isolation Site[z]	2011			2012		
	Fungi Isolated[y]			Fungi Isolated[y]		
	F. solani	*M. phaseolina*	Others	*F. solani*	*M. phaseolina*	Others
Top	50.0 a*	10.0 b	15.0 b	32.5 a	12.5 bc	22.5 a
Soil Line (inoculation site)	50.0 a	37.5 a	25.0 b	37.5 a	45.0 a	27.5 a
Bottom	50.0 a	7.5 b	12.5 b	45.0 a	30.0 ab	27.5 a
Roots	47.5 a	0.0 b	37.5 b	45.0 a	17.5 bc	30.0 a
Storage Roots	45.0 a	0.0 b	75.0 a	40.0 a	5.0 c	7.5 a
F Value	0.0800	11.8500	14.9300	0.4600	6.3800	1.8900
Pr>F	0.9889	<.0001	<.0001	0.7640	<.0001	0.1142

[z] Top (stem 5 cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.
[y] Fso (*Fusarium solani*), Mp (*Macrophomina phaseolina*), and Others (other *Fusarium* species).
* Conservative Tukey-Kramer Grouping for Isolation Site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

There was not a significant difference in recovery rate of *F. solani* between isolation sites within treatment in each year (Table 7 A and B). The highest recovery rate of *M. phaseolina* was from the soil line (the inoculation site) and the lowest was from storage roots followed by roots in the treatments *M. phaseolina* and Fso+Mp in both years (Table 7 A and B). Other *Fusarium* species were frequently isolated from storage roots in all treatments in 2011 (Table 7 A). However, this pattern was not observed in 2012 where their distribution was similar among all isolation sites (Table 7 B).

TABLE 7. Toothpick inoculation experiment. Percent recovery of fungi from different isolation sites in each treatment in 2011 **(A)** and 2012 **(B)**.

A

Treatment[z] / Site of Isolation[y]	Fungi Isolated											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Top	0 b*	100 a	0 b	100 a	0 c	0 c	10 c	30 bc	10 c	0 c	50 abc	0 c
Soil Line	0 b	100 a	0 b	100 a	0 c	0 c	100 a	50 b	30 bc	0 c	70 ab	0 c
Bottom	0 b	100 a	0 b	100 a	0 c	0 c	30 bc	0 c	0 c	0 c	50 abc	0 c
Roots	0 b	90 a	0 b	100 a	0 c	0 c	0 c	0 c	50 abc	10 c	80 ab	10 c
Storage Roots	0 b	90 a	0 b	90 a	0 c	0 c	0 c	0 c	50 abc	90 a	70 ab	90 a
F Value	0.67				10.60				3.23			
Pr>F	0.7818				<0.0001				0.0003			

B

Treatment[z] / Site of Isolation[y]	Fungi Isolated											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Top	0 b*	70 a	0 b	60 a	0 d	0 d	30 cd	20 cd	30 abc	0 c	60 ab	0 c
Soil Line	0 b	90 a	0 b	60 a	0 d	0 d	100 a	80 ab	40 abc	0 c	70 a	0 c
Bottom	0 b	90 a	0 b	90 a	0 d	0 d	60 abc	60 abc	40 abc	0 c	70 a	0 c
Roots	0 b	90 a	0 b	90 a	0 d	0 d	50 bc	20 cd	40 abc	0 c	70 a	10 bc
Storage Roots	0 b	90 a	0 b	70 a	0 d	0 d	20 cd	0 d	0 c	0 c	30 abc	0 c
F Value	0.82				4.02				0.83			
Pr>F	0.6267				<0.0001				0.6209			

[y] Top (stem 5 cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.

[z] Plants inoculated with *Fusarium solani* (Fso), plants inoculated with *Macrophomina phaseolina* (Mp), plants inoculated with *Fusarium solani* and *Macrophomina phaseolina* (Fso+Mp), and non-inoculated plants (Control).

* Means with the same letter within the same column are not significantly different by the Conservative Tukey-Kramer Grouping for Isolation Site*Treatment Least Squares Means (Alpha=0.05).

Storage roots with end rot symptoms were found in over 30% of the storage roots from all treatments in both years, after three months in storage. However, more rotting storage roots were observed in 2011 than in the 2012 in all treatments and there was no statistical difference between treatments within a year, for both years (Figure 18 A and B). Also, neither *F. solani* nor *M. phaseolina* were isolated from rotting roots from the control treatment in the years (Table 8). *F. solani* was isolated more often from Fso treatment than rotting storage roots from Fso+Mp treatment in 2011; however, this difference was not statistically significant. In 2012, *F. solani* was isolated from 20% of the rotting roots in both treatments. However, this percentage was not statistically different from treatments were *F. solani* was not isolated, control and Mp (Table 8). *M. phaseolina* was isolated from rotting storage roots from Mp and Fso+Mp treatments in both years. Nevertheless, these isolation rates were not statistically significant from the failure to isolate *M. phaseolina* from the control or Fso treatments (Table 8). Furthermore, *F. pallidoroseum, F. concentricum, F. proliferatum,* and *Aspergillus* spp. (Other Fungi) were frequently isolated from rotting storage roots from all treatments in both years (Table 8). However, these fungi failed to cause any significant necrosis or decay in sweetpotato storage root slices in the pathogenicity test (data not shown).

3.3.2. Soil Infestation

F. solani was frequently recovered from Fso and Fso+Mp treatments in both years (Table 9). In 2011, *F. solani* recovery was significantly greater from Fso treatment than from Fso+Mp (Table 9). However, this trend was not observed in 2012 (Table 9).

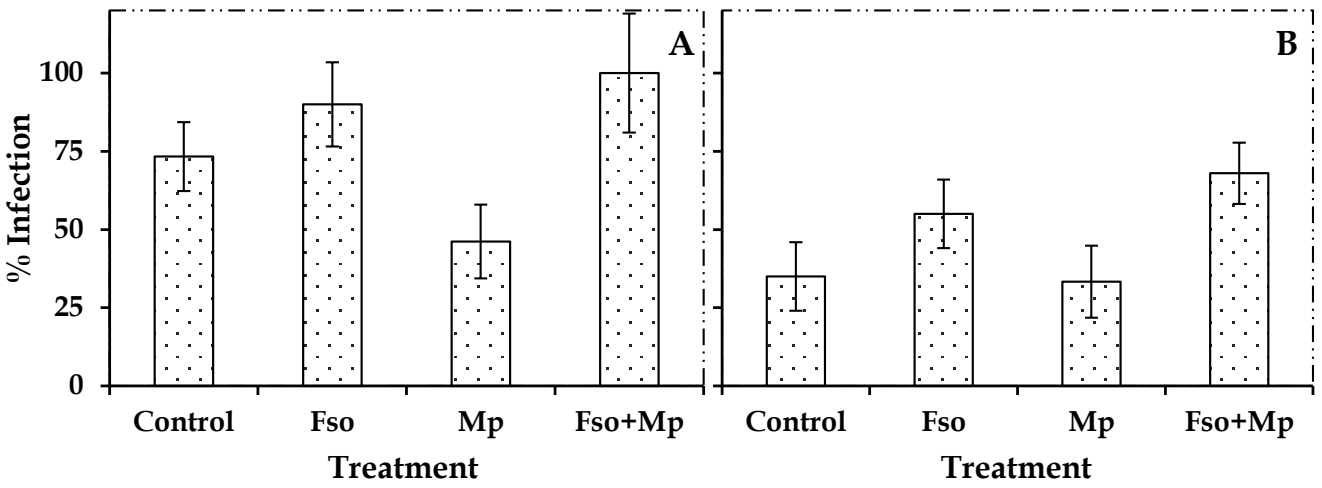

Figure 18 - Toothpick inoculation experiment**,** 2011 **(A)** and 2012 **(B).** Total percentage of sweetpotato storage roots with end rot symptoms in each treatment [plants inoculated with *F. solani* (Fso), plants inoculated with *M. phaseolina* (Mp), plants inoculated with *F. solani* and *M. phaseolina* (Fso+Mp), and non-inoculated plants (Control)]. Conservative Tukey-Kramer Grouping for Treatments Least Squares Means (Alpha=0.05). LS-means within year were not significantly different.

TABLE 8. Toothpick inoculation experiment. Percentage of fungi recovered from sweetpotato storage roots with end rot symptoms from each treatment.

Treatment[z]	2011			2012		
	Fungi Isolated			Fungi Isolated		
	F. solani	***M. phaseolina***	**Other Fungi**	***F. solani***	***M. phaseolina***	**Other Fungi**
Control	0.0 b*	0.0 a	73.3 a	0.0 a	0.0 a	35.0 a
Fso	70.0 a	0.0 a	20.0 b	20.0 a	0.0 a	35.0 a
Mp	0.0 b	7.7 a	38.5 ab	0.0 a	5.6 a	27.8 a
Fso+Mp	40.0 ab	20.0 a	40.0 ab	20.0 a	8.0 a	40.0 a
F Value	15.03	1.39	2.78	3.01	1.01	0.22
Pr>F	<.0001	0.2610	0.0535	0.0349	0.3915	0.8822

[z]Plants inoculated with *F. solani* (Fso), plants inoculated with *M. phaseolina* (Mp), plants inoculated with *F. solani* and *M. phaseolina* (Fso+Mp), and non-inoculated plants (Control).
*Conservative Tukey-Kramer Grouping for Isolated Fungi Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

Percent recovery of *M. phaseolina* was not significantly different between the treatments Mp and Fso+Mp in 2011 (Table 9), whereas in 2012 its recovery was significantly greater from the plants inoculated with *M. phaseolina* alone than combined inoculation with *F. solani* (Table 9). Additionally, *M. phaseolina* was found in about 10% of the plants from the Fso treatment, which was not significantly different from the failure to isolate it from the control plants (Table 9). Others were found in all treatments in both years and were isolated at significantly different rates among treatments within each year (Table 9).

TABLE 9. Soil infestation experiment. Percentage of fungi recovered from sweetpotato plants in each treatment.

Treatment[z]	2011			2012		
	Fungi Isolated			Fungi Isolated		
	F. solani	***M. phaseolina***	**Others**	***F. solani***	***M. phaseolina***	**Others**
Control	0.0 c*	0.0 b	25.6 b	0.0 b	0.0 c	53.3 a
Fso	92.2 a	0.0 b	1.1 c	94.4 a	2.2 c	3.3 b
Mp	0.6 c	57.7 a	47.2 a	0.0 b	75.6 a	10.0 b
Fso+Mp	79.4 b	55.0 a	4.4 c	95.6 a	53.3 b	51.1 a
F Value	734.34	154.61	66.33	1128.14	111.04	40.13
Pr>F	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

[z] Soil infested with *F. solani* (Fso), soil infested with *M. phaseolina* (Mp), soil infested with *F. solani* and *M. phaseolina* (Fso+Mp), and non-infested soil (Control).
*Conservative Tukey-Kramer Grouping for Treatments Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

Except for a lower frequency of isolation from storage roots in 2011, the rate of *F. solani* recovery was not statistically different among isolation sites (Table 10). In contrast, *M. phaseolina* was recovered at higher frequency at isolation sites located in the infested soil in both years (Table 10). Others were isolated from all parts of the plants and their isolation rate was not significantly different among isolation sites (Table 10).

TABLE 10. Soil infestation experiment. Percentage of fungi isolated from different isolation sites in sweetpotato plants.

Isolation Site[z]	2011			2012		
	Fungi Isolated			Fungi Isolated		
	F. solani	*M. phaseolina*	Others	*F. solani*	*M. phaseolina*	Others
Top	43.1 a*	16.3 c	16.9 a	41.3 a	16.3 b	25.0 a
Soil Line	42.5 a	26.9 bc	19.4 a	48.8 a	33.8 ab	25.0 a
Bottom	48.8 a	36.3 ab	23.1 a	50.0 a	41.3 a	32.5 a
Roots	50.0 a	41.9 a	20.0 a	48.8 a	41.3 a	30.0 a
Storage Roots	18.8 b	11.3 c	17.5 a	50.0 a	30.0 ab	40.0 a
F Value	6.31	11.26	0.56	0.41	3.94	1.01
Pr>F	<.0001	<.0001	0.6893	0.8017	0.0038	0.4039

[z]Top (stem 5 cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.
* Conservative Tukey-Kramer Grouping for Isolation Site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

Although recovery rates of *F. solani*, *M. phaseolina*, and Others differed slightly but significantly among sampling times in 2011, they did not significantly increase from week 1 to harvest (Table 11). In 2012, *F. solani* was recovered at the same rate in week 1 and at harvest, while the recoveries of *M. phaseolina* and Others increased significantly from week 1 to harvest (Table 11).

While *M. phaseolina* was recovered less frequently from the storage roots in 2011, the analysis of the interaction effects between treatments and isolation sites revealed a tendency of *M. phaseolina* to be isolated from parts of the plants that were in contact with the infested soil (bottom, roots, and storage roots). On the other hand, the distribution of *F. solani* and Others were scattered in the plant (Table 12 A and B).

TABLE 11. Soil infestation experiment. Percentage of fungi recovered from sweetpotato plants in each sampling time.

Sampling Time[z]	2011			2012		
	Fungi Isolated			Fungi Isolated		
	F. solani	*M. phaseolina*	Others	*F. solani*	*M. phaseolina*	Others
1º Week	46.9 ab*	27.5 ab	13.8 b	45.6 a	25.0 b	8.8 b
2º Week	50.0 a	32.5 ab	30.0 a	ND**	ND	ND
3º Week	34.0 b	20.5 b	8.5 b	ND	ND	ND
Harvest	43.5 ab	33.0 a	27.0 a	49.0 a	39.0 a	46.0 a
F Value	3.64	3.24	12.95	0.40	8.04	70.70
Pr>F	0.0127	0.0218	<.0001	0.5253	0.0048	<.0001

[z] Week 1 (1 week after the planting day), week 2 (2 weeks after the planting day), week 3 (3 weeks after the planting day), and harvest (at harvest day).
* Conservative Tukey-Kramer Grouping for each Sampling Time Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.
** Non-determined, plants were not sampled at these times.

There was a statistical difference in the recovery rate of *F. solani*, *M. phaseolina*, and Others between sampling times in each treatment within a year. However, the results were dispersed and they did not show a tendency to increase or decrease the recovery rate of these fungi with the age of the plant (sampling times) in each treatment (Table 13 A and B). Furthermore, the recovery rate of *F. solani*, *M. phaseolina*, and others did not differ statistically between site of isolation in each sampling time within a year (Table 14 A and B).

End rot disease developed in stored storage roots from all treatments in 2011 and 2012 (Figure 19 A and B). There was a higher incidence of this disease in storage roots from 2011 than from 2012 in all treatments and the disease incidence was significantly different among treatments in 2011, in which the Fso treatment had the highest incidence (Figure 19 A and B).

TABLE 12. Soil infestation experiment, percentage of fungi recovered from different isolation sites in each treatment in 2011 **(A)** and 2012 **(B)**.

A

Treatment[z] / Site of Isolation[y]	Percent Recovery											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Top	0 d*	98 a	0 d	75 b	0 d	0 d	35 c	30 cd	20 cde	0 e	43 abc	5 de
Soil Line	0 d	98 a	0 d	73 b	0 d	0 d	70 b	38 c	23 bcde	0 e	50 ab	5 de
Bottom	0 d	100 a	3 d	93 a	0 d	0 d	73 ab	73 ab	30 abcde	0 e	58 a	5 de
Roots	0 d	100 a	0 d	100 a	0 d	0 d	73 ab	95 a	23 bcde	3 e	50 ab	5 de
Storage Roots	0 d	40 c	0 d	35 c	0 d	0 d	20 cde	25 cde	40 abcd	5 de	25 abcde	0 e
F Value	12.91				10.09				1.35			
Pr>F	<0.0001				<0.0001				0.1863			

B

Treatment[z] / Site of Isolation[y]	Percent Recovery											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Top	0 c*	85 ab	0 c	80 b	0 d	0 d	40 dc	25 d	40 bc	0 c	10 bc	50 ab
Soil Line	0 c	95 a	0 c	100 a	0 d	0 d	85 ab	50 bcd	40 bc	0 c	5 c	55 ab
Bottom	0 c	100 a	0 c	100 a	0 d	0 d	95 a	70 abc	60 ab	0 c	15 bc	55 ab
Roots	0 c	95 a	0 c	100 a	0 d	10 d	85 ab	70 abc	50 ab	10 bc	10 bc	50 ab
Storage Roots	0 c	100 a	0 c	100 a	0 d	0 d	70 abc	50 bcd	100 a	10 bc	10 bc	40 bc
F Value	1.78				2.64				1.43			
Pr>F	0.0493				0.0022				0.1489			

[y]Top (stem 5cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.

[z]Soil infested with *F. solani* (Fso), soil infested with *M. phaseolina* (Mp), soil infested with *F. solani* and *M. phaseolina* (Fso+Mp), and non-infested soil (Control).

*Means with the same letter within the same column are not significantly different by the Conservative Tukey-Kramer Grouping for Isolation Site*Treatment Least Squares Means (Alpha=0.05).

TABLE 13. Soil infestation experiment, percentage of fungi recovered from different sampling times in each treatment in 2011 **(A)** and 2012 **(B)**.

A

Treatment[Z] / Time of Sampling[Y]	Percentage of Recovery											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Week 1	0 d*	100 a	3 d	85 ab	0 c	0 c	48 ab	63 a	8 cd	0 d	48 ab	0 d
Week 2	0 d	100 a	0 d	100 a	0 c	0 c	60 a	70 a	33 bc	3 d	65 a	20 cd
Week 3	0 d	80 b	0 d	56 c	0 c	0 c	52 ab	30 b	12 cd	2 d	20 cd	0 d
Harvest	0 d	92 ab	0 d	82 b	0 c	0 c	70 a	62 a	48 ab	0 d	60 a	0 d
F Value	6.67				3.69				6.27			
Pr>F	<.0001				0.0002				<.0001			

B

Treatment[Z] / Time of Sampling[X]	Percentage of Recovery											
	F. solani				*M. phaseolina*				Others			
	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp	Control	Fso	Mp	Fso+Mp
Week 1	0 c*	93 ab	0 c	90 b	0 c	0 c	73 a	28 b	18 b	0 b	13 b	5 b
Harvest	0 c	96 ab	0 c	100 a	0 c	4 c	78 a	74 a	82 a	6 b	8 b	88 a
F Value	2.10				10.30				46.41			
Pr>F	0.1004				<.0001				<.0001			

[Y]Week 1 (1 week after the planting day), week 2 (2 weeks after the planting day), week 3 (3 weeks after the planting day), and harvest (at harvest day).

[X]Week 1 (1 week after the planting day) and harvest (at harvest day).

[Z] Soil infested with *F. solani* (Fso), soil infested with *M. phaseolina* (Mp), soil infested with *F. solani* and *M. phaseolina* (Fso+Mp), and non-infested soil (Control).

*Means with the same letter within the same column are not significantly different by the Conservative Tukey-Kramer Grouping for Sampling Time*Treatment Least Squares Means (Alpha=0.05).

TABLE 14. Soil infestation experiment, percentage of fungi recovered from different isolation sites in each sampling time in 2011 **(A)** and 2012 **(B)**.

A

Sampling Time[Z] / Site of Isolation[Y]	Percent Recovery											
	F. solani				*M. phaseolina*				Others			
	Week 1	Week 2	Week 3	Harvest	Week 1	Week 2	Week 3	Harvest	Week 1	Week 2	Week 3	Harvest
Top	48 a*	50 a	38 ab	38 ab	23 abc	13 abc	10 bc	20 abc	13 a	25 a	8 a	23 a
Soil Line	38 ab	50 a	40 a	43 a	15 abc	38 abc	18 abc	38 abc	15 a	28 a	10 a	25 a
Bottom	53 a	50 a	43 a	50 a	28 abc	40 ab	33 abc	45 a	20 a	33 a	10 a	30 a
Roots	50 a	50 a	50 a	50 a	45 a	40 ab	38 abc	45 a	8 a	35 a	8 a	30 a
Storage Roots	ND	ND	0 b	38 ab	ND	ND	5 c	18 abc	ND	ND	8 a	28 a
F Value	1.08				0.88				0.30			
Pr>F	0.3714				0.5489				0.9810			

A

Sampling Time[Z] / Site of Isolation[Y]	Percent Recovery					
	F. solani		*M. phaseolina*		Others	
	Week 1	Harvest	Week 1	Harvest	Week 1	Harvest
Top	35 a*	48 a	5 b	28 ab	5 c	45 ab
Soil Line	50 a	48 a	30 ab	38 a	5 c	45 ab
Bottom	50 a	50 a	35 ab	48 a	18 bc	48 a
Roots	48 a	50 a	30 ab	53 a	8 c	53 a
Storage Roots	ND	50 a	ND	30 ab	ND	40 ab
F Value	0.34		0.54		0.45	
Pr>F	0.7958		0.6570		0.7172	

ND = Not determined, storage roots were not formed yet at this times.

[y] Top (stem 5 cm above the soil line), soil line (stem at the soil line), bottom (stem 5 cm below the soil line), roots (pencil roots), and storage roots.

[z] Week 1 (1 week after the planting day), week 2 (2 weeks after the planting day), week 3 (3 weeks after the planting day), and harvest (at harvest day).

[x] Week 1 (1 week after the planting day) and harvest (at harvest day).

[*] Means with the same letter within the same column are not significantly different by the Conservative Tukey-Kramer Grouping for Isolation Site*Sampling Time Least Squares Means (Alpha=0.05).

Neither *F. solani* nor *M. phaseolina* were isolated from rotting roots from the control treatment in either year (Table 15). The isolation rate of *F. solani* was significantly higher from rotting storage roots from the Fso treatment than rotting storage roots from the Fso+Mp treatment in both years. Furthermore, the isolation rate of *F. solani* was much higher in 2011 than in 2012 in both treatments, Fso and Fso+Mp (Table 15). Even though the recovery of *M. phaseolina* did not differ statistically between treatments in 2012, this fungus was isolated slightly more often from rotting storage roots from the Mp treatment than from rotting roots from the Fso+Mp treatment, in 2011 (Table 15). Additionally, *F. pallidoroseum, F. concentricum,* and *Aspergillus* spp. were frequently isolated from rotting storage roots from all treatments in both years (Table 15), but they did not cause necrosis or decay on sweetpotato slices in the pathogenicity test (data not shown).

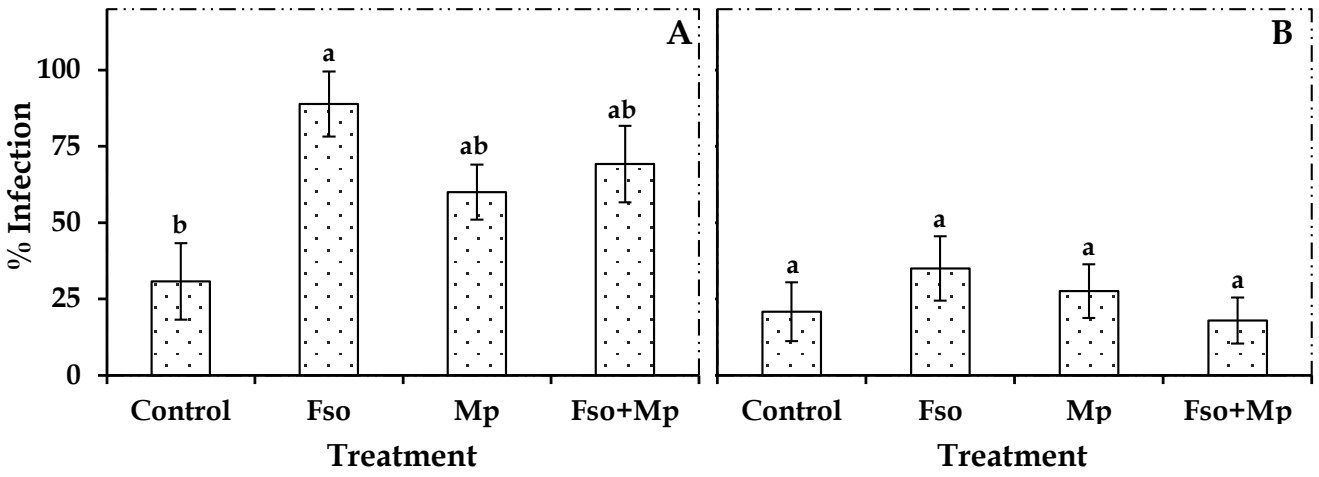

Figure 19 - Soil infestation experiment**,** 2011 **(A)** and 2012 **(B).** Total percentage of sweetpotato storage roots presenting end rot symptoms in each treatment [soil infested with *F. solani* (Fso), soil infested with *M. phaseolina* (Mp), soil infested with *F. solani* and *M. phaseolina* (Fso+Mp), and non- soil infested (Control)]. Conservative Tukey-Kramer Grouping for Treatments Least Squares Means (Alpha=0.05). LS-means with the same letter for the same treatment within year are not significantly different.

TABLE 15. Soil infestation experiment. Percentage of fungi recovery from sweetpotato storage roots developing end rot in each treatment.

Treatment[z]	2011			2012		
	Fungi Isolated[y]			Fungi Isolated[y]		
	F. solani	*M. phaseolina*	Other Fungi	*F. solani*	*M. phaseolina*	Other Fungi
Control	0 c*	0 b	31 a	0 b	0 a	21 a
Fso	89 a	0 b	0 a	25 a	0 a	10 a
Mp	0 c	42 a	15 a	0 b	10 a	18 a
Fso+Mp	39 b	31 ab	0 a	13 ab	0 a	6 a
F Value	44.62	6.44	3.33	4.43	3.08	1.39
Pr>F	<.0001	0.0007	0.0247	0.0056	0.0306	0.2489

[z]Soil infested with *F. solani* (Fso), soil infested with *M. phaseolina* (Mp), soil infested with *F. solani* and *M. phaseolina* (Fso+Mp), and non- infested soil (Control).
*Conservative Tukey-Kramer Grouping for Isolated Fungi Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

3.4. DISCUSSION:

The results of this study provide insight into how *F. solani* and *M. phaseolina* invade sweetpotato plants and storage roots, adding information crucial for developing strategies for end rot disease complex management. The outcomes from toothpick inoculation experiments make clear that both fungi are capable of moving from the vines of the plant to storage roots. This finding has important implications in controlling initial infection of these fungi; since the propagation material, sweetpotato vine cuttings, can be infected with them prior to planting and they could move to storage roots during the natural development of the plant, potentially causing subsequent end rot. Therefore, using propagation plant material free of those pathogens could be an efficient way to reduce the initial inoculum concentration and consequently reducing disease incidence.

It was also found that both fungi infected sweetpotato plants and storage roots from infested soil as early as the first week after planting. This suggests that controlling the

population of these fungi in the soil by methods such as soil fumigation, would be an essential approach to reduce the incidence of latent pathogens inside of the storage roots, thus potentially reducing the occurrence of end rot infections. In another experiment, we found that soil fumigated with 1% Chloropicrin has a great potential to reduce the incidence of *M. phaseolina* in symptomless sweetpotato storage roots (Table A4). This is an indication that it is worthwhile investing some time to find out the best concentration of this compound to be used and also to test other chemical compounds for this purpose.

Likewise, our results make it evident that *F. solani* and *M. phaseolina* can be simultaneously present inside of sweetpotato plants and they do not seem to influence the spread of each other in plants whether they are inoculated together in the vines or infested together in the soil in comparison to treatments where they are introduced alone. This helps to explain why both pathogens have frequently been isolated at the same time from sweetpotato storage roots with end rots.

The higher recovery rate of *F. solani*, especially at sites distant to the site of inoculation, in relation to *M. phaseolina* in both experiments suggests that *F. solani* is more aggressive in colonizing sweetpotato plants than *M. phaseolina*. *F. solani* was commonly recovered from all parts of the plants in both experiments, while *M. phaseolina* was mostly recovered from the inoculation sites and from plant parts exposed to infested soil. Furthermore, the spread and penetration of these fungi into sweetpotato plants seems to happen fast, within the first week after transplanting, and they do not seem to be correlated with the three developmental stages of sweetpotato plants as first hypothesized. Furthermore, recovery rates did not significantly increase from the first week after inoculation until harvest.

The dramatically reduced frequency of isolation of other *Fusarium* species from treatments in which *F. solani* was present suggests that *F. solani* has some means of competitively colonizing the plant and reducing colonization by the other fusaria. In contrast, *M. phaseolina* seems to have a positive biological interaction with other *Fusarium* species as these species were isolated more often in some treatments where *M. phaseolina* was inoculated. Further inoculation studies in which the other *Fusarium* species are inoculated alone or simultaneously with *F. solani* or *M. phaseolina* should be conducted to test these interactions. Furthermore, the fact that *F. solani* is more aggressive at colonizing sweetpotato plants suggests that it might be more likely than *M. phaseolina* to induce end rots when environmental conditions favor their development.

Even though *F. oxysporum* and *F. pallidoroseum* (synonym *F. incarnatum*) have been found to cause surface rot in sweetpotato storage roots (Harter and Weimer, 1919; Martin and Person, 1951; Ray *et al.*, 1996), isolates of these species collected in this study did not cause significant symptoms on inoculated sweetpotato slices. The other isolates of *F. solani* also did not cause significant rot in inoculated slices (Figure 20). In addition, those *F. solani* isolates differed significantly from our standard isolate (M-10), used in this research. While M-10 colonies produced a blue to blue-green pigment, sparse aerial mycelia, abundant micro- and macroconidia in PDA, and aerial microconidia from long monophialides, matching the morphological characteristics described by (Clark, 1980); the isolates found in our studies had a cream color on the upper surface of PDA and also produce microconidia on mono- and polyphyalides (data not shown). The identification of these isolates was confirmed to be *F. solani* by comparing their TEF gene sequences with the FUSARIUM-ID database (Geiser *et al.*, 2004). A more in depth study should be conducted to evaluate the role of the endophytic

Fusarium species in sweetpotatoes and also to better characterize those isolates that are members of the *F. solani* polytypic species complex (VanEtten and Kistler, 1988) now known as *F. solani* species complex (FSSC) (O'Donnell, 2000). It is possible that many of these isolates, members of FSSC, found in sweetpotatoes can be characterized as new *formae speciales* or even as new species as discovered by Nalim and his colleagues (2011).

The hypothesis proposed by Clark (1980) that *F. solani* requires a wound for infection of storage roots, such as occur during harvest, was not supported by our studies as we discovered that this pathogen can be found inside of symptomless storage roots prior to harvest. Although not required, such harvest-related wounds, may still provide a major site of entrance for these pathogens since curing, which promotes healing of the wounds, is well known to reduce the incidence of end rots significantly (Artschwager and Starrett, 1931; Weimer and Harter, 1921). Furthermore, the presence of pathogens inside of symptomless storage roots does not necessarily mean that end rot disease will develop as many roots from inoculated plants failed to develop the disease.

Considering that other fungal species isolated from the rotting storage roots from our experiments failed to cause rotting symptoms in our pathogenicity tests, these fungi may be opportunistic pathogens that develop end rots only under certain environmental conditions. Storage roots stored for extended periods of three months or more, or those grown in greenhouse conditions, as well as those grown in flooded soils are more likely to develop end rot diseases (daSilva, unpublished data). End rot of sweetpotato storage roots may also be linked with physiological changes in the plant, as it has been shown that incidence of tip rot, a type of end rot, in Mississippi is associated with ethylene-induced stress and pre-harvest foliar applications of ethephon (Arancibia *et al.*, 2013). Likewise, pathogen genotypes, plant

cultivars, plant nutrition, storage roots desiccation, and storage conditions may all play a significant role in the development of end rot diseases in sweetpotato storage roots.

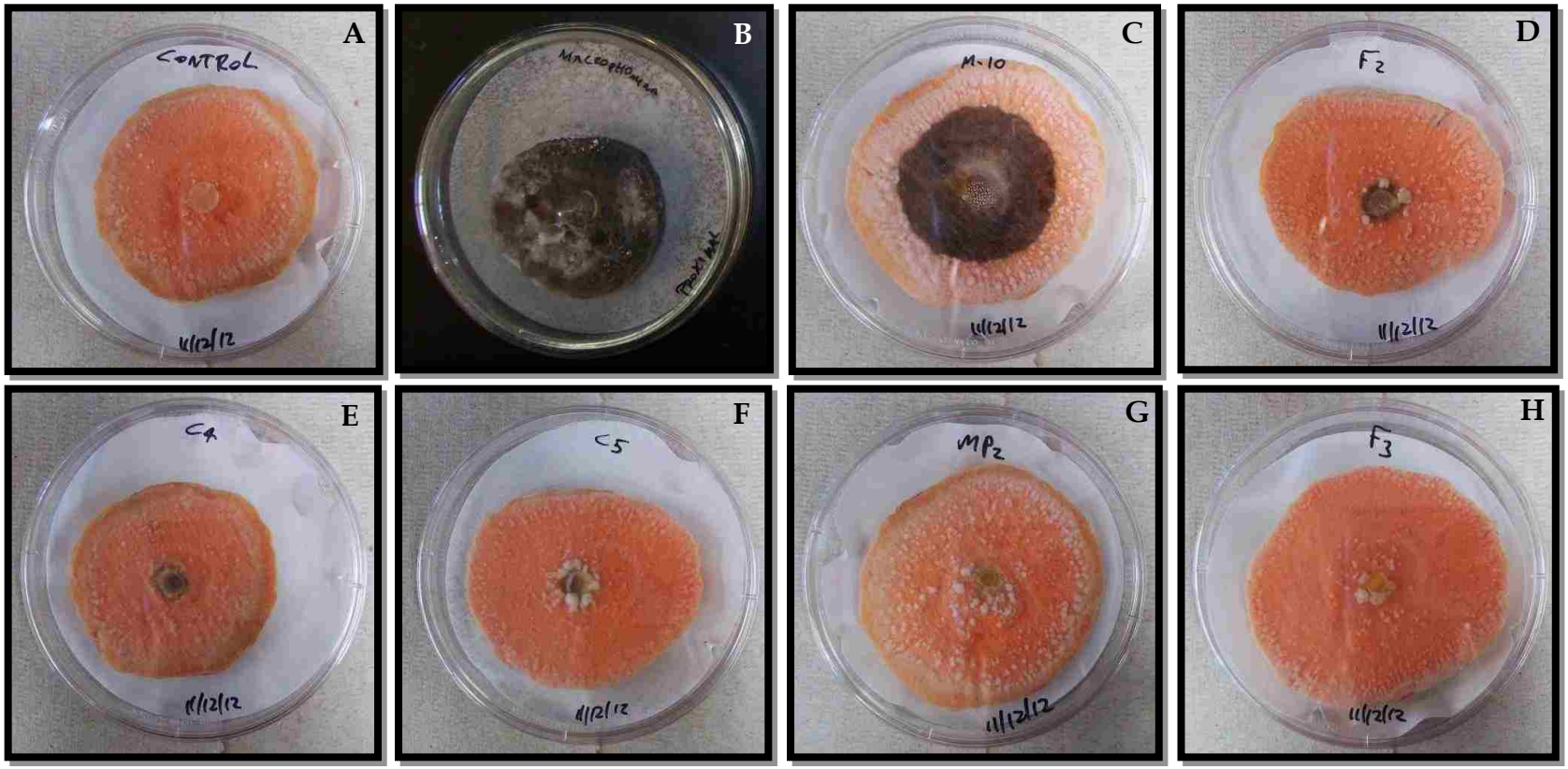

Figure 20 - Pathogenicity test. Slices of sweetpotato storage root inoculated with 0.5 cm diameter agar plugs: Sterile agar control (A), *M. phaseolina* isolate CK-7 (B), *F. solani* isolate M-10 (C), *F. solani* isolate F2 (D), *F. solani* isolate C4 (E), *F. solani* isolate C5 (F), *F. solani* isolate Mp2 (G), and *F. solani* isolate F3 (H). Plates were incubated in the dark for two weeks at 28°C.

CHAPTER 4: CONCLUSIONS

Flooding-associated bacterial soft rot and fungal end rots are among the main diseases that threaten sweetpotato production in the southern United States. Little is known about the etiology of these disease complexes making management of them difficult. Knowing the causal agent of these diseases and how those agents enter the sweetpotato storage roots are vital steps to maximize integrated control measures.

Two anaerobic bacteria were isolated from soft rotting sweetpotato storage roots growing in flooded fields. The isolates were identified as *Clostridium* spp. and caused severe soft rot symptoms when inoculated in sweetpotato storage roots, which provided strong support to the given hypothesis that *Clostridium* spp. are involved in the flooding-associated bacterial soft rot of sweetpotato storage roots. Furthermore, these two *Clostridium* isolates were found to be phylogenetically distinct from the human pathogenic Clostridia according to the comparison analyses of the 16s rDNA sequences.

The two end rot pathogens studied, *F. solani* and *M. phaseolina,* were found inside of symptomless storage roots and they were proven to be capable of moving from infested soil to sweetpotato plants and spreading inside of plants to storage roots, potentially causing end rot disease. Strong evidence was provided that sweetpotato storage root infections by these fungi can occur prior to harvest, which completely requires adapting earlier approaches to management and control of this disease complex. An orthodox approach for managing the disease is to cure the storage roots as it was believed that the sites of entrance of these pathogens were uniquely through wounds created during the harvest process. Since the evidence implicates infected propagating material as a source of these fungi, it is worth

considering using sweetpotato transplants free of these pathogens and efficient ways to reduce soil infestation by these fungi, prior to planting, as additional control measures.

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APPENDIX

Experiment A1.

Flooding, ethephon, and incidence of end rots on sweetpotato storage roots

Rationale

This experiment was primarily designed to investigate if flooding and/or ethephon affected the incidence of end rot disease pathogens on sweetpotato storage roots.

Material and Methods

There were six treatments: Plots not flooded and not treated with ethephon, plots not flooded and treated with ethephon, plots flooded one day prior to harvest and not treated with ethephon, plots flooded one day prior to harvest and treated with ethephon, plots flooded five days prior to harvest and not treated with ethephon, and plots flooded five days prior to harvest and treated with ethephon. The sweetpotato cultivar Beauregard was used in this assay. After harvest, the storage roots were cured at 29 +/- 2^{o}C and 85-90% RH for 5 days then stored at 15 +/- 2^{o}C until they were analyzed for end rot symptom development. Every two weeks a storage root without end rot symptoms was taken from each treatment for isolations to discover if end rot pathogens were present inside the storage roots and where in the root they would be. The isolations were attempted from different parts of the storage roots (Figure A1).

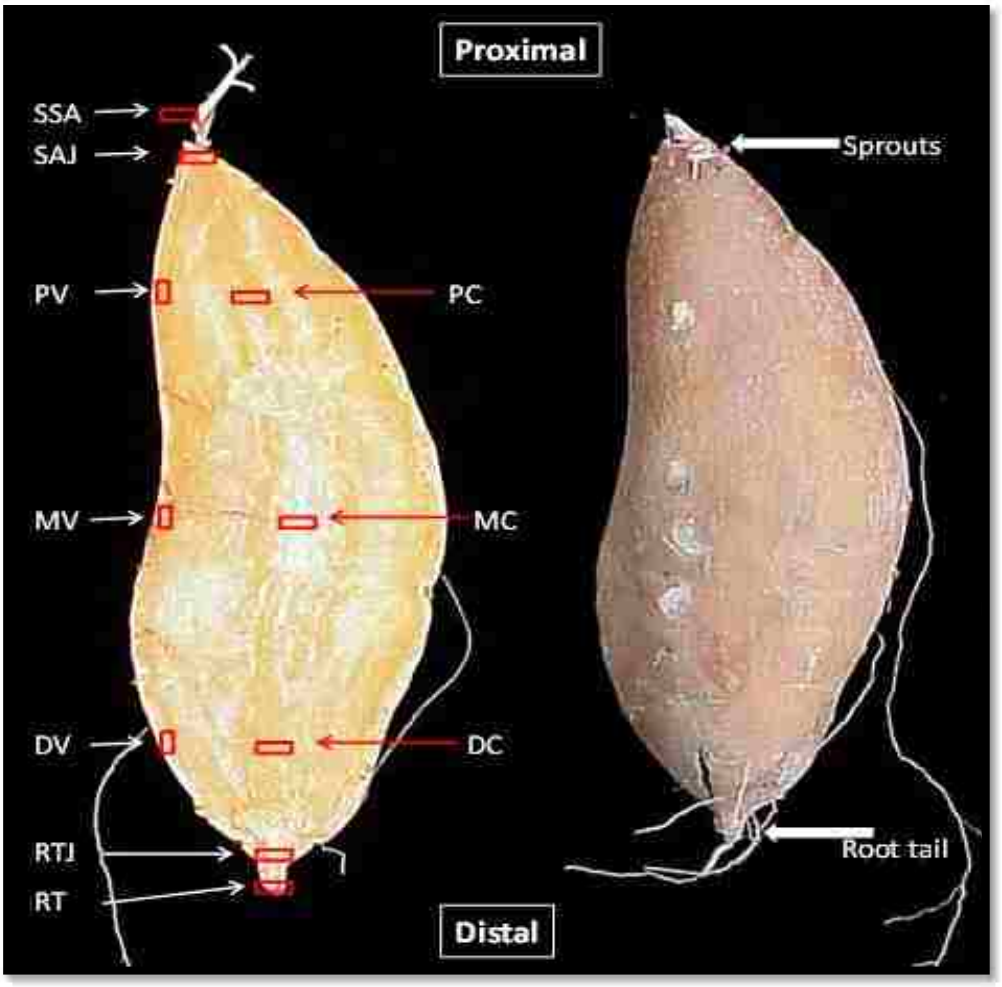

Figure A1 - Figure of sweetpotato storage roots showing the areas where isolations were attempted. SSA = slender stem attachment, SAJ = slender stem attachment junction with main storage root, PV = proximal vascular, PC = proximal central, MV = mid-root vascular, MC = mid-root central, DV = distal vascular, DC = distal central, RTJ = root tail junction with main storage root, RT = slender distal end of root.

Results

Fusarium sp., *Macrophomina phaseolina,* and other fungi were found inside symptomless storage roots in all isolation sites (Table A1). While the incidence of *M. phaseolina* did not differ statistically between isolation sites, *Fusarium* sp. and other fungi were isolated more frequently from the proximal and distal ends than from the isolation sites located toward the center of the storage roots (Table A2). Furthermore, the incidence rate of these fungi did not differ statistically between treatments in this experiment (Table A3).

					Isolation Site						
Isolations	**Treatments**	**SSA**	**SAJ**	**PV**	**PC**	**MV**	**MC**	**DV**	**DC**	**RTJ**	**RT**
1	**BN-X**	BC	Y, Y, Y								F
	BN+X	Y,CV	P, F							F	
	B1-X	L	F, L							B	Y
	B1+X	B	B							F, Y, BC, B	Y
	B5-X	B	F							Y, BC	B
	B5+X	B, Y, F	B, F			F				FY	F
2	**BN-X**	Y, F, P					F			P	
	BN+X	P	P							F, MP, T	P, F
	B1-X	Mp								L	L
	B1+X	F, F, B	Mp							Y	CV
	B5-X	B	B							Y	CV
	B5+X	F, L	L, F, PP							Y	BC
3	**BN-X**	F, F	F, F							F	P
	BN+X	A, Mp, F	BP, Mp,							Mp	Mp
	B1-X	F	A, F, Y	Y		Y, B				Mp	Mp
	B1+X	F, F	P, BM	Y		F		F		Mp, Y	F
	B5-X	F	F			F				Mp	
	B5+X	P	Mp, F	Mp	P, Mp	CV, CV		F		B, F, P, Y	F, F
4	**BN-X**	F	CV, Y							F, F	Y
	BN+X	CV, Mp	F, A							F	F, CV
	B1-X	F, F	CV, L							F, CV	Y
	B1+X	F, BM	B, F								L, BM
	B5-X		Y								
	B5+X	F	F							L	Mp

TABLE A1 – Results of the healthy storage root isolations. B= Beauregard Cultivar, N= Plants not flooded, 1= Plants flooded one day prior to harvest, 5= Plants flooded five day prior to harvest, - = Plants not treated with ethephon, + = Plants treated with ethephon, X= Replication.

Colonies description

B= Bacteria
F= *Fusarium* sp.
T= *Trichoderma*-like
BC= Black mycelia
A= *Aspergillus*-like
Mp= *Macrophomina phaseolina*
P= *Penicillium*-like
BM= Brown mycelia
Y= Yeast
CV= *Curvularia* sp.
L= *Lasiodiplodia* sp.
BP= *Bipolaris* sp.

TABLE A2. Binary LS-means (0 and 1, 0= not present and 1= present) of the incidence of fungi isolated from different locations in healthy storage roots*.

[a] SSA = slender stem attachment, SAJ = slender stem attachment junction with main storage root, PV = proximal vascular, PC = proximal central, MV = mid-root vascular, MC = mid-root central, DV = distal vascular, DC = distal central, RTJ = root tail junction with main storage root, and RT = slender distal end of root.

[b] F= *Fusarium* sp., Mp= *Macrophomina phaseolina,* and Others= Other fungi species.

* Conservative Tukey-Kramer Grouping for root location Least Squares Means (Alpha=0.05). LS-means in a column with the same letter are not significantly different.

	Fungi Isolated[b]		
Root Location[a]	**Fso**	**Mp**	**Others**
SSA	0.167 a,b	0.125 a	0.750 a
SAJ	0.167 a,b	0.125 a	0.833 a
PV	0.000 b	0.042 a	0.083 b
PC	0.000 b	0.042 a	0.042 b
MV	0.083 a,b	0.000 a	0.125 b
MC	0.042 b	0.000 a	0.000 b
DV	0.042 b	0.000 a	0.042 b
DC	0.000 b	0.000 a	0.000 b
RTJ	0.125 a,b	0.208 a	0.708 a
RT	0.125 a,b	0.125 a	0.542 a
F Value	3.350	2.200	26.89
Pr>F	0.0007	0.0230	< 0.0001

TABLE A3. Binary LS-means (0 and 1, 0= not present and 1= present) of the incidence of fungi isolated from different treatments in healthy storage roots produced in plots with different flood treatments*.

[a] B= Beauregard Cultivar, N= Plants not flooded, 1= Plants flooded one day prior to harvest, 5= Plants flooded five day prior to harvest, - = Plants not treated with ethephon, + = Plants treated with ethephon, and X= Replication.

[b] F= *Fusarium* sp., Mp= *Macrophomina phaseolina,* and Others= Other fungi species.

* Conservative Tukey-Kramer Grouping for treatment Least Squares Means (Alpha=0.05). LS-means in a column with the same letter are not significantly different.

	Fungi Isolated[b]		
Treatment[a]	**Fso**	**Mp**	**Others**
BN-X	0.100 a	0.000 a	0.275 a
BN+X	0.075 a	0.150 a	0.300 a
B1-X	0.075 a	0.075 a	0.300 a
B1+X	0.050 a	0.050 a	0.425 a
B5-X	0.050 a	0.025 a	0.250 a
B5+X	0.200 a	0.100 a	0.325 a
F Value	1.5300	1.9000	0.6800
Pr>F	0.1807	0.0947	0.6397

TABLE A4. Percentage of fungi isolated at harvest from symptomless sweetpotato storage roots produced in plots that were fumigated or not fumigated before planting.

Treatment[z]	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Fumigated	32.50 a*	2.50 b	22.50 a	18.33 a	20.00 a	1.67 b
Non-Fumigated	42.50 a	22.50 a	37.50 a	40.00 a	10.00 a	20.00 a
Pr>F	0.3570	0.0051	0.1516	0.0506	0.3191	0.0033

[z] Fumigated (Storage roots from soil fumigated with 1% Chloropicrin) and Non-Fumigated (storage roots from soil non-fumigated with Chloropicrin).

[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.

[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.

* Percentages were calculated from 5 roots collected from each plot for isolation. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A5. Percentage of fungi isolated from symptomless sweetpotato storage roots from each plant source at harvest.

Plant Source[#]	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Beds	40.00 a*	17.50 a	27.50 a	33.33 a	16.67 a	10.00 a
Greenhouse	35.00 a	7.50 a	32.50 a	25.00 a	13.33 a	11.67 a
Pr>F	0.6444	0.1531	0.6306	0.4473	0.7391	0.7835

[#] Beds (plants originated from sprouts grown in beds) and Greenhouse (plants originated from sprouts grown in greenhouses).
[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.
[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.
* Percentages were calculated from 5 roots collected from each plot for isolation. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A6. Comparison of the percentage of fungi isolated from symptomless sweetpotato storage roots from each treatment in each plant source at harvest.

Treatment[z]*Plant Source[#]	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Beds*Fumigated	40.00 a*	0.00 b	20.00 a	16.67 a	23.33 a	0.00 a
Beds*Non-Fumigated	30.00 a	35.00 a	35.00 a	50.00 a	10.00 a	20.00 a
Green.*Fumigated	25.00 a	5.00 b	25.00 a	20.00 a	16.67 a	3.33 a
Green. *Non-Fumigated	55.00 a	10.00 ab	40.00 a	30.00 a	10.00 a	20.00 a
Pr>F	0.0677	0.0336	1.0000	0.2882	0.7391	0.7835

[z] Fumigated (Storage roots from soil fumigated with 1% Chloropicrin) and Non-Fumigated (storage roots from soil non-fumigated with Chloropicrin).

[#] Beds (plants originated from sprouts grown in beds) and Greenhouse (plants originated from sprouts grown in greenhouses).

[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.

[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.

* Percentages were calculated from 5 roots collected from each plot for isolation. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A7. Percentage of fungi isolated from rotting sweetpotato storage roots in each treatment after storage roots being stored for three months at 15°C.

Treatment[z]	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Fumigated	0.50 a*	0.50 a	0.00	0.33 a	1.17 a	0.67 a
Non-Fumigated	0.50 a	0.00 a	0.00	0.00 a	0.00 a	0.54 a
Pr>F	1.0000	0.3179	0.00	0.4202	0.1262	0.8501

[z] Fumigated (Storage roots from soil fumigated with 1% Chloropicrin) and Non-Fumigated (storage roots from soil non-fumigated with Chloropicrin).

[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.

[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.

*Percentages were calculated from 20 roots collected from each plot for storing. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A8. Percentage of fungi isolated from rotting sweetpotato storage roots from each plant source after storage roots being stored for three months at 15°C.

Plant Source#	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Beds	0.50 a*	0.50 a	0.00	0.17 a	1.17 a	0.71 a
Greenhouse	0.50 a	0.00 a	0.00	0.17 a	0.00 a	0.50 a
Pr>F	1.0000	0.3179	0.00	1.0000	0.1262	0.7472

Beds (plants originated from sprouts grown in beds) and Greenhouse (plants originated from sprouts grown in greenhouses).
[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.
[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.
* Percentages were calculated from 20 roots collected from each plot for storing. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A9. Comparison of the percentage of fungi isolated from rotting sweetpotato storage roots from each treatment in each plant source after storage roots being stored for three months at 15°C.

Treatment[z]*Plant Source[#]	Chase[a]			Gilbert[b]		
	Fungi Isolated			Fungi Isolated		
	Fusarium solani	*Macrophomina phaseolina*	Other fusaria	*Fusarium solani*	*Macrophomina phaseolina*	Other fusaria
Beds*Fumigated	1.00 a*	1.00 a	0.00	0.33 a	2.33 a	1.00 a
Beds*Non-Fumigated	0.00 a	0.00 a	0.00	0.00 a	0.00 a	0.00 a
Greenhouse*Fumigated	0.00 a	0.00 a	0.00	0.33 a	0.00 a	0.33 a
Greenhouse *Non-Fumigated	1.00 a	0.00 a	0.00	0.00 a	0.00 a	1.00 a
Pr>F	0.1581	0.3179	0.00	1.0000	0.1262	0.1788

[z] Fumigated (Storage roots from soil fumigated with 1% Chloropicrin) and Non-Fumigated (storage roots from soil non-fumigated with Chloropicrin).

[#] Beds (plants originated from sprouts grown in beds) and Greenhouse (plants originated from sprouts grown in greenhouses).

[a] Plants cultivated at sweetpotato research station, Chase, LA-USA.

[b] Plants cultivated at McLemore Farm, Gilbert, LA-USA.

* Percentages were calculated from 20 roots collected from each plot for storing. Conservative Tukey-Kramer Grouping for isolation site Least Squares Means (Alpha=0.05). LS-means with the same letter within the same column are not significantly different.

TABLE A10. Number of isolates of other fusaria isolated from **(A)** symptomless sweetpotato storage roots at harvest and **(B)** rotting sweetpotato storage roots after storage roots being stored for three months at 15°C, from each treatment and each plant source#.

	Fusarium species	Chase[a]				Gilbert[b]			
		Non-Fum.*Green.	Fum.*Green.	Non-Fum.*Beds	Fum.*Beds	Non-Fum.*Green.	Fum.*Green.	Non-Fum.*Beds	Fum.*Beds
A	*F. pallidoroseum*	8	5	7	4	2	1	2	0
	F. oxysporum	0	0	0	0	0	0	0	0
B	*F. pallidoroseum*	0	0	0	0	0	0	0	0
	F. oxysporum	0	0	0	0	1	1	0	3

Non-Fum.*Green. (storage roots from plants originated from sprouts grown in greenhouses and planted in soil non-fumigated with Chloropicrin), Fum.*Green (storage roots from plants originated from sprouts grown in greenhouses and planted in soil fumigated with 1% Chloropicrin), Non-Fum.*Beds (storage roots from plants originated from sprouts grown in beds and planted in soil non-fumigated with Chloropicrin), Fum.*Beds (storage roots from plants originated from sprouts grown in beds and planted in soil fumigated with 1% Chloropicrin)

[a]Plants cultivated at sweetpotato research station, Chase, LA-USA.

[b]Plants cultivated at McLemore Farm, Gilbert, LA-USA.

TABLE A11. List of *Fusarium* isolates, from this study, that had the TEF 1-alpha gene partially sequenced and deposited in the NCBI Database.

Isolate	Species	Isolation_source	Isolation Site	GenBank Accession Number
F1	*F. pallidoroseum* (synonym *F. incarnatum*)	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820972
F7	*F. pallidoroseum* (synonym *F. incarnatum*)	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820973
F9	*F. pallidoroseum* (synonym *F. incarnatum*)	Plant Stem	Exp_Infested Soil/Treat_Control	KC820974
G2	*F. oxysporum*	Storage Root	Asymptomatic Storage Root	KC820980
G3	*F. oxysporum*	Storage Root	Asymptomatic Storage Root	KC820981
G5	*F. oxysporum*	Plant Stem	Exp_Infested Soil/Treat_Control	KC820979
G6	*F. nygamai*	Plant Stem	Exp_Toothpick/Treat_Mp	KC820978
G7	*F. proliferatum*	Plant Stem	Exp_Toothpick/Treat_Mp	KC820975
G9	*F. commune*	Storage Root	Surface Rot	KC820971
F-89.021*	*F. denticulatum*	Leaf Tissue	Chlorotic Leaf Distortion	KC820969
C	*F. proliferatum*	Plant Stem	Exp_Infested Soil/Treat_Fso+Mp	KC820976
D	*F. concentricum*	Storage Root	Exp_Toothpick/Treat_Control	KC820970
E	*F. proliferatum*	Storage Root	Surface Rot	KC820977
F2	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820963
F3	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820964
F4	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820965
F5	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820966
F6	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC820967
F8	*F. solani*	Plant Stem	Exp_Infested Soil/Treat_Mp	KC825359
B	*F. solani*	Rotting Storage Root	Exp_Infested Soil/Treat_Fso	KC820968

* Not from this study.

VITA

Washington Luis da Silva was born in the city of Contagem, in Minas Gerais state, Brazil. When he was five years old, he and his family moved to a farm in the small village of Divinolândia de Minas, in Minas Gerais state, where he attended primary and higher secondary school. In March 2003, he started his undergraduate major in Agronomy Engineering at Universidade Federal de Viçosa (UFV) and promptly started working with ecology and behavior of leaf-cutting ants. He was awarded a prestigious scholarship from the Programa Institucional de Bolsas de Iniciação Científica (Institutional Program of Scientific Initiation, Minas Gerais State Science Scholarship) - FAPEMIG PROBIC/UFV, that lasted from August 2003 to May 2005. Soon thereafter, he was awarded another scholarship from the Programa Institucional de Bolsas de Iniciação Científica (Institutional Program of Scientific Initiation, National Brazilian Science Scholarship) - PIBIC/CNPq, which lasted from June 2005 to March 2007. From March 2007 to September 2008 he participated in an exchanged program in the United States, MAST international program at the University of Minnesota, where he interned at the Mahoney's Garden Center in Winchester Massachusetts. During this time, he also worked as a museum guide volunteer at Harvard Museum of Natural History and he took classes on the taxonomy, anatomy, and evolution of *Cetacea*. In February of 2009, he resumed his classes at UFV and started working as a research assistant with fluorescence microscopy studying the effects of silicon on the defense system of wheat against infection by *Pyricularia grisea*. Washington was awarded another scholarship from the Programa Institucional de Bolsas de Iniciação Científica (Institutional Program of Scientific Initiation, National Brazilian Science Scholarship) - PIBIC/CNPq, extending from July 2009 to July 2010.

He completed his bachelor of science from UFV in July 2010 and in August of that year he joined the Department of Plant Pathology and Crop Physiology at Louisiana State University to pursue his Master's degree in Plant Health under the guidance of Professor Christopher A. Clark. His Master's project focused on studying the etiology and biology of end rot and soft rot disease complexes in sweetpotato storage roots. He served as the treasurer and also member of the executive committee of the Plant Pathology and Crop Physiology Graduate Student Association for two years. He was also a member of the Committee of Courses and Curriculum of the Plant Pathology and Crop Physiology Department from 2011 to 2012. During his Master's studies, he received four travel awards, two from American Phytopathological Society (APS), one from the Department of Plant Pathology and Crop Physiology at LSU, and one from the LSU graduate school, to present his research results at the APS professional meetings. Washington and his wife Rachel, whom he married in March 2009, had their first daughter, Aurelia, in January 2013 while attending graduate school.