PREVENTION AND DETECTION OF SOUR SKIN OF ONION (*ALLIUM CEPA)* BY CROP ROTATION, MICRONUTRIENT MANIPULATION, AND VOLATILE COMPOUND DETECTION

by

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(Under the Direction of Ron Gitaitis)

Abstract

Sour skin of onion (*Allium cepa* L.), caused by the soil-borne bacterium *Burkholderia cepacia*, (ex Burkholder 1950) Yabuuchi et al. 1993, is a devastating disease of onion and is responsible for post-harvest losses annually. Current management strategies are to avoid contaminated fields and adjust planting dates to avoid high temperatures during harvest. In addition, harvesting onions at optimum maturity, field-curing a minimum of 48 h, sorting and grading to remove infected onions and storing onions under proper conditions are also used to manage sour skin. However, these strategies have not adequately reduced losses when conditions are favorable for disease. Other strategies, such as chemical control and resistance, are not viable options for controlling sour skin. Furthermore, infected onions may go undetected during the sorting and grading process. Such bulbs have the potential of infecting healthy onions in storage. Innovative and integrated management practices aimed at preventing disease in the field and improving early detection are needed to effectively manage sour skin of onion.

This research evaluated management practices focused on prevention of sour skin in the field by rotating or double-cropping with crops that had a negative impact on *B. cepacia* populations *in vitro*. Under controlled conditions in the laboratory, populations of *B. cepacia* declined in the soil as a result of direct contact with root exudates of pearl millet (*Pennisetum glaucum* (L.) R. Brown). Initially, double-cropping onion with pearl millet reduced sour skin incidence and severity in field trials. However, by the fourth year of continuous double-cropping of onion with pearl millet, the beneficial effects of reducing sour skin were virtually non-existent. Since the crops used in the rotation and double-cropping treatments deplete different soil nutrients, the role that mineral nutrition plays on plant disease development also was investigated. In 2012 and 2014, field-grown onion bulbs and soils were evaluated for mineral composition. Data were analyzed with stepwise and maximum R^2 improvement regression using sour skin (incidence or severity) as the dependent variable. In 2012, a tissue model (P=0.0002; adj. R^2=0.51) was developed that included ratios of copper: iron, zinc: iron and sulfur: aluminum as well as manganese and nitrogen as independent variables. Likewise, a soil model (P = 0.00006 adj. R^2 = 0.57) that included the ratios of zinc: iron, iron: manganese and manganese: zinc as well as copper and potassium as independent variables were also developed to predict sour skin severity. Due to a total crop loss in 2013, onions purchased from grocery stores were inoculated, incubated, graded for disease severity, and used for mineral analysis. The grocery store model (P=0.00001; adj. R^2=0.43) also contained a ratio of copper: iron, and the minerals aluminum, potassium, nitrogen, and sulfur as independent variables, all of which were components of the models developed in 2012. In 2014, a tissue model (P=0.00002; adj.

R^2=0.34) based on natural infections in the field contained the ratios of copper: iron, sodium: iron, manganese: zinc, and the elements calcium, manganese, and nitrogen as the independent variables. The elements copper, iron, manganese, and zinc consistently occurred in nine different sour skin models developed over a 3 year period. These elements are cofactors of three superoxide dismutases (SODs) in plants that play a key role in reactive oxygen species (ROS) detoxification and systemic acquired resistance (SAR). Preliminary results suggest that the effects these elements have on sour skin is by ultimately affecting SODs and the SAR pathway.

In addition to field studies, investigations on early disease detection were conducted to improve current sorting and grading processes. Improvements are needed for the onion industry to eliminate onions with internal infections from going in to storage or entering the marketplace. Targeting the detection of volatile organic compounds (VOCs) using zNose technology was evaluated. When numbers of infected onions ranged from 10-50% and the volume of the container was $\leq$ 2 L, an increase in the level of VOCs could be detected, but qualitative differences could not be discerned due to the inability of zNose technology to distinguish closely related compounds. In addition, when the level of infected onions was decreased to 1% and the storage area was increased to 11,000 L, neither increased levels of VOCs nor a distinct profile could be detected. However, VOCs such as dipropyldisulfide and propyl-1-propenyldisulfide, unique to onions with sour skin, were identified using GCMS. These compounds could be used as targets for early disease detection.

INDEX WORDS: Vidalia onion, *Allium cepa*, Sour skin disease, *Burkholderia cepacia*, crop rotation, superoxide dismutase, volatile organic compounds, zNose technology

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DEDICATION

I would like to dedicate this dissertation to my children (Ella Leighton Selph, Katherine Olivia Selph, and Andrew Hendrix Selph); you are my reason for being, my reason for persevering, and my reason for reaching my goals. This dissertation is also dedicated to my husband (Michael Wayne Selph) and my parents (Wylie Roger Watson, and Cynthia Kerrance Watson), without your continued support and encouragement none of this would be possible. You kept me sane and grounded throughout this entire process and for that I thank you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Onion Production in Georgia

Onion (*Allium cepa)* is a monocot in the genus *Allium* and a member of the order *Asparagales* and the family *Alliaceae*. Onion is one of 18 vegetable species in the genus *Allium* that are grown commercially throughout the world (Davis, 2008). The bulb onion has been cultivated since approximately 3,000 B.C. and its origins can be traced back to central Asia (Davis, 2008). Onions are currently produced in 170 countries with approximately 1.5 million ha being harvested annually (National Onion Association, 2011). Approximately 60 million metric tons of onions are produced annually worldwide; the U.S. is one of the primary producers (AgMRC, 2016). The onion is the fifth most valuable vegetable crop in the U.S. with a fresh market value of $969 million USD in 2014 (AgMRC, 2016). Georgia onion production accounts for approximately 14% of the U.S. crop with a fresh market value in 2014 of approximately $138 million USD (Wolfe and Stubbs, 2014).

There are three distinct categories of onions currently cultivated, which are classified by color; yellow, red, and white onions (National Onion Association, 2011). For the purposes of this dissertation we will primarily focus on sweet

yellow onions grown in Georgia; this onion type is referred to as the “Vidalia onion”, however the research conducted is applicable to the entire onion industry. Currently, Vidalia onions are cultivated for either fresh-market sale or short-term storage. Fresh-market onions can be consumed directly as a raw or cooked vegetable, pickled, or as a seasoning in numerous recipes. In addition, processed Vidalia onions are used in sauces, relishes, and salad dressings.

Vidalia onions are known nationally and internationally as a specialty crop from the state of Georgia. In 2014, the Georgia farm gate report listed onions as the most valuable vegetable crop and the 18th most valuable agricultural commodity (Wolfe and Stubbs, 2014). Vidalia onions are considered a sweet, short-day, hybrid, yellow onion with the cultivar Granex in its pedigree. The cultivars grown as a Vidalia onion change over time as the Georgia Commissioner of Agriculture makes an annual recommendation on the varieties allowed to be marketed as a Vidalia onion. His endorsement is made after consultation with the Director of the Experiment Station of the College of Agricultural and Environmental Sciences from the University of Georgia. The Director’s input is developed from results of an annual variety trial that use ratings of different variables such as maturity, color, shape, disease resistance, insect tolerance, bolting, and several different flavor characteristics. Approximately 20 different cultivars representing different maturity groups meet those standards annually and can be approved to be grown and marketed as a Vidalia onion. To be on the approved list, each onion cultivar must be evaluated for a minimum of 3 years (Boyhan and Kelley, 2014). In addition, the onion must

be grown in a legally specified region in Georgia. This region, designated by both State and Federal mandates, lies within 20 counties surrounding Toombs County. This region is characterized as having soils with low sulfur content and having a moderate climate (Clemens, 2002; Howard, 2002). Soil and climate characteristics are important for the development of the Vidalia onion's sweet flavor. In 2014, Vidalia onions were grown on approximately 5,000 ha (Wolfe and Stubbs, 2014).

Vidalia onions are susceptible to a variety of diseases; due, possibly, to the onion's lack of pungency compounds, soft texture, and thin skin. This dissertation focuses on one of the main post-harvest diseases of onion in Georgia, sour skin, caused by the bacterium *Burkholderia cepacia*. This disease can result in significant pre- and post-harvest yield losses annually (Howard, 2002).

Sour Skin of Onion

Burkholderia cepacia, the causal organism of sour skin, is one of the most important post-harvest pathogens associated with Vidalia onions. This bacterium is a gram-negative rod belonging to the ß-proteobacterium group. Sour skin was first described in New York in 1950 by Walter Burkholder at Cornell University and has since been increasing in importance worldwide (Burkholder, 1950 and Davis, 2008). Losses resulting from sour skin can be as high as 50% annually (Burkholder, 1950; Davis, 2008). The bacteria gain entry into the onion bulb through a variety of ways. Bulbs can become infected as a result of wounding

from wind-blown sand, hail, insect feeding, farm equipment used to undercut and lift onions from the ground, or cutting the necks at harvest to remove foliage. Potentially, young leaves can become infected allowing the bacterium to move down the leaf blade into the onion bulb resulting in bulb infections, but this is rarely observed in Georgia (Davis, 2008). Generally, onions infected with *B. cepacia* exhibit soft rot symptoms. The rot is generally brown to reddish brown in color and has a prominent vinegary odor (Burkholder, 1950; Davis, 2008). In many instances, a conspicuous yellow, granular material consisting of bacterial cells and macerated onion tissue accumulates in affected tissues. This disease is favored by warm and humid conditions, which typically occur during harvest in southern Georgia.

Current Management of *Burkholderia cepacia* and its Shortcomings

B. cepacia is a unique organism that utilizes a wide variety of carbon sources, inhabits a range of habitats, and has a diverse host range; making sour skin difficult to manage (Coenye and Vandamme, 2007). At present, there are no effective chemical-based practices to control sour skin of onion. Sour skin is typically managed through cultural practices aimed at minimizing exposure to *B. cepacia*. The following recommendations are aimed at managing sour skin of onion.

Research conducted in California indicates that when over-head irrigation is used throughout the growing season, there is an increase in sour skin incidence. Over-head irrigation increases the probability that inoculum from one

infected onion plant will be splash-dispersed to other onion plants and increase sour skin incidence. In-furrow irrigation is recommended; however, avoidance of over-head irrigation in Georgia is ineffective due to the amount of natural rainfall growers receive during the growing season (Teviotadle et al., 1990; Diaz-Perez et al., 2004; Boyhan and Kelley, 2007).

Additionally, it is recommended that onion bulbs be harvested at the optimum stage of maturity. This is assessed by evaluating the percentage of onion tops that are down, plant tops that have fallen over due to a breakdown of the plant tissue directly above the bulb. The percentage of fallen tops range from 20% to 50% at plant maturity (Boyhan and Kelley, 2007). The fallen tops are a sign that when the onion bulbs are undercut (lifted from the soil to break the connection between the plant roots and soil) the neck region tissue will desiccate and reduce pathogen invasion during harvest (Boyhan et al., 2005; Boyhan and Kelley, 2007). If implemented correctly, this management strategy reduces the incidence of sour skin; however, early in the season some growers will harvest immature onions in an attempt to be first to market to obtain a higher price. This leads to onion bulbs with green necks that can serve as an entry point for *B. cepacia* invasion and increased post-harvest yield loss.

It is also recommended that onion bulbs be cured in the field for 1 to 3 days or by forced-air drying to dry the neck region of the onion bulbs to prevent inoculation and spread of the pathogen during the clipping process (Boyhan and Torrance, 2002; Boyhan and Kelley 2007). These management practices can be problematic if weather conditions are unfavorable for adequate curing of onion

tissues (warm and wet weather will exacerbate any disease present during this process). Additionally, forced-heated air-drying is not recommended as it will increase the severity of sour skin (Boyhan and Torrance, 2002; Boyhan and Kelley, 2007; Davis, 2008; Schroeder, Humann, and du Toit, 2012).

Storage of onions at optimum conditions is essential for preventing the development and spread of sour skin disease storage. There are several recommended storage options for onion growers. The most common are circulated fresh air (RH ~85%), refrigerated (~0°C and RH between 65-70%), and controlled atmosphere (~3% oxygen, ~5% carbon dioxide, ~92% nitrogen, <1°C, and RH between 70-80%). The type of storage used is determined by the length of time the onions will be held. Refrigerated and controlled atmosphere are the most effective in preventing the advancement and spread of sour skin in storage (Myers, 1999; Boyhan and Kelley, 2007). The effectiveness of any type of storage hinges on the amount of infected onions inadvertently introduced into storage. Introducing infected onions into storage is unavoidable; unfortunately, because all sorting and grading is based on the onion bulb's external characteristics, which does not account for latent infections.

Traditional methods for detecting post-harvest diseases include non-destructive hand grading and culling of diseased onions prior to storage and during storage as needed (Flentje et.al., 1963; Li et.al., 2009). These methods do not detect all infected onions; early stages of disease and internal rots are often overlooked because they do not exhibit visible disease symptoms. Infected but asymptomatic onion bulbs are stored with healthy onions. This is highly

problematic because the latently infected onions may develop symptoms and spread inoculum in storage. Any disease developed in storage can result in the loss of entire storage units. Storage units can hold up to 1,100 metric tons of onions, resulting in a potential loss of approximately $2 million USD per unit.

It is also important to note that Vidalia onions are almost exclusively harvested by hand to reduce damage to onions (Boyhan and Kelley, 2007). It is during this process that infected onions can be missed and included with healthy onions and when onions can be accidently inoculated with *B. cepacia*.

As mentioned, current disease management recommendations and detection strategies do not adequately prevent pre- and post-harvest yield loss attributed to sour skin of onion. This dissertation addresses research to develop management strategies to effectively reduce disease incidence and severity prior to and during storage with crop rotation and double-cropping, mineral nutrient manipulation, and volatile organic compound detection.

Crop Rotation and Pathogen Reduction as Disease Management

Crop rotation has been defined as growing economic plants in recurring succession and in a specific sequence on the same land (Curl, 1963). These types of rotations have been explored extensively to reduce pathogen populations in the soil by rotating host crops with non-host crops (Agrios, 2005).

In this dissertation, crop rotation is restricted to the use of a crop within the same time period of the growing season. Since onion is a winter crop in Georgia, the term crop rotation is limited to other winter crops such a carrot or turnip. A

cash crop grown during the summer between onion crops will be referred to as double-cropping. Currently, onion growers' choices as a double-crop behind onions are based on planting date and profitability of the crop and not on potential impacts the crop may have on soil-borne pathogens.

Research conducted by Haundenshield and Lorbeer (2003) in New York showed that when certain crops are grown in closed systems with *B .cepacia*, the bacterial populations are reduced in the soil compared to treatments when crops known to support *B. cepacia* growth were grown. Two crops shown to reduce *B. cepacia* populations in the soil were pearl millet (*Pennisetum glaucum*) and carrot (*Daucus carota*) (Haudenshield et.al, 2003; Haudenshield et.al., 2003). The reduction in pathogen populations was confirmed using plate count procedures and *Pseudomonas cepacia*, azelaic acid, tryptamin (PCAT) medium. Nischwitz et al. showed that when winter-grown onions were double-cropped with pearl millet in Georgia, there was a reduced incidence of sour skin (Nischwitz et al., 2007). However, a significant reduction in bacterial populations was not shown. This research will determine if *B*. *cepacia* populations and subsequent levels of sour skin are differentially affected by different plant species used in rotation and double-cropping strategies with Vidalia onions.

Mineral Nutrition and Plant Disease

The relationship between mineral nutrition and plant disease is quite complex. The interaction between specific nutrients and diseases can vary depending on an array of factors (Huber and Haneklaus, 2007 and Dordas,

2008). Some important factors that contribute to this complex interaction are the environment (temperature, humidity, time of year, soil characteristics, and altitude), the plant (species, cultivar, and growth stage), the pathogen (species, virulence, mode of entry, location, etc.), and the nutrient (composition, activity, amount) (Huber and Haneklaus, 2007 and Dordas, 2008). Despite this complexity, when compared to a plant grown under conditions of relatively poor nutrition, a healthy plant receiving balanced mineral nutrition is less likely to succumb to disease (Huber and Haneklaus, 2007 and Dordas, 2008).

For onion, several mineral nutrients are key for producing an optimal crop. These include the macronutrients calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P), potassium (K), boron (B), zinc (Zn), and sulfur (S). Additionally, application of the micronutrients manganese (Mn), iron (Fe), copper (Cu), molybdenum (Mo), and chlorine (Cl) are vital for onion growth and production (Boyhan and Kelley, 2007).

Examples can be found for the association of each of these elements with both the increase and decrease in disease severity or incidence in numerous plant species. The following mineral-disease interactions demonstrate the varied effects observed in these relationships (Agrios, 2005; Huber and Haneklaus, 2007; Dordas, 2008).

Calcium application has been shown to protect plants from infection by *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Botyrits*, and *Fusarium* (Huber, 1980; Graham,

1983). However, Ca is utilized by the pathogen, *Colletotrichum trifolli*, and aids in tissue maceration and increased disease severity (Kiraly, 1976).

Magnesium application has been linked to the decrease in several diseases including bacterial blight of cotton, caused by *Xanthomonas citri* pv. *malvacearum*, and soft rot of potato, caused by *Pectobacterium atrosepticum*, (Batson, 1971; Kelman et al., 1989; McGuire and Kelman, 1986). However, Mg is also associated with an increase in bacterial spot of pepper and tomato, caused by *Xanthomonas euvesicatoria*, and pod rot of peanut, caused by *Fusarium*, *Pythium*, and *Rhizoctonia* spp., (Woltz and Jones, 1979; Csinos and Bell, 1989; Halleck and Garren, 1968).

Nitrogen treatments have been linked with the increase in diseases caused by obligate parasites such as *Puccinia graminis*, *Blumeria graminis*, and *Tobacco mosaic virus*, as well as *Pseudomonas syringae* (Howard et al., 1994; Büschbell and Hoffmann, 1992; Singh, 1970; Hoffland et al., 2000). This increase in disease is associated with an increase in green tissue that is essential for growth of obligate parasites (Dordas, 2008). N has also been linked with the decrease in diseases caused by *Xanthomonas euvesicatoria*, *Alternaria solani*, and *Fusarium oxysporum* (Chase, 1989; Blachinski et al., 1996; Woltz and Engelhar, 1973).

Phosphorus is no exception when it comes to conflicting interactions with plant disease. The application of P decreased the incidence of bacterial blight of broad bean, caused by *Pseudomonas syringae* pv. *syringae* (Abd El Moneem et

al., 1994). However, P tends to increase the susceptibility of plants to viruses in general. This increase in susceptibility is attributed to the virus' need for P during replication (Prabhu et al., 2007).

In general, for bacterial diseases, the application of K decreases disease; with a few exceptions. Potassium application has been shown to increase fire blight of apple, caused by *Erwinia amylovora*, and tomato canker, caused by *Clavibacter michignaense* subsp. *michiganensis* (Chase et al., 1968; Walker J.C., 1969). The interaction between K and other pathogens is more variable than for bacterial pathogens.

Boron application is generally associated with a decrease in disease, and B deficiency is linked with increased pathogenicity of the causal pathogen (Stangoulis and Graham, 2007). Zinc has been shown to decrease *Xanthomonas euvesicatoria* on pepper; however, Zn is crucial for the production of tabtoxin by *Pseudomonas syringae*, thus playing a critical role in disease (Adaskaveg and Hine, 1985; Durbin and Uchytil, 1985). Sulfur fertilizer application reduced incidence and severity of several fungal diseases, whereby the decrease in disease has been attributed to the induction of sulfur-induced resistance (SIR) (Haneklaus et al., 2007).

As previously mentioned, the exact role that these elements play in plant disease and resistance is not completely understood. Each element plays a distinct role in plant growth and development that varies greatly depending on plant species, environment, and pathogen involved. Understanding this

interaction as it pertains directly to the plant/pathogen system of interest is essential. Once this interaction is established, the mode of action can be explored. There are several proposed explanations for the interaction between specific nutrients and the decrease or increase in plant disease. One such explanation is the role these nutrients play in the activation of plant resistance, specifically systemic acquired resistance (SAR).

The role that nutrients play in SAR is not well understood, but great strides are being made to better understand and utilize these interactions. Previous research conducted at the University of Georgia on tobacco showed a direct relationship between Cu:Fe ratios and plant disease incidence (Gitaitis et al., 2013). Similarly, research conducted on cucumber downy mildew indicated that foliar applications of micronutrient solutions prior to pathogen inoculation, SAR could be induced (Reuveni et.al., 1997). Additionally, silicon root treatments were shown to increase plant resistance to cucumber powdery mildew (Liang et al., 2005).

One objective of this dissertation is to better understand interactions between mineral nutrition and sour skin of onion. Nutrient analysis of plant tissues and soils where plants are grown were conducted in an attempt to determine optimum nutrient levels associated with reduced sour skin incidence and severity. In addition, the mode of action by which these nutrients affect disease will be explored. Often, the mode of action suggested when exploring relationships between nutrition and disease is the direct activity of each nutrient

on the plant tissues or on the pathogen. However, other modes of action will be explored like the interaction of nutrients amongst themselves as well as SAR.

Volatile Organic Compounds and Disease Detection

Post-harvest diseases of onions will always pose a problem for producers and processors. In addition to management strategies that reduce disease incidence and severity there is a need for a rapid detection protocol that can be easily implemented in current processing practices. This rapid detection protocol should be implemented prior to and during storage to prevent yield loss associated with *B. cepacia* infected but asymptomatic onions.

One area of recent interest is the detection of disease-associated volatile organic compounds (VOCs) for rapid detection of plant disease. VOCs have been of interest in several fields of science including medicine and plant pathology (Jansen et al., 2011). Research has shown that when plants are diseased they produce volatile compounds that can be unique to the plant patho-system. Examples of this include when apple and pear trees are infected with, *Erwinia amylovora*; when grapevines are infected with *Agrobacterium tumefaciens*, and when tobacco is infected with *Pseudomonas syringae pv. tabaci* (Jansen et al., 2011). Additionally, when onions are infected with *B. cepacia* unique volatile organic compounds are produced (Vikram et al., 2005; Li et.al.,2009; Li et al., 2011). However, this research could be expanded in two primary areas: 1) only one strain of *B. cepacia* was utilized previously and this could present a problem due to the extreme diversity of the bacterium; 2) the

sensor enose technology used to detect VOCs is easily contaminated or overwhelmed by VOCs and a better alternative must be explored. An alternative to this technology is zNose technology that can rapidly detect VOCs (Staples, 2000).

The ZNose GC analyzer is an instrument that evaluates head-space samples for the presence of volatile compounds. A sample of the gas is passed through the surface acoustic wave (SAW) sensor analyzer and a volatile compound profile identifying compounds is produced in approximately 60 s (Staples, 2000). Storage units currently used by onion processors provide the type of sealed space that is conducive for headspace gas accumulation.

The volatile compound profile of a product can provide valuable information regarding its quality. However, a highly sensitive instrument is needed to perceive subtle differences in products based on these compounds. The zNose GC analyzer is highly sensitive in its ability to detect differences in volatile compound profiles produced by different substances. To date, this technology has not been used to evaluate onions. However, based on previous research conducted with this technology, it may be applicable for use with onions. Several researchers have used a zNose GC analyzer for the evaluation of flavor profiles of vegetable oil and honey, as well as the differentiation of physically damaged and whole (healthy) apples (Gan et al., 2005; Lammertyn et.al., 2004; Li et.al., 2007; Li et.al., 2009).

Detection of subtle differences in products, normally not detected by traditional means, has been possible through the use of zNose technology. Lammertyn *et al.* (2004) classified nine different honey varieties by type according to their volatile compound profiles. The type of honey was representative of the nectar, or plant, from which each honey was derived. These differences in varieties were observed when evaluated by the zNose GC analyzer (Lammertyn et.al, 2004).

Vegetable oils have been analyzed with zNose technology to evaluate flavor and quality. Based on volatile compound profiles, 16 vegetable oils were differentiated. Profiles were gathered over a period of time and deterioration of the oils was observed. Vegetable oil quality was determined based on the volatile compounds produced and as a result, fresh oil was distinguished from old oil (Gan et al., 2005).

Apple quality was also evaluated with zNose technology. Li *et al.* (2007) conducted a series of experiments in which volatile compound profiles were produced from healthy apples and those with wounds, or internal defects. The zNose GC analyzer detected differences in the volatile compounds produced by each of the apples. Healthy apples could be differentiated from those with bruises or internal defects when apples were sampled in small containers with a small volume to sample ratio (Li et.al, 2007).

One objective of this dissertation is to determine the ability of zNose technology to detect sour skin-associated volatile compounds.

Justification and Goals

The primary goal of this dissertation research was to design and implement novel disease management and detection strategies to prevent and rapidly detect sour skin infected onions through the following objectives.

1. Implement a double-cropping/crop rotation program that would effectively reduce disease incidence and thereby reduce in-field yield loss and the amount of infected onions going into storage.

2. Further our understanding of the relationship between plant nutrition, disease, and disease resistance in an effort to develop disease management strategies based on cultural practices.

3. Explore the applicability of detection of disease-associated volatile organic compounds as an early detection strategy for sour skin of onion prior to and during storage. Additionally, determine differences in volatile compounds produced by different strains of *B. cepacia*.

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Chapter 2

EFFECT OF CROPPING HISTORY ON SOUR SKIN INCIDENCE OF ONION AND POTENTIAL MODE OF ACTION AGAINST *BURKHOLDERIA CEPACIA*.[1]

[1] Watson, A., Dutta, B., and Gitaitis, R. To be submitted to Plant Disease

Abstract

Sour skin, caused by *Burkholderia cepacia*, is a major disease of onion that is responsible for yield losses annually. This disease is primarily a post-harvest disease with infection and symptom development beginning at harvest and continuing into storage. There are few effective management strategies for this soil-borne pathogen. The lack of effective bactericides and resistant cultivars make cultural practices one of the few options to reduce sour skin of onion. This research utilized crop rotation and double-cropping programs with non-host crops that were previously reported to reduce disease. A crop-rotation scheme involving onion (*Allium cepa*) and carrot (*Daucus carota* subsp. *sativus*) and a double-cropping scheme using either pearl millet (*Pennisetum glaucum*), corn (*Zea mays*), peanut (*Arachis hypogaea*), or soybean (*Glycine max*) were evaluated. In 2010, sour skin incidence was significantly reduced in onions following pearl millet. A significant increase in marketable yield (% healthy bulbs) occurred for onions following pearl millet in 2011. Overall, there was a trend of decreased disease incidence and increased marketable yield for onions following pearl millet in three out of four years of this field study. The mode of action by which pearl millet reduces disease incidence is unknown, but may be due to a reduction of soil-borne *B. cepacia* populations. Bacterial growth assays were conducted to explore the interaction of corn, pearl millet and onion root exudates on *B. cepacia* populations *in vitro*. Results showed a significant reduction in area under the growth curve data for *B. cepacia* growing in pearl millet root exudates relative to corn root exudates.

Introduction

Onion (*Allium cepa*) is one of the most important vegetable commodities in the U.S. and in Georgia. In 2014 onions were the 5th most valuable vegetable commodity in the nation with a total value of approximately $1 billion USD and the most valuable vegetable commodity in the state of Georgia with a value of $138 million USD (Wolfe and Stubbs, 2014). Several diseases pose problems for onion growers worldwide, but in the southeastern U.S. the most problematic diseases in sour skin. This disease is caused by the soil-borne bacterium, *Burkholderia cepacia*, and causes up to 50% yield loss annually (Davis, 2008). Adequate prevention of disease development in the field is essential for reducing post-harvest yield losses. However, there are limited control strategies available to combat the pathogen due to its plasticity in terms of host-range, habitat, and substrate utilization (Vermis et al., 2003). Currently, there are no effective chemical applications or resistant cultivars available for the management of sour skin. As a result**,** new management strategies must be developed.

Double-cropping and crop rotation are some of the oldest cultural practices used to combat soil-borne diseases (Curl, 1963). The premise behind these practices is that non-host crops will reduce the pathogen population in the soil and thereby reduce inoculum and subsequent disease incidence. *In vitro* studies conducted in New York found that carrot and millet supported the lowest *B. cepacia* populations in muck soil, whereas corn supported the highest (Haudenshield and Lorbeer, 2003; Haudenshield et al., 2003). Additionally, research conducted in Georgia showed that onions double-cropped with pearl

millet had significantly less sour skin and higher marketable yield than onions following corn (Gitaitis et al., 2005). These findings are problematic for many Vidalia onion farmers, as corn is one of the primary crops grown during the summer following onion. In this study, we evaluated a double-cropping and crop-rotation program that utilized crops previously shown to reduce *B. cepacia* populations in the soil. It was hoped that this inoculum reduction could reduce sour skin disease incidence and severity in subsequent onion crops. In addition, the mode of action by which bacterial populations were being reduced was explored through *in vitro* experiments.

Materials and Methods

Double-Cropping/Crop Rotation

Vidalia onions were grown under standard grower practices in an area that was under continuous onion production for more than 15 years at the Blackshank Farm near Tifton, GA. Field plots were arranged in a randomized complete block design with four replications. Rotation treatments consisted of an onion-carrot rotation and continuous onion production. Double-crop treatments following either rotation consisted of corn, peanut, pearl millet or soybean. To better evaluate the impact of these crops on *B. cepacia* populations, the plot plan established in 2010 was used for the duration of the study. By not changing the layout of the different double-crop treatments, indirect effects due to changes in the composition of total soil microflora, and changes in nutrient concentrations and composition affected by those crops would be minimized. In total, there were

eight treatments and four replicates of each treatment. Onion transplants (cvs. Century and Sweet Vidalia) were obtained from the Vidalia Onion Research Farm near Reidsville, GA. Transplants were set in pegged holes (~15 cm within-row-spacing) on Dec. 10, Dec. 3, Dec. 2, and Nov. 14 in 2009, 2010, 2011, and 2013 respectively. Each rotation treatment consisted of four, four-row beds ~20 m in length, and each double-cropping treatment consisted of two four row beds ~ 20 m in length. There was 1.8-m spacing between the centers of beds. Plots were fertilized as follows: 168 kg ha^{-1} of 18-46-0 (DAP) and 1120 kg $ha^{-1}$of 5-10-15 with 5% sulfur at time of transplanting, 336 kg $ha^{-1}$of 6-12-18 with 4% sulfur at 4 weeks after transplanting (WAT), 336 kg ha $^{-1}$ of 6-12-18 with 4% sulfur at 6 WAT, 224 kg ha^{-1} of calcium nitrate at 8 WAT, and 224 kg ha^{-1} of calcium nitrate 10 WAT. Pendimethalin (Prowl 3.3 EC) at 2.4 LHa^{-1} and oxyfluorfen (Goal 2E) at 2.4 LHa^{-1} were applied at transplanting for weed control. Iprodione (Rovral) at 1.8 LHa^{-1} at 6 and 11 WAT, pyraclostrobin and boscalid (Pristine) at 1.1 kg ha^{-1} AT 7, 10 AND 15 WAT, 15% mancozeb and 46.1% cupric hydroxide (Mankocide) at 2.8 $KgHa^{-1}$ AT 8 WAT, fluopicolide (Presidio) at 1.1 $KgHa^{-1}$ AT 14 WAT, dimethomorph (Forum) at 0.42 $KgHa^{-1}$ AT 15 WAT, and manganese zinc ethylenebisdithiocarbamate (Manzate) at 3.4 kg ha^{-1} at 14 and 15 WAT were applied for fungal disease control. Methomyl (Lannate) at 3.6 l ha-1 at 8, 12, and 14 WAT was applied for insect control. Onion plants were undercut when approximately 70-80% of onion tops were down and bulbs were allowed to dry in the field for 48 h; necks and roots were clipped; and onion bulbs were bagged on May 14, May 2, April 27, and May 9 in 2010, 2011, 2012, and 2014, respectively.

Onions were stored at ambient temperature at the UGA Vidalia Onion Lab for 2 weeks to simulate packing-house conditions for the time it may take to fill a commercial cold room. Onion bulbs were visually rated for disease incidence and severity both externally and internally by cutting bulbs. Severity was evaluated on a 0 to 5 scale, with 0 indicating healthy bulbs, 1=< 20%, 2 =< 40%, 3=<60%, 4=<80%, and 5 indicating bulbs with 100% disease severity. Marketable yield was calculated by subtracting the number of total rotten bulbs from the number of total bulbs harvested and then calculating the percent. Results were compiled and analyzed using PROC GLIMMIX and LSD separation of means. All data analyses were performed using SAS version 9.3 (SAS Institute Inc., Cary, NC).

Grow-Out Assay

To evaluate the mode of action for the reduction of *B. cepacia* populations, a grow-out assay was developed to determine how *B. cepacia* would respond to different crop roots in a closed system. Ten milliliters of autoclaved gravel was placed in sterile 250 ml beakers and layered with 25 g pasteurized potting soil. Pearl millet, onion, corn, and carrot seed were planted, and 1 ml of a bacterial suspension (1×10^6 CFUml^{-1}) of *B. cepacia* strain BC 98-4 (24 h culture grown on King's Medium B at 30°C) was mixed into each beaker. The beakers were placed in sterile 2 L bottles with screw caps to allow for watering, while preventing contamination. Four replicates of each treatment were arranged randomly on the benchtop. Plants were grown for 6 weeks under fluorescent growth lights with 12 h light and 12 h dark periods in the laboratory at room temperature; at the end of the growth period the plants were removed and contents were suspended in

sterile tap water. Serial dilutions (1:9) ranging from 10^0 to 10^{-7} were prepared in triplicate. Aliquants (100 µl) were plated on a modified oxidation fermentation polymyxin bacitracin lactose agar (OFPBL) and incubated at 30°C for 7 days (Henry et al., 1999). Following incubation, the colonies were counted and mean numbers of CFU ml^{-1} were determined for the soil suspension of each plant type. This experiment was repeated once. Data were analyzed with ANOVA and LSD mean separation using SAS version 9.3 (SAS Institute Inc., Cary, NC) to determine statistical differences between plant types and the corresponding bacterial populations.

Bioscreen Assay

A Bioscreen C Automated Microbiology Growth Curve Analysis System (Growth Curves USA, Piscataway, NJ) was used to measure the growth of *B. cepacia* population in the presence of root exudates of corn and pearl millet. Pearl millet (cv. TiftGrain 102) and corn were grown hydroponically for 6 weeks, after which the water was collected and filter-sterilized through a 0.22 µm Millipore filter. A representative strain of *B. cepacia* used in previous studies, strain BC 98-4, was used to determine the interaction between *B. cepacia* at different concentrations of root exudates of millet and corn (Table 2.1). Each of the root exudates was prepared by filter-sterilization to remove potential bacterial contamination according to previously reported methods (Yoshitomi and Shann, 2001). All components were prepared aseptically and sterilized prior to use, and Bioscreen plates were loaded aseptically under a laminar flow hood. Controls, including sterilized water, nutrient broth, and root exudates were included, as well

as a nutrient broth standard (8 g nutrient broth/l of de-ionized water) and an experiment standard (25% nutrient broth standard). Bacterial growth in root exudates was compared with growth in the standards listed above.

This experiment was divided into different runs due to the limitations of space, as the Bioscreen C only holds two plates per run. The duration of each "run" was 5 days and data were collected every 30 min. This experiment was repeated once. Data were compiled and the mean of 10 replications was used to develop one growth curve for each treatment. Area under the growth curve (AUGC) for each replication in each treatment was calculated in SAS version 9.3 (SAS Institute Inc., Cary, NC) then statistical differences between treatments were determined by ttest using SAS.

Results

Double-Cropping/Crop Rotation

From 2010-2014 onions grown after pearl millet consistently had a lower sour skin incidence and a higher level of marketable yield (% healthy bulbs) than did onions associated with other double-cropping treatments. However, the treatments were not significantly different in all years. In 2010 sour skin incidence was significantly less (P = 0.03) in onions double-cropped after pearl millet with a mean sour skin incidence of 5.4% compared to peanut with 21.4% and corn with 18.5% sour skin incidence (Fig. 2-1). In 2011, onions following pearl millet had significantly greater (P = 0.01) percent marketable bulbs (66.9%) than onions following soybean (51.7%) or peanut (56.1%). However, percent marketable

bulbs in onions following pearl millet was not significantly different (LSD = 9.2) from percent marketable bulbs in onions following corn (61.2%) (Fig. 2-2). No field data were collected during the 2013 growing season due to severe bolting of onions and subsequent crop failure. In 2012, percent marketable bulbs for onions following pearl millet, soybean, peanut or corn were 30.6%, 29.2%, 20.9% and 20.9%, respectively but were not significantly different (LSD = 17.4). In 2014, percent marketable bulbs for onions following pearl millet, soybean, peanut or corn were 57.1%, 58.1%, 54.6% and 59.4%, respectively but were not significantly different (LSD = 9.7) (Fig. 2-2). In all years, there were no significant differences in the total yield (Kg/Ha), number of bulbs, and size of bulbs in onions following all crop types.

Grow-Out Assay

When plants were grown in closed container systems, similar to those reported previously, (Haudenshield and Lorbeer 2003), mean populations (1.51 10^8 CFU/g of soil) of *B. cepacia* were significantly higher (*P* =0.03) in soils containing onion roots than with carrot, corn or pearl millet roots. Populations of *B. cepacia* associated with carrot, corn and pearl millet were 1.54×10^7 CFU/g soil, 6.01×10^7 CFU/g soil, and 6.05×10^6 CFU/g soil, respectively but were not significantly different (Fig. 2-3).

Bioscreen Assay

Burkholderia cepacia growth was reduced when grown in root exudates relative to the nutrient broth standard and was similar to the growth curve

associated with the 25% nutrient broth treatment. Increased bacterial growth occurred as corn root exudate concentrations increased from 25% to 50% and from 50% to 75%. In contrast, rate of *B. cepacia* growth decreased as pearl millet root exudate concentrations increased from 25% to 50% and from 50% to 75%. The 75% corn treatment and the 75% millet treatment were the only root exudate treatments that were significantly different. AUGC values for the 75% corn and millet root exudates treatments were 3.9 and 1.3, respectively and were significantly different (P=0.02).

Discussion

Throughout this study, we observed a consistent trend that sour skin incidence and *B. cepacia* populations were lower in onions following pearl millet than in onions following other crops. However, there were only significant differences in the field for sour skin incidence in 2010 and for number of marketable bulbs in 2011 with P values of 0.03 and 0.01, respectively. Significant differences in sour skin incidence in 2010 and marketable yield in 2011, combined with the overall trends of the data, indicate that pearl millet affected *B. cepacia* in some manner. These observations also support the previous observations of Haudenshield and Lorbeer (2003) and Gitaitis (2005). Although there appeared to be a steady decline in the effectiveness of pearl millet over the course of this study, other factors must be considered before dismissing pearl millet as a double-crop alternative for onion growers. These include the continuous onion production where this study was conducted, which probably created higher disease pressure compared to what most growers would

experience. Continuous onion production would most likely support increased pathogen growth throughout the years. In addition to the increased inoculum load present in the soil, higher temperatures and increased moisture levels (data not shown) in 2012 most likely attributed to the increased levels of sour skin observed that year. In addition, there was the potential for introduction of new inoculum via onion transplants. In the years prior to this study, when corn and pearl millet were the only two double-cropping treatments, onions were direct-seeded at the site used for this study. During the time that direct-seeding was used, sour skin levels in onions following corn were significantly higher from that in onions following pearl millet. However, beginning in 2008, the switch was made from direct-seeding to using transplants to provide better weed control. Onion transplants were grown at the UGA Vidalia Onion and Vegetable Research Farm near Reidsville, GA and were transplanted by hand in the research plots at Tifton, GA. Such plants could be exposed to different bacterial strains than what are found in the fields where they are transplanted. This might have introduced new strains of *B. cepacia* surviving as rhizosphere inhabitants on onion transplants. Previous research has shown that *B. cepacia* is an excellent soil survivor and it has the capacity to inhabit plant roots as an endophyte or colonize the rhizosphere (Chiarini et al., 2000; Tabacchioni et al., 2002; Compant et al., 2008). In 2016, field-grown onion transplants produced at the same location as those used in this study developed sour skin when transplanted into potting soil and grown in the green house (personal communication Spencer Stumpf). Thus, the introduction of new strains of *B.*

cepacia may be possible. New strains have the potential to affect the outcome of the study for a couple of reasons. First, newly introduced strains would not have been exposed to the crop rotation/double-cropping treatments. The endogenous strain could have been affected through unidentified selection pressures as a consequence of the double-cropping treatments. For example, there could have been a change in the composition of the microflora. Second, the crop rotation/double-cropping system could have reduced the inoculum load in the soil and the introduction of a new strain on transplant roots would have subverted the benefit of a reduced inoculum load. In the case of direct-seeding, the roots of developing seedlings in soils with a reduced inoculum load would be less likely colonized with soil-borne populations of *B. cepacia* and there is no evidence that this bacterium is seed-borne. Thus, a reduction of soil inoculum levels would be beneficial. In comparison, transplants harboring pathogenic bacterial populations on the rhizoplane would put the crop at a distinct disadvantage. These factors would override the effects pearl millet on bacterial populations if fresh inoculum was introduced annually on transplants. As previously mentioned in years prior to this study when direct-seeding was used, there was a consistently significant difference between pearl millet and corn in terms of percent sour skin and marketable yield (Gitaitis, et al., 2005). These particular facets are possibilities for future research, particularly the documentation that the roots of transplants may already be contaminated early in the season. It has generally been assumed that the source of inoculum for sour skin has been soil-borne in production fields and not associated with transplants.

Additionally, a direct interaction between pearl millet roots and *B. cepacia* appears to occur. The *in vitro* assay showed that pearl millet supported the lowest *B. cepacia* population when compared to onion, which supported the highest population. BioScreen C assays also showed that pearl millet roots supported the lowest pathogen populations when compared to corn root exudates. Preliminary studies using filter paper disks saturated with root exudates indicated no visible toxic interaction between root exudates and *B. cepacia*; however, the results from the BioScreen C study indicated a direct effect of root exudates on bacterial growth. Additional work confirming these observations, identifying the chemical composition of the root exudates, and related aspects may result on a better understanding of the interactions between *B. cepacia* and biological as well as physical components of the soil environment.

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Tables

Table 2-1. Treatments evaluated with a BioScreenC unit to study the effects of pearl millet and corn root exudates on the growth of *Burkholderia cepacia.*

Bioscreen Treatments				
Description	Root Exudate µl	Sterile Water µl	Nutrient Broth µl	Bacterial Suspension µl
100 % Millet	180	0	0	20
75% Millet	135	0	45	20
50% Millet	90	45	45	20
25% Millet	45	90	45	20
100% Corn	180	0	0	20
75% Corn	135	0	45	20
50% Corn	90	45	45	20
25% Corn	45	90	45	20
Root Exudate Control	180 millet	20	0	0
Root Exudate Control	180 corn	20	0	0
Experiment Standard	0	135	45	20
Nutrient Broth Control	0	155	45	0
Standard	0	0	180	20
Sterile Water Control	0	200	0	0

Figures

Figure 2-1. Percent sour skin incidence of onions at postharvest in onions double-cropped with pearl millet, soybean, corn, or peanut from 2010-2014.

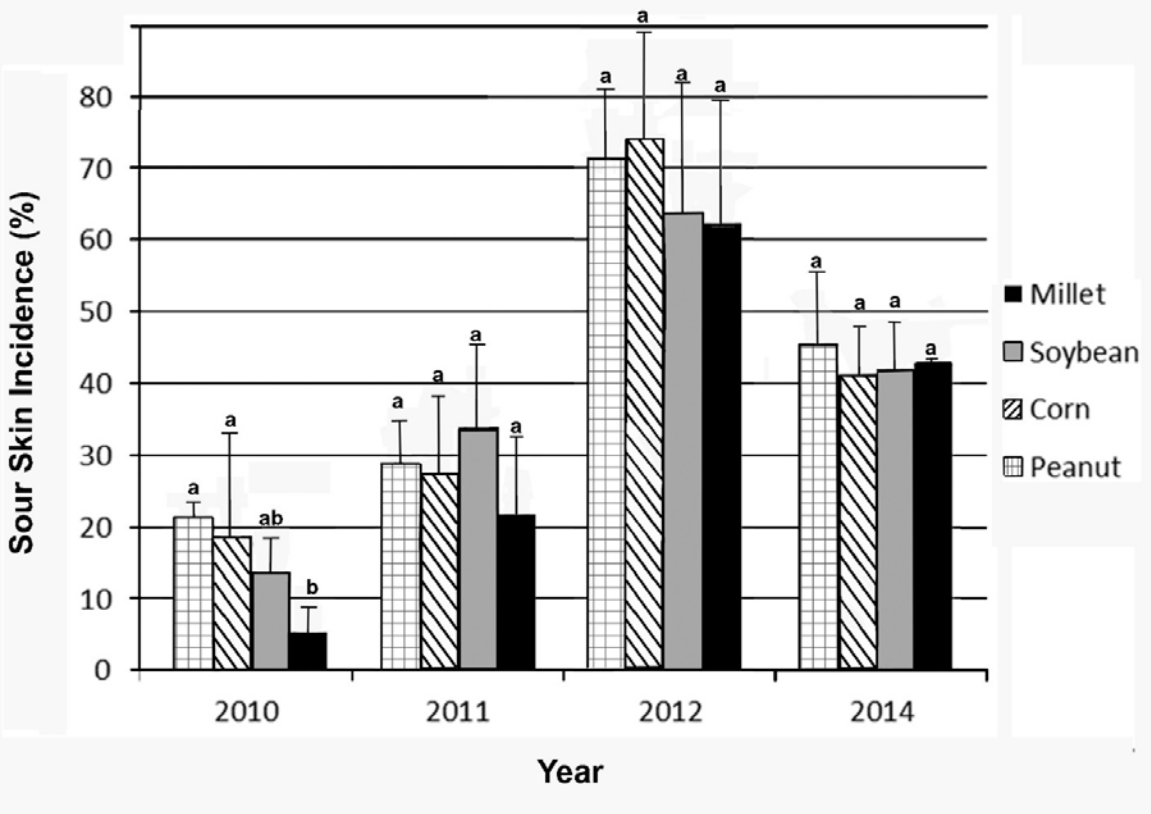


Figure 2-2. Percent marketable yield of onions at postharvest in onions double-cropped with pearl millet, soybean, corn, or peanut from 2010-2014.

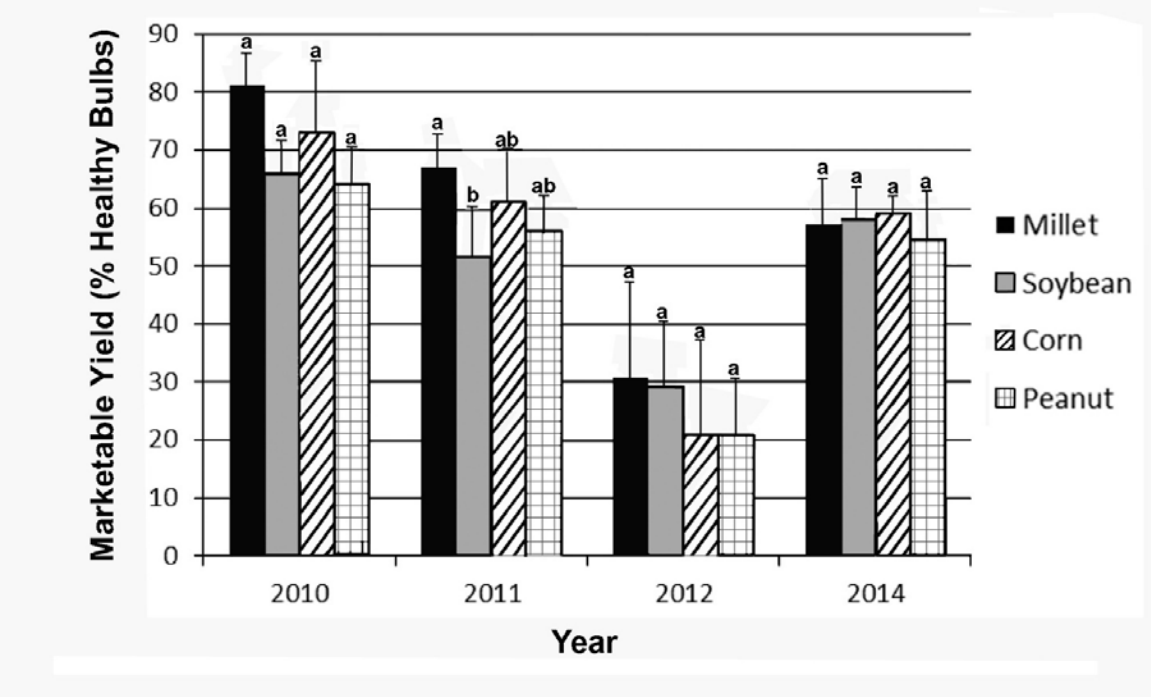


Figure 2-3. Final populations (CFU/g soil) of *Burkholderia cepacia* after $1^{x}10^{6}$ CFU/ml inoculum was added to pot cultures of onion, carrot, corn, or pearl millet. The final population represents the mean of two experiments with four replications per experiment.

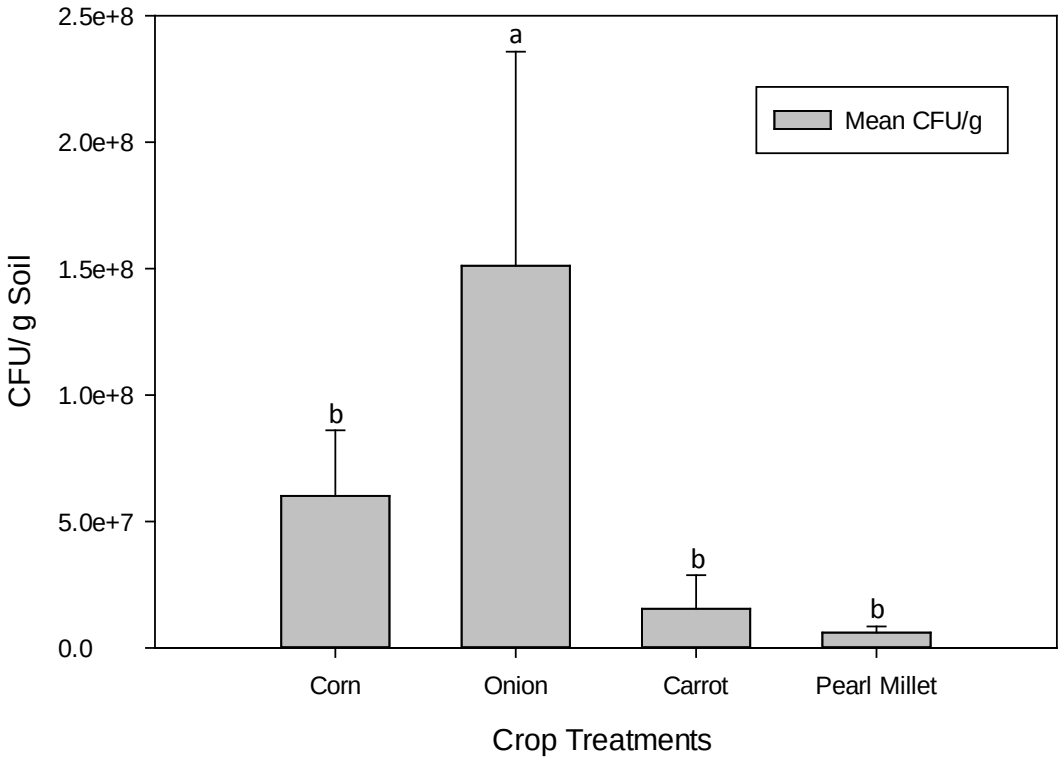
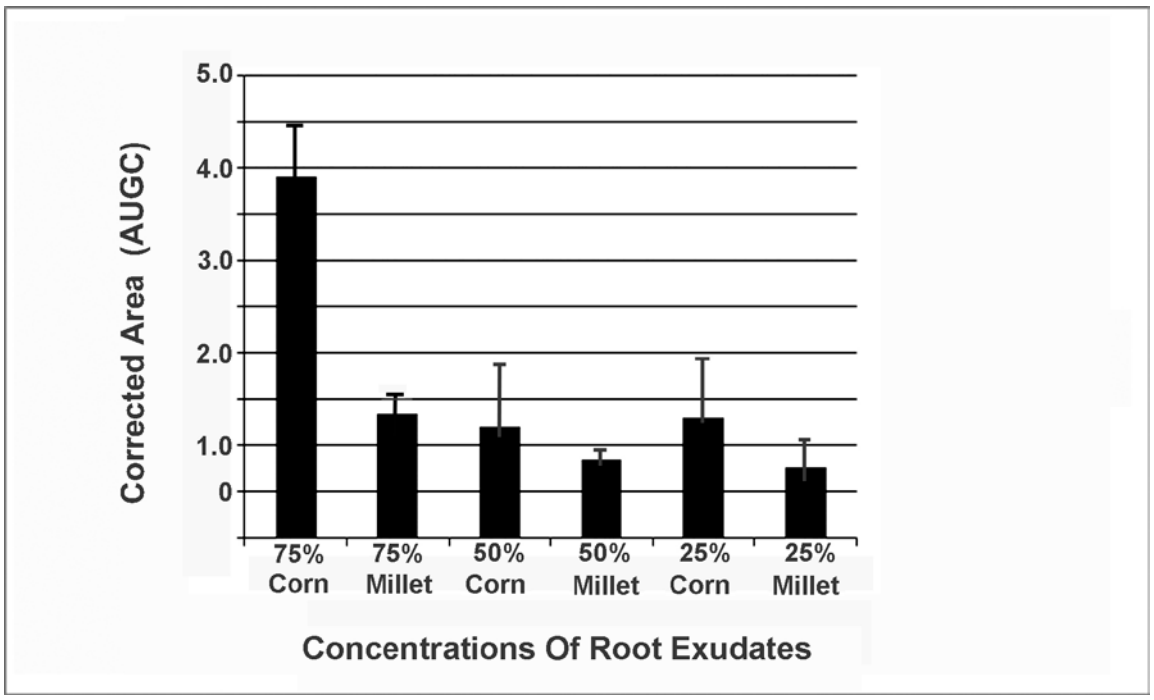


Figure 2-4. Corrected area under growth curve (AUGC) data for *Burkholderia cepacia* (BC 98-4) after 20µl of a bacterial suspension ($1^{x}10^{8}$ CFU/ml) was introduced into different dilutions of root exudates from pearl millet, corn, nutrient broth, or sterile water. Each curve represents the mean of two experiments with 10 physical replications per experiment.



Chapter 3

IDENTIFICATION OF MACRONUTRIENT AND MICRONUTRIENT CONCENTRATIONS AND MICRONUTRIENT RATIOS AS INDEPENDENT VARIABLES IN MULTIPLE REGRESSION MODELS FOR THE PREDICTION OF SOUR SKIN ONION INCIDENCE OR SEVERITY[2]

[2] Watson, A., Dutta, B., and Gitaitis, R. To be submitted to Plant Disease

Abstract

Vidalia onions, *Allium cepa*, are a high-value, specialty crop grown in Georgia that are subject to significant losses annually due to sour skin, caused by the bacterium *Burkholderia cepacia*. The current lack of effective management strategies necessitates new management strategies. The role of micronutrients in disease development was studied using Vidalia onions and *B. cepacia,* as the pathosystem. In 2012 and 2014, onion bulbs were grown in research plots near Tifton, GA and soils were evaluated for mineral composition. Data were analyzed via stepwise and maximum R^2 improvement regression using sour skin incidence or severity as the dependent variable. In 2012 the tissue model (P=0.0002; adj. R^2=0.51) included Cu:Fe, Zn:Fe, Mn, S:Al, and N as independent variables. Due to excessive bolting in the field in 2013, onions purchased from grocery stores were substituted and artificially inoculated with a suspension of ~1 $^{x}10^{8}$ CFU of *B. cepacia*/ml and incubated at 30 °C until symptom expression. The grocery store model (P=0.00001; adj. R^2=0.43) contained Cu:Fe, Al, K, S, and N as independent variables. In 2014, a tissue model (P=0.00002; adj. R^2=0.34) contained Cu:Fe, Na:Fe, Mn:Zn, Mn, Ca, and N as independent variables. The Cu:Fe ratio consistently appeared in most models and was negatively correlated (P=0.004; R^2=0.25) with sour skin in 2012. In addition, Cu, Fe, Zn and Mn, or ratios containing them, occurred as independent variables in all years. In addition, RNA was extracted from healthy bulbs with high Cu:Fe ratios sampled from areas experiencing low sour skin incidence and from healthy bulbs with low Cu:Fe ratios sampled from high sour skin areas and sent for transcriptome

analysis to identify what genes were differentially expressed. Over 30 genes of interest associated with plant disease were identified following transcriptome analysis, including PR1, which was associated with the high Cu:Fe ratio and low disease incidence.

Introduction

Vidalia onions, *Allium cepa*, are a high-value specialty crop grown in southeastern Georgia (Boyhan and Torrence, 2002). This short-day sweet onion, along with all other varieties and types of onion is susceptible to a wide variety of pre- and post-harvest diseases. One disease of significant importance, sour skin of onion, is caused by the soil-borne bacterium *Burkholderia cepacia*, and is responsible for significant yield losses annually (Davis, 2008). Control of this disease centers around cultural practices aimed at reducing exposure to, and spread of the pathogen (Boyhan and Kelley, 2007). There are no chemical control practices or resistant cultivars currently available for the management of sour skin of onion (Boyhan and Kelley, 2007; Davis, 2008). As a result, new management practices need to be developed to prevent in-field infection.

The relationship between plant disease and nutrition has been explored as a means to reduce incidence and severity of many plant diseases (Datnoff et al., 2007 and Dordas, 2009). There are sixteen macro- and micronutrients essential for healthy plant growth (Datnoff et al., 2007). The classification of elements being a macronutrient or a micronutrient is based on the amount of each required by the plant for optimum nutrition. The macronutrients include hydrogen (H),

carbon (C), oxygen (O), nitrogen (N), potassium (K), calcium (Ca), magnesium (Mg), phosphorus (P), and sulfur (S). These elements are required in larger quantities by the plant, although levels may vary by. The micronutrients, which are required in smaller quantities are, chlorine (Cl), boron (B), iron (Fe), manganese (Mn), zinc (Zn), copper (Cu), and molybdenum (Mo) (Agrios, 2005; Datnoff et al., 2007). In onion there are several key mineral nutrients that are important for optimal crop production. These nutrients are Ca, Mg, N, P, K, B, Zn, and S. In addition, the micronutrients; Mn, Fe, Cu, Mo, and Cl affect onion growth and production (Boyhan and Kelley, 2007). The exact interaction between these elements and plant disease varies, based on many factors including the host-pathogen system, environment, plant cultivar, and plant growth stage (Agrios, 2005; Datnoff et al., 2007; Dordas, 2009). Examples can be found for each essential nutrient's association with plant disease incidence and severity (Datnoff et al., 2007). There are numerous examples in the literature on this topic and a few of them are discussed below.

Calcium was shown to reduce disease caused *by Sclerotium rolfsii* Sacc. in carrot by decreasing host susceptibility (Datnoff et al. 2007). However, Ca is utilized by the pathogen *Colletotrichum trifolli* and aids in tissue maceration and increased disease severity in alfalfa (Kiraly, 1976).

Magnesium application has been linked to a decrease of several diseases including bacterial blight of cotton, caused by *Xanthomonas campestris* pv. *malvacearum*, and soft rot of potato, caused by *Erwinia carotovora* pv. *atroseptica*, (Batson, 1971; Kelman et al., 1989; McGuire and Kelman, 1986).

However, Mg is also associated with an increase in bacterial leaf spot of pepper and tomato, caused by *X. campestris* pv. *vesicatoria*, and pod rot of peanut, caused by *Fusarium*, *Pythium*, and *Rhizoctonia* spp., (Woltz and Jones, 1979; Csinos and Bell, 1989).

Nitrogen treatments have been linked to an increase in diseases caused by obligate parasites like *Puccinia graminis*, *Erysiphe graminis*, and Tobacco mosaic virus, as well as the facultative pathogen *Pseudomonas syringae* (Howard et al., 1994; Büschbell and Hoffmann, 1992; Singh, 1970; Hoffland et al., 2000). This increase in disease is associated with an increase in green tissue that is essential for the growth of obligate parasites (Dordas, 2008). Nitrogen has also been linked with decreases in diseases caused by *X. euvesicatoria*, *Alternaria solani*, and *F. oxysporum* (Chase, 1989; Blachinski et al., 1996; Woltz and Engelhar, 1973).

As with other nutrients, P is no exception when it comes to conflicting interactions with plant disease. Phosphorus application decreased the incidence of bacterial blight of broad bean plants, caused by *P. syringae* pv. *syringae*, (Abd El Moneem et al., 1994). However, P tends to increase the susceptibility of plants to viruses in general. This increase in susceptibility is attributed to the need for P during viral replication (Prabhu, Fageria, Berni, and Rodrigues).

In general, bacterial disease severity decreases in response to increased levels of potassium with a few exceptions. Potassium application has been shown to increase fire blight of apple, caused by *E.amylovora*, and bacterial

canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Chase et al., 1968; Walker J.C., 1969). The interaction between K and other pathogen groups is more varied than with bacterial pathogens (Datnoff et al., 2007).

Increased levels of B generally are associated with a decrease in disease and B deficiency is linked with increased pathogen virulence (Stangoulis and Graham, 2007). Zinc has been shown to decrease bacterial leaf spot severity caused by *X. euvesicatoria* on pepper. However, Zn is crucial in the production of tabtoxin by *P. syringae*, thus playing a critical role in pathogenicity (Adaskaveg and Hine, 1985; Durbin and Uchytil, 1985). Sulfur fertilizer application reduced incidence and severity of several fungal diseases. The decrease in disease has been attributed to the induction of sulfur-induced resistance (SIR) (Haneklaus, Bloem, and Schnug, 2007).

The majority of these studies have evaluated the effect(s) of a single element on plant disease severity, while very few have looked at total nutrient profiles in association with disease. Because of the varied interactions and sometimes contradictory effects observed with each element, and the lack of research on multiple nutrient interactions, it is important to investigate effect of the interaction of nutrients on pathosystems of interest. Our overall research objective was to determine which elements play an essential role in the relationship between *B.cepacia* and Vidalia onions. This was achieved through onion bulb and soil analysis in conjunction with disease ratings. A potential mode of action was explored through transcriptome analysis to identify genes, including

possible defense-related genes, which may be involved in nutrient-host-pathogen interactions.

Materials and Methods

Research was conducted over a 3-year period to investigate what effects mineral levels in soils and tissues have on sour skin incidence and severity.

2012 and 2014 Field Trials

Vidalia onions were cultivated under standard grower practices in an area that had been under continuous onion production for more than 15 years at the Blackshank Farm in Tift County, GA. Field plots were arranged in a randomized complete block design with four replications. Treatments consisted of a rotation of onion and carrot or continuous onion production in the winter. Both rotation treatments were double-cropped with corn, peanut, pearl millet or soybean in the summer. To better evaluate the impact that the crops selected for the double-cropping study may have on *B. cepacia* populations, the plot plan established in 2010 was used for the duration of the study. By maintaining the layout of the different double-crop treatments, effects due to changes in the composition of other soil microflora, as well changes in nutrient concentrations and composition affected by those crops could be minimized.

Onion transplants (cvs. Century and Sweet Vidalia) were obtained from the Vidalia Onion & Vegetable Research Farm near Reidsville, GA Toombs Co. Transplants were set in pegged holes (~15 cm within-row-spacing) on Dec. 2 in 2011 and Nov. 14 in 2013. Each rotation treatment consisted of four, four-row

beds ~20 m in length, and each double-cropping treatment consisted of two four row beds ~ 20 m in length. Prepared beds were spaced 1.8-m apart between centers. Plots were fertilized as follows: 168 $KgHa^{-1}$ of 18-46-0 (DAP) and 1120 $KgHa^{-1}$of 5-10-15 with 5% sulfur at time of transplanting, 336 $KgHa^{-1}$of 6-12-18 with 4% sulfur at 4 weeks after transplanting (WAT), 336 $KgHa^{-1}$ of 6-12-18 with 4% sulfur at 6 WAT, 224 $KgHa^{-1}$ of calcium nitrate at 8 WAT, and 224 $KgHa^{-1}$ of calcium nitrate 10 WAT. Pendimethalin (Prowl 3.3 EC) at 2.4 LHa^{-1} and oxyfluorfen (Goal 2E) at 2.4 LHa^{-1} were applied at transplanting for weed control. Iprodione (Rovral) at 1.8 LHa^{-1} at 6 and 11 WAT, pyraclostrobin and boscalid (Pristine) at 1.1 $KgHa^{-1}$ AT 7, 10 AND 15 WAT, 15% mancozeb and 46.1% cupric hydroxide (Mankocide) at 2.8 $KgHa^{-1}$ AT 8 WAT, fluopicolide (Presidio) at 1.1 $KgHa^{-1}$ AT 14 WAT, dimethomorph (Forum) at 0.42 $KgHa^{-1}$ AT 15 WAT, and manganese zinc ethylenebisdithiocarbamate (Manzate) at 3.4 $KgHa^{-1}$ at 14 and 15 WAT were applied for fungal disease control. Methomyl (Lannate) at 3.6 LHa-1 at 8, 12, and 14 WAT was applied for insect control. Onion plants were undercut when approximately 70-80% of onion tops were down and bulbs were allowed to dry in the field for 48 h. Necks and roots were clipped, and onion bulbs were bagged on April 27, and May 9 in 2012, and 2014, respectively. Onions were stored at ambient temperature at the UGA Vidalia Onion Lab for 2 wks. Onion bulbs were visually rated for disease incidence and severity externally and internally by cutting bulbs. Severity was evaluated on a 0 to 5 scale with 0 indicating healthy onions, 1 = < 20%, 2=<40%, 3=<60%, 4=<80% and 5 indicating onions with 100% severity. In addition, at harvest two

healthy-appearing onion bulbs were sampled from each replication of each treatment for nutrient analysis. Composite soil samples were collected from each replication in each treatment by taking 10 samples with a soil probe in an X pattern across plant beds and compiling them (approximately 1 lb. soil). Both soil and bulb samples were transported to the Plant, and Water Laboratory in Athens, GA for tissue nutrient analysis. These analyses consisted of: A P1 basic plant test for nitrogen, sulfur, potassium, phosphorus, calcium, magnesium, manganese, iron, aluminum, boron, copper, zinc, and nickel; a S1 routine soil test for pH, phosphorus, potassium, calcium, magnesium, manganese and zinc, and a S2 test for sodium, iron, copper, chromium, molybdenum, nickel, cadmium and lead.. Organic matter was oxidized by exposing tissues to high temperatures and the ash was dissoluted with hydrochloric acid. The concentrations of the elements in the digest were determined by atomic absorption spectrometry. Results were compiled and analyzed by standard and multiple regression where sour skin incidence and sour skin severity were the dependent variables and nutrients and nutrient ratios were the independent variables. This analysis was done to evaluate regression models to identify specific nutrients that correlated with disease incidence and severity. Initially all nutrients and their ratios were screened for significance in their association with sour skin incidence and severity. Several models were generated for each year of the study and from the group of models generated, some were selected for further evaluation based on: 1) models consistently containing similar nutrients or nutrient ratios; 2) significant values; 3) reasonably high adjusted R^2 values; and 4) models with variance

inflation factor (VIF) values below 5, to reduce problems with collinearity. All statistical analyses were conducted using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA).

2013

In 2013 there was a complete crop loss due to bolting, so an alternative study was conducted. Sweet onion bulbs were purchased from grocery stores in Tifton, GA and brought to the lab where they were stored at 4°C. Prior to inoculation bulbs were cleaned by removing all dry skin, basal roots, and necks. Onion bulbs were then surface disinfested by soaking in a 10% sodium hypochlorite solution for 1 minute and then rinsed with sterilized water to remove chemical residue. Bulbs were inoculated with an aqueous suspension (1×10^8 CFU/ml) of *B. cepacia* culture grown overnight on King's medium B (KMB) at 30°C. Then 500µl of inoculum was injected into the shoulder of each onion using a hypodermic needle and syringe. Onions were incubated at 30°C for 72 h then rated for disease severity. Severity was determined by slicing onion bulbs in half to obtain a healthy half and a diseased half. The volume of rot was calculated by measuring the length, width, and depth of the decayed area and inserting the values in to the equation for the volume of an ellipsoid ($\frac{4}{3}\pi abc$) and dividing by two. The healthy half of the onion bulb was sent to the UGA Soil, Plant, and Water Laboratory in Athens, GA for tissue nutrient analysis. These analyses consisted of a P1 basic plant test for nitrogen, sulfur, potassium, phosphorus, calcium, magnesium, manganese, iron, aluminum, boron, copper, zinc, and nickel. Organic matter was oxidized by exposing tissues to high temperatures

and the ash was dissoluted with hydrochloric acid. The concentrations of the elements in the digest were determined by atomic absorption spectrometry. Results were compiled and analyzed via standard and multiple regression via SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) where sour skin severity was the dependent variable and mineral data were the independent variables. This experiment was conducted three times and each experiment consisted on twelve replications.

Transcriptome Analysis

In 2012, 2013, and 2014 the copper to iron ratio (Cu:Fe) consistently occurred in significant models and was negatively correlated with sour skin incidence and severity (Fig. 3-3). Based on this finding, and previous observations with Tomato Spotted Wilt (TSW) in tobacco and bacterial leaf spot in pepper (data not shown), transcriptome analysis was conducted to explore the interaction between mineral content of plant tissues and plant defense. To determine the role nutrition played in plant disease resistance transcriptome analysis was used to identify potential defense-related gene targets of interest for future studies.

Six onion bulbs were selected in 2014 for transcriptome analysis based on Cu:Fe ratios and on sour skin ratings for the plots from which they were harvested. Three bulbs were selected from plots with low disease ratings and high Cu:Fe ratios and three bulbs were selected from plots with low Cu:Fe ratios (Fig. 3-7). RNA extraction was done using Qiagen Plant Mini RNA extraction kit according to manufacturer's instructions and RNA samples were sent to Science

Exchange, Palo Alto, CA for RNA sequencing and construction of an RNAseq library. Further analysis was conducted by the Quantitative Biology Consulting Group, Athens, GA. Briefly, raw data were transferred from the University of Rochester Genomics core, then *De Novo* assemblies were performed using Trinity assembler. Data obtained were filtered and mapped to identify differentially expressed genes. The differentially expressed genes were normalized and annotated via BlastX against SwissProt and RegSeq databases.

Results and Discussion

In 2012 a tissue model was developed with sour skin incidence as the dependent variable containing the ratios of Cu:Fe, Zn:Fe, and S:Al and the elements Mn and N as the independent variables (P=0.0002; adj.R^2=0.51) (Fig.3-1). A soil model was also developed with sour skin incidence as the dependent variable (P=0.00006; adj. R^2=0.57) that included the ratios of Zn:Fe, Fe:Mn, and Mn:Zn, as well as the elements Cu and K as the independent variables (Fig 3-2). In 2013 a grocery store tissue model was developed with sour skin severity as the dependent variable and the ratio Cu:Fe and elements Al, K, N, and S as the independent variables (P=0.00001; adj. R^2=0.43)(Fig. 3-4). Additionally, in 2014 a tissue model was developed with sour skin severity as the dependent variable and contained the independent variables Cu:Fe, Na:Fe, and Mn:Zn ratios and Ca, Fe, Mn, and Zn (P=0.00002; adj. R^2=0.34) (Fig.3-5). A final model was developed in 2014 with sour skin incidence as the dependent variable and the ratios Cu:Mn and Fe:Mn as well as Mn, Ca, and N as independent variables (P=0.00004; adj. R^2 0.34) (Fig. 3-6).

The elements Cu, Fe, Zn, and Mn were consistently associated with sour skin incidence and severity throughout this study. These elements are of particular interest as they are metal cofactors for the three superoxide dismutase (SOD) enzymes found in plants. The three SODs found in plants are the Cu/Zn SOD, FeSOD, and MnSOD (Giannopolitis and Ries, 1977; Mehendy, 1994). SOD enzymes detoxify reactive oxygen species (ROS) within the plant, these ROS are byproducts of normal cellular function and oxidative bursts associated with the infection process (Durrant and Dong, 2004). When these ROS are detoxified H_2O_2 is produced, that can initiate the production of salicylic acid. In turn SA, activates plant defense genes and systemic acquired resistance (Durrant and Dong, 2004). These SOD enzymes are partially regulated by the availability of their metal cofactors. It has been shown that Cu/Zn SOD expression is regulated by Cu availability within the plant. Fe SOD expression can also be regulated by Cu availability, or deficiency (Cohu and Pilon, 2007).

Research conducted on bacterial leaf spot (BLS) of pepper and tomato spotted wilt (TSW) of tobacco support the interaction of these micronutrients with SOD enzymes and plant disease resistance (Gitaitis et al., 2013). BLS severity correlated with a similar mineral model in pepper that also contained Cu, Fe, Mn and Zn but a ratio of Zn/Fe instead of Cu/Fe. BLS severity also was significantly correlated with levels of expression of Cu/Zn SOD, Fe SOD and Mn SOD genes with R^2 values of 0.97, 0.86 and 0.79, respectively (Gitaitis personal communication). Furthermore, expression of the plant defense gene *NPR1* also was significantly correlated with BLS severity with a R^2 value of 0.88 and with

expression of all three SOD genes with an R^2 of 0.93 (Gitaitis and Dutta personal communication). With TSW of tobacco, a micronutrient model that relied heavily on the Cu:Fe ratio, predicted high risk sites and low risk sites in two consecutive years (data not shown).

Transcriptome analysis generated 209,187 transcript isoforms; these were filtered down to 76,439 transcripts. Following analysis and annotation approximately 300 transcript annotations were identified as being significantly differentially regulated in onions with low Cu:Fe ratio and high disease vs. those with high Cu:Fe ratio and low disease. Of these transcripts, 30 were identified as being associated with disease resistance in the literature (Tables 3-2 and 3-3.). Additionally nine transcripts were up-regulated in onions with high Cu:Fe ratios when compared to onions with low Cu:Fe ratios. The most important of these transcripts was associated with the *PR1* gene, which is involved in the systemic acquired resistance pathway (Cameron, 1999; Van Loon and Van Strien, 1999). Quantifying the effects on plant disease resistance genes such as *NPR1*, *PR1*, or the SOD genes has been a challenge with onion as the sequences of most of those genes were unknown at the time the data in this study were collected. Hence, it was impossible to evaluate gene expression by qPCR.

Serendipitously, an interesting trend was identified; onion samples obtained from areas where pearl millet was previously grown as a summer double-crop had 23 up-regulated defense-related transcripts that were not found in onions grown after corn or soybean. This observation could be considered independent from the effects of the Cu/Fe ratio as onion bulbs from the millet

plots were represented by both a high and low Cu/Fe ratio. This suggests that pearl millet is affecting differential gene expression on subsequent crops of onion in a manner possibly unrelated to micronutrients or in a way micronutrients are not associated with our mineral models.

The exact interaction between micronutrients and sour skin incidence and severity is not completely understood, but these data indicate a relationship between mineral makeup in the soil, plant tissues and disease development. Furthermore, based on the preliminary findings of the transcriptome analysis, we speculate that micronutrients may be affecting activity of plant disease resistance genes. Evidence for the involvement of SAR as a mode of action has been observed in the form of a consistent interaction between Cu, Fe, Zn, and Mn and plant disease has been established over the course of this study. As previously mentioned, these nutrients are the metal cofactors for plant SOD enzymes as well as the up-regulation of PR1, which is a component of the SAR pathway in plants with high Cu:Fe ratios and low disease.

The relationships identified here warrant further research that could result in the development of risk prediction systems and eventually the development of management strategies through the application of specific levels of micronutrients. In order to develop a risk prediction system, the models developed in this study will be evaluated in the future using samples obtained from a survey of fields from the Vidalia onion growing region in Georgia. By validating the models, one cohesive model that can accurately predict sour skin risk based on soil micronutrients will be established. In addition to validating the

models, sour skin incidence and severity, soil and tissue micronutrients, and PR1 activity will be evaluated to strengthen the association between micronutrients and plant resistance. This will be done by utilizing qPCR with primers developed by ElMorsi et al (2015) for PR1 in onion.

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Tables

Table 3-1. Results of multiple regression analysis from 2012 – 2014 when sour skin incidence was the dependent variable and micronutrient concentrations or ratios of micronutrient concentrations in bulb tissues or soil served as independent variables.

2012 Field Trial Models			
Source	**Model**	***P* value**	**Adjusted R^2**
Tissue	sour skin incidence= -139Cu:Fe – 14.7Zn:Fe + 0.7Mn + 0.21S:Al – 0.002N + 107.9	0.0002	0.51
Soil	sour skin incidence= -57Zn:Fe + 40Cu – 5.6 Fe:Mn + 2.2Mn:Zn – 0.46K + 97.7	0.00006	0.57
CuFe	sour skin incidence= -144.5Cu:Fe + 83.7	0.004	0.25
2013 Grocery Models			
Source	**Model**	***P* value**	**Adjusted R^2**
Tissue	sour skin severity= -17.9Cu:Fe – 0.34Al + 0.003K – 0.001N + 2.93	0.0001	0.43
2014 Field Trial 1 Models			
Source	**Model**	***P* value**	**Adjusted R^2**
Tissue	sour skin severity= -353Cu:Fe + 35Na:Fe + 27.8Mn:Zn – 1.77Mn + 0.04Ca + 0.003N – 28.8	0.00002	0.34
Tissue	sour skin incidence= -49.9Cu:Mn – 1.56Fe:Mn – 1.06Mn + 0.024 Ca + 0.0013N + 24.52	0.00004	0.34

Table 3-2. Transcriptome analysis of healthy onion bulbs. These genes of interest were defense-related and up-regulated in response to elevated Cu:Fe ratios. The genes are arranged in order of highest to lowest fold change, which is indicative of the degree of up-regulation when compared to all other onion samples. Also presented in this table is *P* value, false discovery rate (FDR), the description of the gene, and references linking each gene with plant disease resistance.

Fold Change	*P* Value	FDR	Description	Reference
5002	6.02E-06	0.0128	Pathogenesis-related protein 1A	Cameron, R.K. et al., 1999; Van Loon, L.C. and Van Strien, E.A., 1999
4733	4.60E-10	5.78E-06	B2 protein	Shorrosh et al., 1993; Oh et al., 2010
2365	1.02E-05	0.00875	SCY1-like protein 2	
2188	5.50E-08	0.00038	Probable protein NAP1	Luo, M. et al., 2011
1879	2.34E-07	0.00046	Probable serine/threonine-protein kinase WNK4	Song, W-Y. et al., 1995
1524	1.12E-06	0.00248	Cytochrome c-type biogenesis ccda-like chloroplastic protein 2	
1370	2.50E-06	0.0043	Two-component response regulator ARR8	
1045	1.82E-05	0.0172	Probable receptor-like protein kinase At5g15080	
38	2.35E-05	0.03284	F-box protein At1g78280	Hondo et al., 2007; Craig et al., 2009; Dielen et al., 2010; Santer A. and Estelle, M., 2010; Schumann et al., 2011; Sadanandom et al., 2012

Table 3-3. Transcriptome analysis of healthy onion bulbs. These genes of interest were differentially up-regulated in onion bulbs grown following a summer double-crop of pearl millet when compared to onion bulbs grown following corn and soybean. The genes are arranged in order of highest to lowest fold change, which is indicative of the degree of up-regulation when compared to all other onion samples. Also presented in this table is *P* value, false discovery rate (FDR), the description of the gene, and references linking each gene with plant disease resistance.

Fold Change	*P* Value	FDR	Description	Reference
6468	9.46E-09	3.09E-05	Ubiquitin-conjugating enzyme E2	Hondo et al., 2007; Craig et al., 2009; Dielen et al., 2010; Santer A. and Estelle, M., 2010; Schumann et al., 2011; Sadanandom et al., 2012
5309	5.84E-14	6.31E-10	Universal stress protein A-like protein	Kerk, D. et al., 2003
3991	2.05E-12	1.71E-08	Glutaredoxin-C4	Foyer, C.H. et al., 2003
3775	2.69E-06	0.00337	HVA22-like protein k	Su, J. et al., 1998
3357	9.22E-12	6.94E-08	Cytochrome P450 85A1	
2276	1.73E-09	8.12E-06	Cytochrome P450 85A1	
2056	5.27E-09	1.98E-05	Cytochrome P450	Li, X. et al., 2010
2007	8.56E-06	0.00767	Transmembrane E3 ubiquitin-protein ligase	Hondo et al., 2007; Craig et al., 2009; Dielen et al., 2010; Santer A. and Estelle, M., 2010; Schumann et al., 2011; Sadanandom et al., 2012
1752	4.01E-08	0.00011	Cytochrome P450 85A1	Li, X. et al., 2010
1751	2.35E-06	0.00414	Probable cinnamyl alcohol dehydrogenase	Logeman, E. et al., 1997; Tranchet, M. et al., 2010
1358	5.12E-07	0.00087	Monocopper oxidase-like protein	Chen et al. 2016
1330	6.88E-07	0.00108	G-type lectin S-receptor-like serine/threonine-protein kinase SD2-2	Song, W-Y. et al., 1995
1113	1.38E-05	0.01433	Probable transmembrane ascorbate ferrireductase 2	Bérczi, Su, Asard 2007
1078	4.77E-06	0.00501	Metal tolerance protein C1	Zhang, M. et al., 2011; Ricachenevskey, F.K. et al.,

				2013
952	3.42E-05	0.02727	BTB/POZ domain-containing protein NPY2	Hondo et al., 2007; Craig et al., 2009; Dielen et al., 2010; Santer A. and Estelle, M., 2010; Schumann et al., 2011; Sadanandom et al., 2012
866	6.72E-05	0.04506	Myb-related protein 3R-1	Liu, Schiff, Dinesh-Kumar 2004
859	2.69E-05	0.01921	Myb-related protein 3R-1	
844	3.36E-05	0.0224	Serine/threonine-protein kinase EDR1	Song, W-Y. et al., 1995
788	5.13E-05	0.03112	Cytochrome P450 85A1	Li, X. et al., 2010
766	6.42E-05	0.03657	Zinc phosphodiesterase ELAC protein 2	
759	6.76E-05	0.03824	Kelch-like protein 12	Hondo et al., 2007; Craig et al., 2009; Dielen et al., 2010; Santer A. and Estelle, M., 2010; Schumann et al., 2011; Sadanandom et al., 2012
706	9.50E-05	0.04928	Purple acid phosphatase 2	
45	7.17E-05	0.04769	Copper transport protein ATX1	

Figures

Figure 3-1. Fit of observed vs. predicted percent sour skin incidence from a multiple regression analysis model where sour skin incidence was the dependent variable and the Cu:Fe ratio in tissues was the independent variable from a field study in 2012.

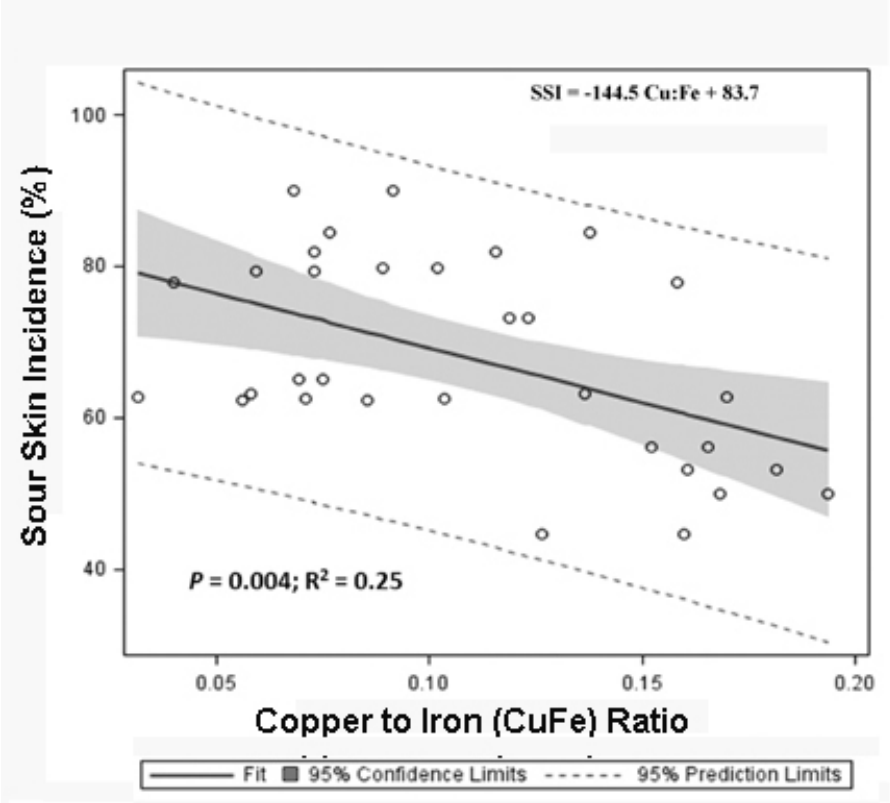


Figure 3-2. Onion bulbs selected for transcriptome analysis on the basis of bulbs with a low Cu:Fe ratio coming from an area with high levels of sour skin incidence (OZ3, OZ4, and OM1) or with a high Cu:Fe ratio coming from areas with low levels of sour skin incidence (OS1, OS2, and OM3), that were selected from the linear regression shown in Fig. 3-3.

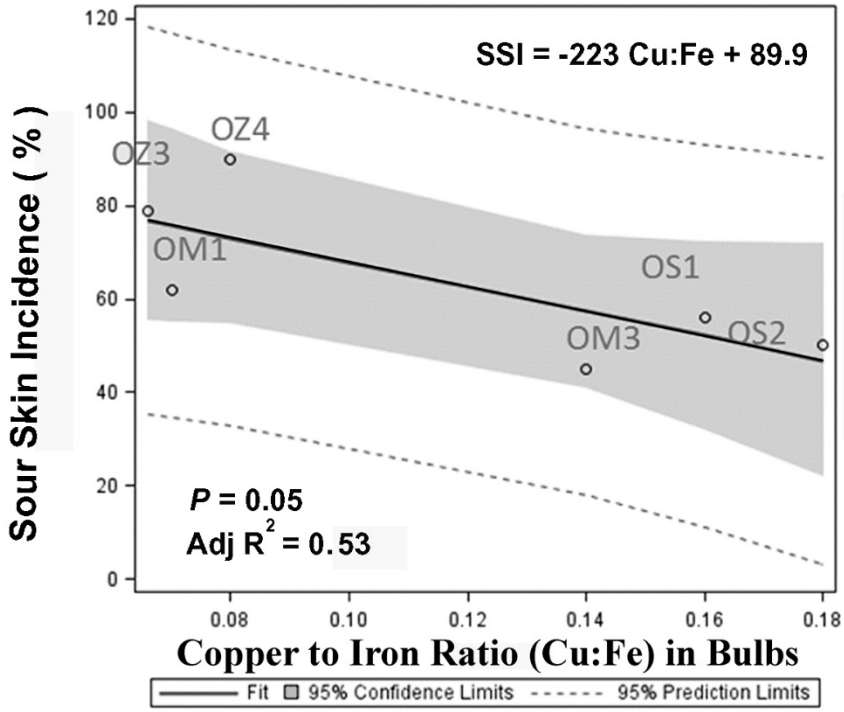


Figure 3-3. Fit of observed vs. predicted percent sour skin incidence from a multiple regression analysis model where sour skin incidence was the dependent variable and micronutrient levels and ratios in tissues were the independent variables from a field study in 2012.

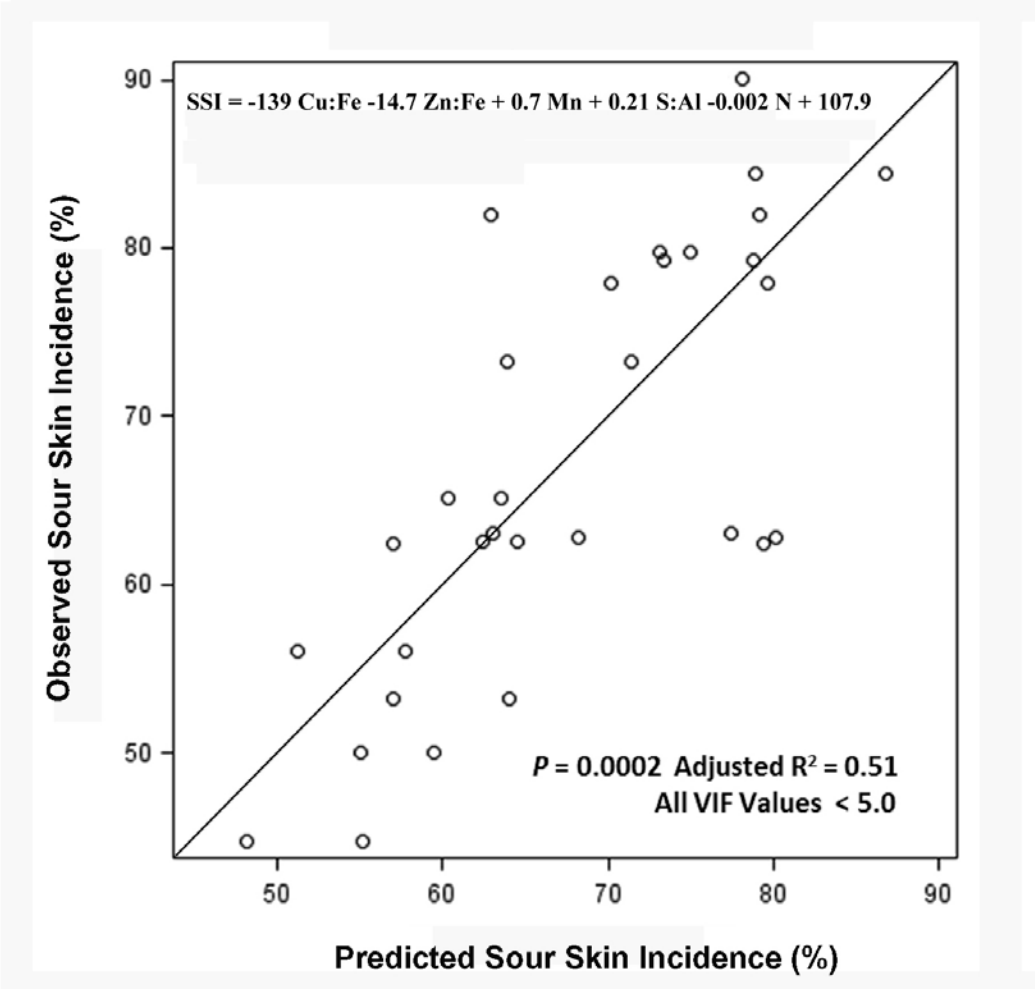


Figure 3-4. Fit of observed vs. predicted percent sour skin incidence from a multiple regression analysis model where sour skin incidence was the dependent variable and micronutrient levels and ratios in soils were the independent variables from a field study in 2012.

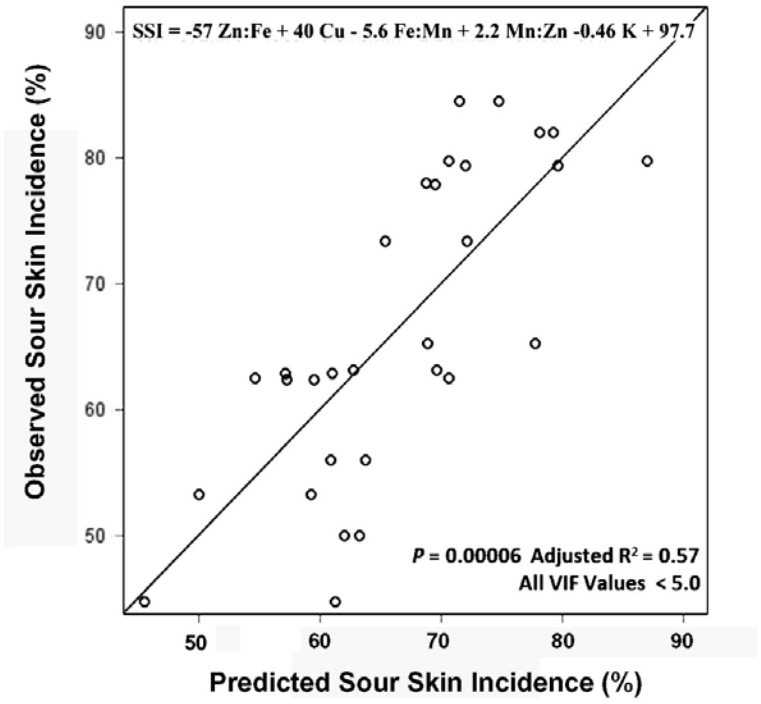


Figure 3-5. Fit of observed vs. predicted percent sour skin severity from a multiple regression analysis model where sour skin severity was the dependent variable and micronutrient levels and ratios in tissues were the independent variables from an *in vitro* study in 2013.

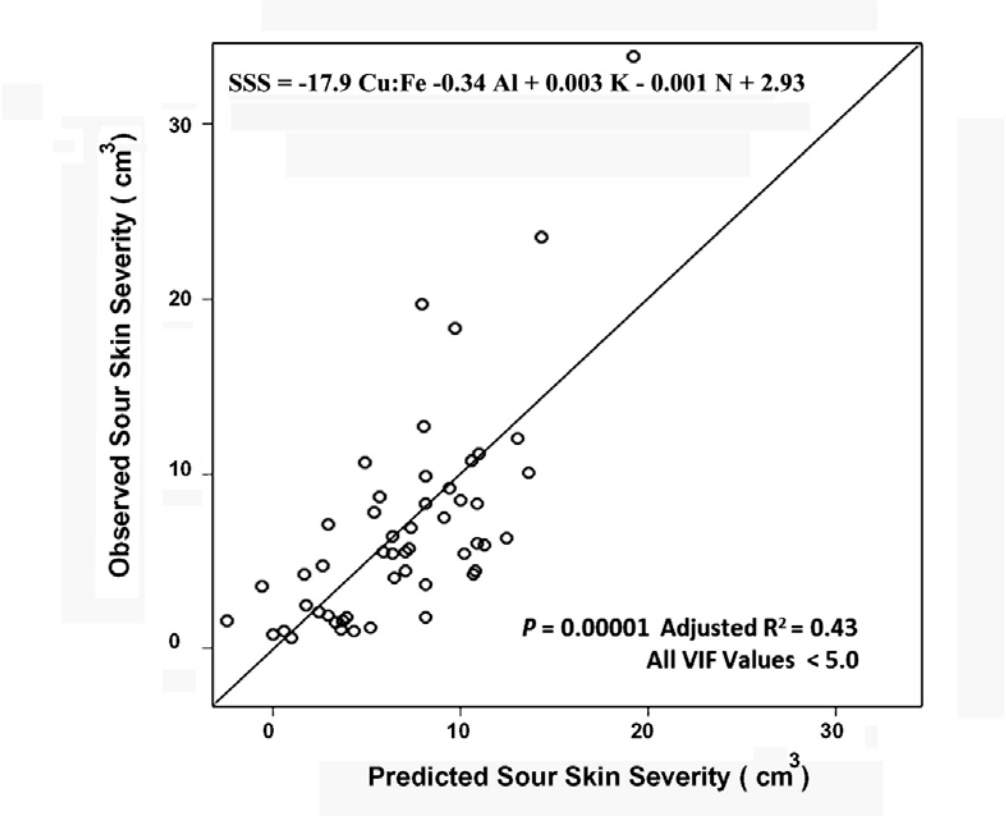


Figure 3-6. Fit of observed vs. predicted percent sour skin severity from a multiple regression analysis model where sour skin severity was the dependent variable and micronutrient levels and ratios in tissues were the independent variables from a field study in 2014.

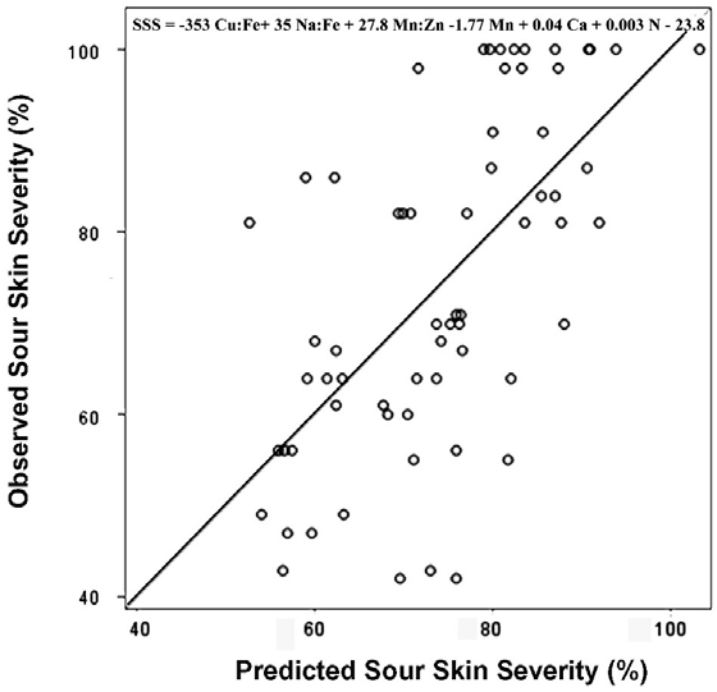
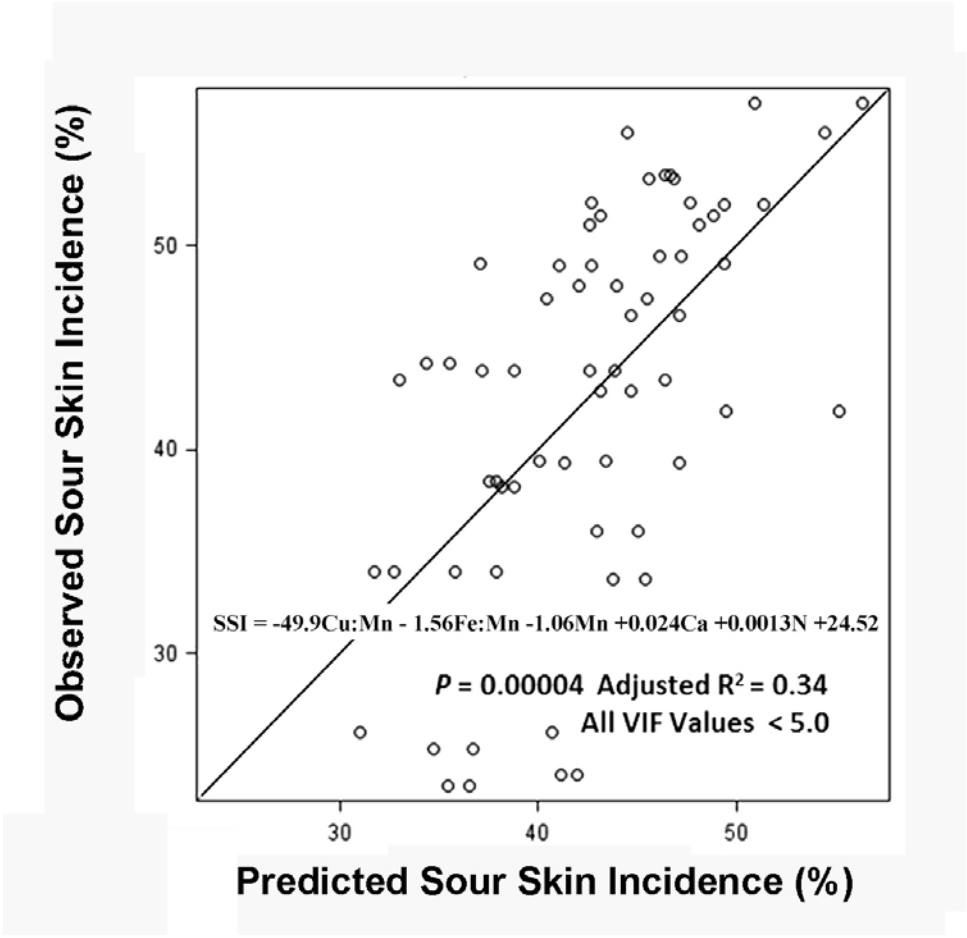


Figure 3-7. Fit of observed vs. predicted percent sour skin incidence from a multiple regression analysis model where sour skin incidence was the dependent variable and micronutrient levels and ratios in tissues were the independent variables from a field study in 2014.



Chapter 4

Early Detection of *Burkholderia cepacia* in Post-Harvest Onions via Disease- Associated Volatile Organic Compounds[3]

[3] Watson, A., Li, C., Schmidt, N., and Gitaitis, R. To be submitted to Food Science and Technology

Abstract

Sour skin of onion is one of the most important post-harvest diseases of Vidalia onions in Georgia. Rapid detection of this disease prior to**,** and during storage, is essential for reducing yield losses attributed to sour skin. The presence of disease-associated volatile organic compounds (VOCs) was evaluated to rapidly detect sour skin in stored onions. Onions were inoculated with *Burkholderia cepacia*, the causal organism of sour skin, and analyzed for the production of disease-associated VOCs using zNose technology. Principal component analysis and linear discriminant analysis of headspace gas quantitatively differentiated diseased onions from healthy onions. Different strains of *B. cepacia* produced different volatile compound profiles, and some strains could be differentiated based on zNose data analysis. Additionally, when the headspace of diseased onions was sampled with carboxen/polydimethylsiloxane (CAR/PDMS) solid-phase microextraction (SPME) units and analyzed by gas chromatography and mass spectrometry (GC-MS), unique VOCs were detected. A total of 32 compounds were present in the headspace samples of *B. cepacia*-infected onions, of which, 16 were exclusive to infected onions and the other 16 compounds were found in the control onions. Furthermore, when the headspaces of onions infected with 10 different *B. cepacia* strains was sampled and analyzed with GC-MS, each strain produced a unique VOC profile indicating variability in VOC production among bacterial strains. Overall, higher levels of VOCs were detected in the headspace samples of diseased onions compared with healthy

onions in a 2 L container when there was 100% disease. However, when the container volume was increased to 7.6 L and the disease incidence was decreased to 10%, only a slight increase in VOCs was observed. VOCs were not detected when the container space was increased to ~11,000 L and disease incidence was either 1% or 100%. It is concluded that zNose technology may have some potential for detecting VOCs produced by sour skin-infected onions. However, modifications in sampling technique, such as concentrating the sample is necessary for zNose technology to be effective

Introduction

Onion (*Allium cepa* L.) is an important agricultural commodity worldwide, and the U.S. is one of the leading producers. Onion is the fifth most valuable vegetable crop in the U.S. with a value of ~ $969 million USD in 2014 (AgMRC, 2015). There is a wide range of onion varieties produced nationwide. In Georgia multiple varieties of short-day, sweet, Spanish onions are typically grown and are collectively known as Vidalia sweet onions (Boyhan and Torrance 2002; Boyhan, Torrance, Cook, Riner, and Hill 2009). In 2014, Vidalia onions were grown on approximately 4,900 ha in 18 counties and were worth an estimated $138 million USD (Wolfe and Stubbs, 2014). This production accounted for approximately 14% of the US onion crop in 2014. These onions are produced for fresh market or short-term storage. Storage is essential in extending the availability of Vidalia onions for consumption and stabilizing market prices. There is a variety of post-harvest storage diseases associated with onions, but sour skin (caused by *Burkholderia cepacia*) is one of the more important diseases associated with

Vidalia onions (Davis, 2008).

There are a limited number of management practices available for the control of sour skin of onion. Current recommendations are to avoid fields with known sour skin problems, avoid overhead irrigation, and harvest onions at optimum maturity so that necks will adequately dry during the curing process. Of these, only the third method is practical in Georgia as *B. cepacia* can survive in soil for years and rain events are frequent enough in most years to negate the benefits of avoiding overhead irrigation. Other recommendations include curing bulbs in the field or in the packinghouse at ambient temperatures to dry onion necks (Boyhan and Torrance, 2002; Boyhan and Kelley, 2007). Following harvest, onions should be thoroughly inspected during the sorting and grading process to remove any diseased onions (Flentje et.al., 1963; Lammertyn et.al., 2004; Li et.al., 2009). Once onions have been sorted and graded, they are stored at ~1-3°C with a relative humidity (RH) between 65-70%, or in a controlled atmosphere (~3% oxygen, ~5% carbon dioxide, ~92% nitrogen, ~ 1°C, and a RH between 70-80%) (Myers, 1999; Boyhan and Kelley, 2007).

Although current grading measures are effective for detecting visually diseased bulbs, problems remain with latently-infected onions going undetected and being stored alongside healthy onions. These onions serve as inoculum sources for secondary pathogen spread during storage. Hence, a non-destructive disease detection method must be developed to effectively detect and eliminate latently infected bulbs prior to and during. One such method may involve disease-associated volatile organic compounds (VOCs).

VOCs have been the target for rapid disease detection in a variety of scientific fields. In the medical field, disease-associated VOCs were used to diagnose respiratory infections like tuberculosis (caused by *Mycobacterium tuberculosis*), *Pseudomonas aeruginosa* in cystic fibrosis patients, and invasive aspergillosis (caused by *Aspergillus fumigatus)* (Sethi et al., 2013). Characteristics of food products have also been evaluated by monitoring specific VOCs. Honey varieties were distinguished based on their plant derivative (Lammertyn et. al., 2003), ripeness of both melon and mango were determined (Vallone et al., 2012; Li et al., 2009), levels of deterioration of apples were ascertained (Li et al., 2007), and quality of vegetable and coconut oil were established through VOC analysis (Gan et al., 2005; Marina et al., 2010).

In addition to research conducted in the medical and food science fields, there has been extensive research to investigate the association between volatile compound emissions in diseased plants vs. healthy plants. Research has shown that when plants are infected with pathogens, the VOCs are different from those emitted by healthy plants (Jansen et al., 2011). Potatoes infected with *Phytopthora infestans* produced three specific VOCs: (E)-2-hexenal, benzene-ethanol, and 5-ethyl-2(5H)-furanone (Laothawornkitkul et al., 2010). Specific VOC mixtures were linked to powdery mildew-infected tomato and cucumber plants (Laothawornkitkul et al., 2008). Additionally, *Sclerotium rolfsii*-infected and beet armyworm-parasitized peanut plants produced unique VOCs when compared with healthy plants. (Cardoza et al., 2002).

Changes in VOC emissions can be observed during the infection process. When willow was infected with leaf rust, there was a decrease in isoprene emissions compared with healthy willow trees (Toome et al., 2010). Silver birch trees infected with *Marssonina betulae* were differentiated from trees damaged by autumnal moth larvae based on VOC profiles (Vuorinen et al., 2007).

In addition to these studies, research has been conducted on VOCs produced by onions infected with *Botrytis allii* and *B. cepacia* (Li et al., 2011). In this study, one representative strain of each pathogen was used. Based on VOCs diseased onions could be differentiated from healthy onions, and the pathogens could be differentiated from each other. E-nose technology was used to detect VOC patterns and gas chromatography mass spectrometry (GC-MS) was used to identify specific compounds. Using E-nose technology the treatments (*B. allii*, *B. cepacia*, and healthy) could be differentiated based on groups of VOCs but not based on individual compounds. Following GC-MS analysis, two compounds (2-nonanone and 3(2H)-furanone, 2-octyl-5-methyl-) that were unique to onions infected with *B. cepacia* were observed. These compounds could be targets for disease detection via VOCs.

This study aimed to expand on previous research by addressing several questions. Namely, could disease-associated VOCs be used to detect sour skin of onion in a commercial storage scenario, and whether there are differences in VOCs produced by different *B. cepacia* strains. As previously discussed, E-nose technology was used previously to rapidly identify VOC patterns associated with different diseases of onion and healthy onions. However, another goal of this

study was to identify individual compounds that might serve as targets for rapid disease detection. zNose technology is a type of VOC detection sensor that rapidly detects specific compounds, unlike enose, and much like GC-MS does (Staples, 2000). To our knowledge this technology has not been used to evaluate onions, but has been used to evaluate the flavor profiles of vegetable oil (Gan et al., 2005), and honey (Lammertyn et al., 2003), as well as the quality of apples (Li et al., 2007), and cantaloupes (Vallone et al., 2012

Materials and Methods

Plant Materials, Pathogen Materials, and Inoculation Protocol

Peruvian sweet onions, Vidalia sweet onions, or generic sweet onions were purchased from commercial sources and stored at 4°C prior to use. Before inoculation**,** dry skins were removed from onion bulbs and basal roots and necks were trimmed with a sterile scalpel. Bulbs were soaked in a 10% sodium hypochlorite solution (NaOCl) for 1 min and then rinsed with sterile water for 1 min. After NaOCl treatment**,** onions were submersed in 70% ethanol and dried.

Burkholderia cepacia strains used in this study were selected from the University of Georgia Coastal Plain Experiment Station's phytopathogenic bacteria collection. Selection of these strains was based on preliminary assays showing that all strains were pathogenic and represented a range of aggressiveness. These strains were: BC 83-1, BC 84-1, BC 88-4, BC 89-3, BC 90-2, BC 92-12, BC 92-33, BC 93-1, BC 93-7, BC 93-11, BC 97-5, BC 98-2, BC

98-4, BC 99-2, and BC 99-3. All bacterial cultures were grown on King's Medium B (KMB) for 24 h at 30°C prior to use.

Onions bulbs were inoculated by harvesting bacteria with the end of a sterile toothpick and inserting the toothpick into the onion tissue ~1 cm deep (Li et al., 2011) Control onions were stabbed with sterile toothpicks to simulate the wounding caused by inoculation. Onions were incubated in sterile aluminum foil-covered 2 L or 7.6 L jars at 30°C for 72 h, in storage units at 4°C and 23.8°C for 7 days, or in storage units at 23.8°C for 6 weeks depending on the experiment prior to data acquisition.

Experimental Design

zNose experiments were conducted in three phases. The first phase was to determine the ability of zNose technology to detect sour skin-associated VOCs. The second phase was to use zNose technology and GC-MS to evaluate 10 *B. cepacia* strains for variability in VOC production. Finally, the third phase was to determine a threshold for detection based on volume to sample ratio and percent disease incidence.

In phases one and two zNose technology was evaluated for efficacy in detecting VOCs associated with onions infected with *B. cepacia* and to determine variability amongst pathogen strains. For this experiment, onions inoculated with 10 different *B. cepacia* strains and four controls (wounded to simulate inoculation) were used. There were 10 replications of each treatment and each replication consisted of two inoculated onions or two control onions placed in a

sterile 2 L jar. Due to numbers of experimental units, the experiment was arranged and conducted as four groups; two groups with two bacterial strains and one control and two groups with three bacterial strains and one control. Onions were sampled at 24 and 48 h post inoculation. This experiment was conducted twice, referred to as trials 1 and 2.

Results obtained from phase 1 were considered in the development of phase 3, as phase 1 represented 100% infection with a small volume to sample ratio.

Phase 3 consisted of two container sizes. The first was a 7.6 L jar and the second was a simulated storage size of ~11,000 l and three different sour skin disease incidence percentages 100%, 10%, and 1%. Initially 10% percent disease incidence in 7.6 L jars was evaluated at 30°C. This scenario was created by using one inoculated onion and nine healthy onions with a control of 10 healthy onions. The simulated storage container then was evaluated at the optimum storage temperature (1.4°C), as well as the optimum disease development temperature (23.8°C) with a 1% disease incidence level. The 1% sour skin incidence scenario was created by placing one inoculated onion bulb among 99 healthy onion bulbs. One hundred healthy onions were used as a control in a separate storage room. There were two replicates for each treatment. Each temperature treatment was evaluated separately with a corresponding control treatment at the same temperature. Storage units were sampled every 24 h for 7 d as outlined below. This experiment was conducted two times. Based on the negative results obtained after the completion of the 1% storage scenario,

disease incidence was increased to 100%. The 100% infection level was created by utilizing 100 diseased onions with a control treatment of 100 healthy onions. The treatments were replicated twice, and the onions were incubated at 23.8°C for optimum disease development. Storage units were sampled once a week for 6 weeks. The experiment was conducted twice. For all experiments conducted in phase 2, *B. cepacia* strain 98-4 was used.

zNose data acquisition

For all experiments when onions were incubated in jars following incubation, the jars were placed at ambient (~21°C) temperature, lids were removed, and jars were flushed with zero air for 30 s to remove volatile compounds accumulated during incubation. Jars were re-sealed and volatiles were allowed to accumulate for 4 h prior to zNose sampling. Data were collected at 48 and 72 h post-inoculation by placing the sampling needle through the aluminum lid into the headspace using the following zNose operational parameters: sensor 40°C, column 40°C, valve 160°C, inlet 200°C, trap 225°C, and maximum column temperature of 200°C. Each sample was collected for 10 s during which time the sample was carried through the zNose apparatus by helium gas. Following each sample, the system was baked at 120°C for 15 s to remove residual VOCs from the previous sample. During the sampling process, VOCs present in the sample were separated and detected by the surface acoustic wave (SAW) sensor in the zNose. The zNose sensor was purged prior to each sample set and upon completion of sampling. The system was calibrated prior to sampling each day the zNose was used.

For all experiments conducted in storage units, the zNose apparatus was placed inside the storage unit and the sample was collected as described above. Samples were collected every 24 h for 7 d or once weekly for 6 weeks depending on the experiment.

zNose data analysis

Data were initially transformed according to methods previously established (Li et al. 2007). Briefly, raw zNose data, which consisted of the vibration frequency of the SAW sensor, were modified using an excel formula to correct any change in data due to sensor drift by selecting a central observation and correcting all data to this observation. The data were then averaged; all samples for each treatment were averaged to obtain a representative data set for each treatment, so that each treatment had one representative zNose chromatogram. The data were graphed for visualization of the peaks. Principal component analysis (PCA) and linear discriminant analysis (LDA) were conducted using the full data set with XLSTAT software. LDA was conducted using data obtained following PCA, the model developed via LDA was trained using two thirds of each sample set and validated with the remaining one third of each sample set.

SPME and GC-MS data acquisition and analysis

Solid phase microextraction devices (SPMEs) were conditioned according to manufacturer's instructions (Supelco Co. Bellefonte, PA) prior to use. The SPMEs were exposed to the headspace of three randomly selected replications

of each treatment in trial one and trial two according to a previously established protocol (Li et al., 2011). The SPMEs were then sent to Dr. Norman Schmidt at Tabor College, Hillsboro, KS, for GC-MS analysis.

Results and Discussion

Phase 1 and 2: Detection of sour skin-associated VOCs comparison among pathogenic strains

zNose data were collected at 48 (data not shown) and 72 HPI. To determine the ability of zNose technology to detect and differentiate sour skin-associated VOCs, strain 98-4 (a well-documented strain of *B. cepacia*) was compared with a control (healthy onions). zNose chromatograms demonstrate the ability of zNose technology to detect disease-associated VOCs (Figs. 4-1 and 4-2). PCA analysis of *B. cepacia* strain 98-4 inoculated onions and control onions from trials 1 and 2 show that sour skin-infected onions grouped separately from control onions (Figs. 4-3 and 4-4). This suggests that there are quantitative differences between diseased and healthy onions. Quantitative differences were further demonstrated through linear discriminant analysis, as diseased onions in trial 1 were correctly classified as diseased 100% of the time and healthy onions were correctly classified 100% of the time (Table 4-1). This trend continued in trial 2 where diseased onions were correctly classified 87.5% of the time and healthy onions 100% of the time (Table 4-2).

zNose chromatograms from trials 1 and 2 demonstrate quantitative differences in VOC production, with greater differences occurring after 72 HPI

(Figs. 4-1 and 4-2). There were no major differences in the quality of volatile compounds produced by healthy and diseased onions based on the location of peaks on the chromatogram, but there were observed differences in the quantity of volatile compounds produced. In general, diseased onions produced substantially larger amounts of these volatile compounds when compared with healthy onions. Principal component analysis from trials 1 (Fig. 4-5) and 2 (Fig.4-6) did not show any distinct groupings between diseased onions. LDA indicates variability in the correct classification between the 10 *B. cepacia* strains and the control groups. However, in both trials there was a high percentage of correct classification of the control onions. In trial 1 96.2% of the control onions were correctly classified as healthy (Table 4-3) and in trial 2 96.0% of the healthy onions were correctly classified (Table 4-4).

GC-MS analysis conducted in trial 2 revealed both quantitative and qualitative differences in VOC production between sour skin infected and healthy onions. There were 32 compounds detected in diseased and healthy onions. Of these, 19 compounds were previously reported to be associated with onions, and there were 16 compounds identified to be uniquely associated with diseased onions (Table 4-5) (Boelens, 1971; Vikram et al., 2005; Li et al., 2011; Løkke et al. 2012). Of those previously reported, only 2-nonanone was consistently associated with diseased onions (Li et al., 2011). Of the remaining compounds, methylene chloride, hexane, toluene, and o-xylene are typical solvents used in labs utilizing GC-MS, and thus may be contaminants absorbed by the SPMEs. Of particular interest was 1,2-dithiolane, which was found only in diseased onions

and is an intermediary in the breakdown of alliin, a compound found in onion. Its presence is most likely due to the digestion of onion tissues by *B. cepacia* (Kubec, 1997). In addition, VOC variability among *B. cepacia* strains was found as each strain produced a unique pattern of VOCs with some overlap among strains (Table 4-6). This variability in VOC production demonstrates the need for an array of pathogenic strains to determine which VOCs are associated with disease. This variability is most likely due to the natural variability of the pathogen (Vermis et al., 2003; Compant et al., 2008). The *B. cepacia* strains selected represented a range of aggressiveness and phenotypic characteristics.

Phase 3: Detection Threshold

zNose technology provided rapid detection of sour skin-associated volatile compounds present in air samples collected from diseased and healthy onions when the sample to volume was lowest (2 L) and the disease incidence was highest (100%) (Figs.4-1 and 4-2). However, as the container volume increased from to 2 L to 7.6 L and then to ~11,000 L and as disease incidence decreased from 100% to 10% and then to 1%, efficiency of detection of VOCs decreased to the point that no VOCs were detected in the highest volume regardless of disease incidence (data not shown).

Conclusion

Post-harvest diseases continue to cause substantial problems in onion production and storage. Detection of VOCs was evaluated as a non-destructive disease detection strategy in stored onion bulbs. zNose technology was chosen

as the sampling apparatus based on its reported accuracy and speed of VOC detection. In this study, a number of compounds were found to be associated with diseased onions; but no one compound was found among all pathogenic strains evaluated. This indicates the need for using several strains of a pathogen when evaluating VOC production. In addition, a group of compounds should be targeted for rapid disease detection that would cover a range of strains. zNose technology detected volatile compounds when the sample volume was at its lowest and the disease incidence was at its highest. However, this technology was not sensitive enough to separate the subtle differences in compounds with similar retention times. GC-MS detected and differentiated the compounds that the zNose was unable to. Finally, zNose technology detected little to no volatile compounds as the container size increased to represent a small storage unit. This suggests that zNose technology, used in a similar manner as in this study, would not be suitable for use in large commercial onion production storage rooms for detecting disease developing in onions in storage. However, further research into the use of disease-associated VOCs, including altering sampling conditions, for example concentrating headspace samples, could improve non-destructive rapid disease detection.

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Tables

Table 4-1. Confusion matrix of the classification results from the linear discriminant model to classify control healthy onions and *Burkholderia cepacia* onions from trial 1. The model was trained using ~ two thirds (27 data sets) of the data sets and validated using ~one third (13 data sets) of the data sets.

True Labels	Estimated Labels			
	BC	C	Total	% correct
BC	6	0	6	100.00%
C	0	7	7	100.00%
Total	6	7	13	100.00%

Table 4-2. Confusion matrix of the classification results from the linear discriminant model to classify control healthy onions and *Burkholderia cepacia* onions from trial 2. The model was trained using ~ two thirds (27 data sets) of the data sets and validated using ~one third (13 data sets) of the data sets.

True Labels	Estimated labels			
	C	BC	Total	% Correct
C	5	0	5	100.00%
BC	1	7	8	87.50%
Total	6	7	13	92.31%

Table 4-3. Confusion matrix of the classification results from the linear discriminant model to classify control healthy onions and 10 different strains of *Burkholderia cepacia* infected onions from trial 1. The model was trained using ~ two thirds (193 data sets) of the data sets and validated using ~one third (93 data sets) of the data sets.

	Estimated Labels												
True Labels	A	B	C	D	E	F	G	H	I	J	K	Total	% Correct
A (BC 83-1)	5	2	0	0	0	0	0	0	0	0	0	7	71.43%
B (BC 84-1)	1	7	0	0	0	0	0	0	0	0	0	8	87.50%
C (Control)	0	0	25	1	0	0	0	0	0	0	0	26	96.15%
D (BC 88-4)	0	0	0	6	0	0	0	0	0	0	0	6	100.00%
E (BC 89-3)	0	1	0	0	4	0	0	0	0	0	0	5	80.00%
F (BC 90-2)	0	0	0	0	0	4	1	0	0	4	1	10	40.00%
G (BC 92-12)	1	0	0	0	0	1	6	0	0	0	0	8	75.00%
H (BC 92-33)	0	0	0	0	0	0	0	5	0	0	0	5	100.00%
I (BC 93-11)	0	0	1	0	0	0	0	0	5	0	0	6	83.33%
J (BC 98-4)	0	0	0	0	0	0	0	0	0	5	0	5	100.00%
K (BC 99-3)	0	0	0	0	0	1	0	0	2	0	4	7	57.14%
Total	7	10	26	7	4	6	7	5	7	9	5	93	81.72%

Table 4-4. Confusion matrix of the classification results from the linear discriminant model to classify control healthy onions and 10 different strains of *Burkholderia cepacia* infected onions from trial 2. The model was trained using ~ two thirds (193 data sets) and validated using ~ one third (93 data sets).

	Estimated Labels												
True Labels	A	B	C	D	E	F	G	H	I	J	K	Total	% Correct
A (BC 93-1)	5	0	0	0	0	0	0	0	0	0	0	5	100.00%
B (BC 93-7)	0	4	1	0	0	0	1	0	0	0	0	6	66.67%
C (Control)	1	0	24	0	0	0	0	0	0	0	0	25	96.00%
D (BC 97-5)	0	0	0	7	0	0	0	0	0	0	0	7	100.00%
E (BC 98-2)	0	0	2	0	4	0	0	0	0	0	0	6	66.67%
F (BC 98-4)	0	0	1	0	0	2	0	0	0	0	0	3	66.67%
G (BC 92-12)	0	3	0	0	0	0	6	0	0	0	0	9	66.67%
H (BC 93-11)	1	0	1	0	2	0	0	5	1	0	0	10	50.00%
I (BC 90-2)	0	0	0	0	0	0	0	0	7	0	0	7	100.00%
J (BC 92-33)	0	0	0	0	0	0	0	2	0	8	0	10	80.00%
K (BC 99-3)	0	0	0	0	0	0	0	0	0	0	5	5	100.00%
Total	7	7	29	7	6	2	7	7	8	8	5	93	82.80%

Table 4-5. Volatile organic compounds identified in the headspace above onions infected with *Burkholderia cepacia* and healthy onions using a gas chromatogram mass spectrometer (GC-MS). Retention time (*rt*) is presented in minutes.

rt	*Compound*	*B. cepacia*	Control	Δ
1.495	Sulfur dioxide +	+		1.04E+10
1.646	Acetone +	+	+	5.31
1.764	Methylene Chloride +	+	+	1.71
1.99	Hexane +	+	+	4.26
2.05	1-Propanethiol +	+	+	3.41
3.492	toluene +	+	+	15.94
4.025	Hexanal	+		9.55E+09
5.455	o-xylene	+		2.23E+10
5.926	o-xylene	+		1.36E+10
6.279	Thiophene, 3,4-dimethyl-	+	+	1.06
6.555	Disulfide, methyl 2-propenyl	+		2.60E+09
7.05	Disulfide, methyl propyl	+	+	10.11
7.178	Disulfide, methyl 1-propenyl	+		2.44E+10
11.349	2-Nonanone	+		3.27E+10
11.451	1,2-Dithiolane	+		8.07E+09
11.641	2-Nonanone	+		3.48E+10
11.766	Heptanoic acid	+		7.82E+10
11.816	Dipropyldisulfide	+	+	2.83
12.112	propyl-1-propenyldisulfide	+	+	3.13
14.043	Pyrimidine, 4-butyl-3,4-dihydro-5-methyl-	+	+	7.14
17.272	2-Undecanone	+	+	2.66
18.238	Trisulfide, dipropyl	+	+	8.67
19.578	2,4-Octanedione	+	+	7.96
19.641	Pyrimidine, 4-butyl-3,4-dihydro-5-methyl-	+	+	10.38
19.769	1-Propanethiol	+		1.50E+10
20.05	3-Isopropyl-5-methyl-5-methylhexan-2-one	+		1.40E+10
20.133	3-isopropyl-5-methylhexan-2-one	+		1.98E+10
20.949	5-oxotetrahydrofuran-2-carboxylic acid	+		4.63E+10
21.057	2(3H)-Furanone, 5-butyldihydro-	+		4.42E+10
21.142	5-oxotetrahydrofuran-2-carboxylic acid	+		2.60E+10

21.307	3(2H)-Furanone, 2-hexyl-5-methyl-	+	+	9.62
22.649	2-Tridecanone	+	+	9.04

Table 4-6. Volatile organic compounds identified in the headspace above onions infected with 10 different strains of *Burkholderia cepacia* using a gas chromatogram mass spectrometer (GC-MS). Retention time (*rt*) is presented in minutes.

rt	Compound	BC 90-2	BC 92-33	BC 99-3	BC 97-5	BC 98-4	BC98-2	BC 92-12	BC 93-11	BC 93-1	BC 93-7	Control
1.495	Sulfur dioxide					7.78E+06		8.53E+06	7.55E+06		1.78E+07	
1.646	Acetone	1.84E+07		1.02E+07			1.23E+07	7.80E+06		1.21E+07	1.50E+07	9.51E+06
1.764	Methylene Chloride	1.36E+08	4.33E+07	1.33E+08						8.12E+07	4.06E+07	1.02E+08
1.99	Hexane	1.28E+07								1.29E+07	8.80E+06	1.08E+07
2.05	1-Propanethiol					8.31E+06			1.29E+07			1.24E+07
3.492	toluene	3.34E+07		8.53E+07								1.49E+07
4.025	Hexanal									9.55E+06		
5.455	o-xylene									1.92E+07	2.55E+07	
5.926	o-xylene									1.24E+07	1.48E+07	
6.279	Thiophene, 3,4-dimethyl-							1.36E+07	1.33E+07		3.90E+07	8.30E+07
6.555	Disulfide, methyl 2-propenyl										2.60E+06	
7.05	Disulfide, methyl propyl	4.31E+07	6.10E+07	8.93E+07	6.91E+07	1.05E+08	7.38E+07	1.59E+07	1.10E+07		8.53E+07	2.44E+07
7.178	Disulfide, methyl 1-propenyl	1.71E+07				1.19E+07					4.41E+07	
11.349	2-Nonanone								2.71E+07	3.81E+07	3.28E+07	
11.451	1,2-Dithiolane	8.07E+06										
11.641	2-Nonanone				3.36E+07	2.96E+07	4.11E+07					
11.766	Heptanoic acid							6.83E+07	2.05E+07	1.07E+08	1.17E+08	
11.816	Dipropyldisulfide	5.01E+07	8.81E+07	1.73E+08	2.23E+08	3.29E+08	2.43E+08	1.46E+08	2.06E+08	4.28E+07	1.39E+08	2.32E+08
12.112	propyl-1-propenyldisulfide	1.12E+07	3.65E+07	3.50E+07	4.00E+07	9.32E+07	5.03E+07	5.35E+07	9.01E+07	5.43E+07	9.67E+07	7.18E+07

14.043	Pyrimidine, 4-butyl-3,4-dihydro-5-methyl-			1.80E+07	4.67E+07	5.56E+07	5.06E+07	6.98E+07	6.65E+07	3.72E+07	1.14E+08	3.21E+07
17.272	2-Undecanone	9.44E+06	1.66E+08	6.72E+07		1.87E+08	9.37E+07	1.98E+08	2.06E+08	2.70E+08	2.57E+08	8.11E+07
18.238	Trisulfide, dipropyl				1.46E+07	2.70E+07		1.51E+07	1.59E+07		2.77E+07	9.25E+06
19.578	2,4-Octanedione				1.86E+08	9.56E+07	1.84E+07		6.35E+07	9.08E+07	1.59E+08	2.57E+07
19.641	Pyrimidine, 4-butyl-3,4-dihydro-5-methyl-					8.36E+06		5.40E+07	2.36E+07	2.33E+07	4.97E+07	1.23E+07
19.769	1-Propanethiol										1.50E+07	
20.05	3-Isopropyl-5-methyl-5-methylhexan-2-one								7.98E+06		2.01E+07	
20.133	3-isopropyl-5-methylhexan-2-one					1.98E+07						
20.949	5-oxotetrahydrofuran-2-carboxylic acid							5.07E+07			4.18E+07	
21.057	2(3H)-Furanone, 5-butyldihydro-							5.69E+07		3.16E+07		
21.142	5-oxotetrahydrofuran-2-carboxylic acid				4.16E+07	1.25E+07	1.51E+07		3.49E+07			
21.307	3(2H)-Furanone, 2-hexyl-5-methyl-	8.22E+06	2.15E+07	2.69E+07	1.61E+08	1.84E+08	8.71E+07		6.29E+07	1.56E+08	1.53E+08	3.98E+07
22.649	2-Tridecanone			5.13E+06	8.00E+07	2.23E+07	9.22E+06	2.26E+07	2.05E+07	5.63E+07	2.82E+07	1.35E+07

Figures

Figure 4-1. Chromatogram generated with a zNose for 10 different *Burkholderia cepacia* strains and four control groups with counts (CTS) on the y-axis and time (seconds) on the x-axis from trial

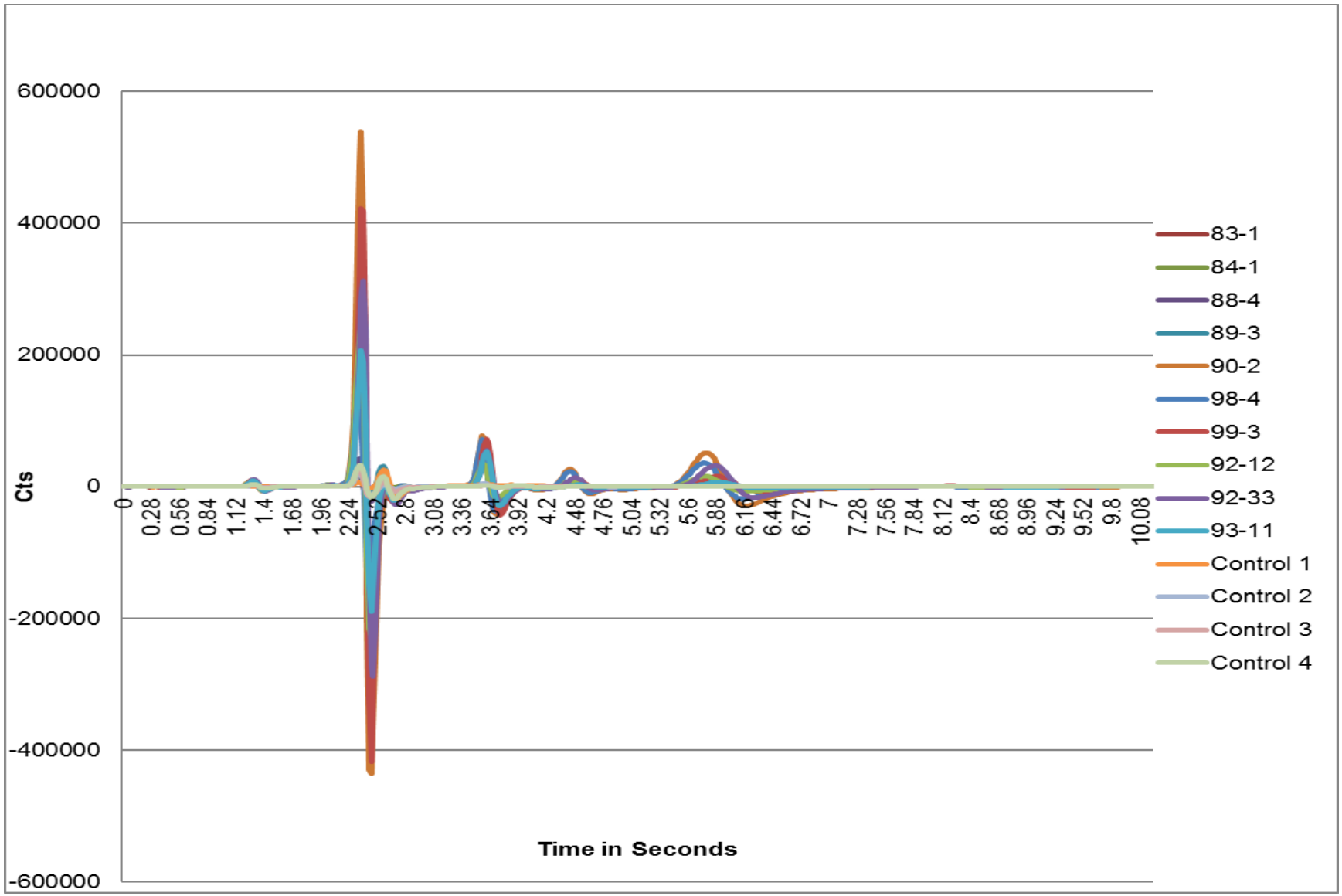

600000
400000
200000
0
-200000
-400000
-600000
Cts
0
0.28
0.56
0.84
1.12
1.4
1.68
1.96
2.24
2.52
2.8
3.08
3.36
3.64
3.92
4.2
4.48
4.76
5.04
5.32
5.6
5.88
6.16
6.44
6.72
7
7.28
7.56
7.84
8.12
8.4
8.68
8.96
9.24
9.52
9.8
10.08
Time in Seconds
83-1
84-1
88-4
89-3
90-2
98-4
99-3
92-12
92-33
93-11
Control 1
Control 2
Control 3
Control 4

Figure 4-2. Chromatogram generated with a zNose for 10 different *Burkholderia cepacia* strains and four control groups with counts (CTS) on the y-axis and time (seconds) on the x-axis from trial 2.

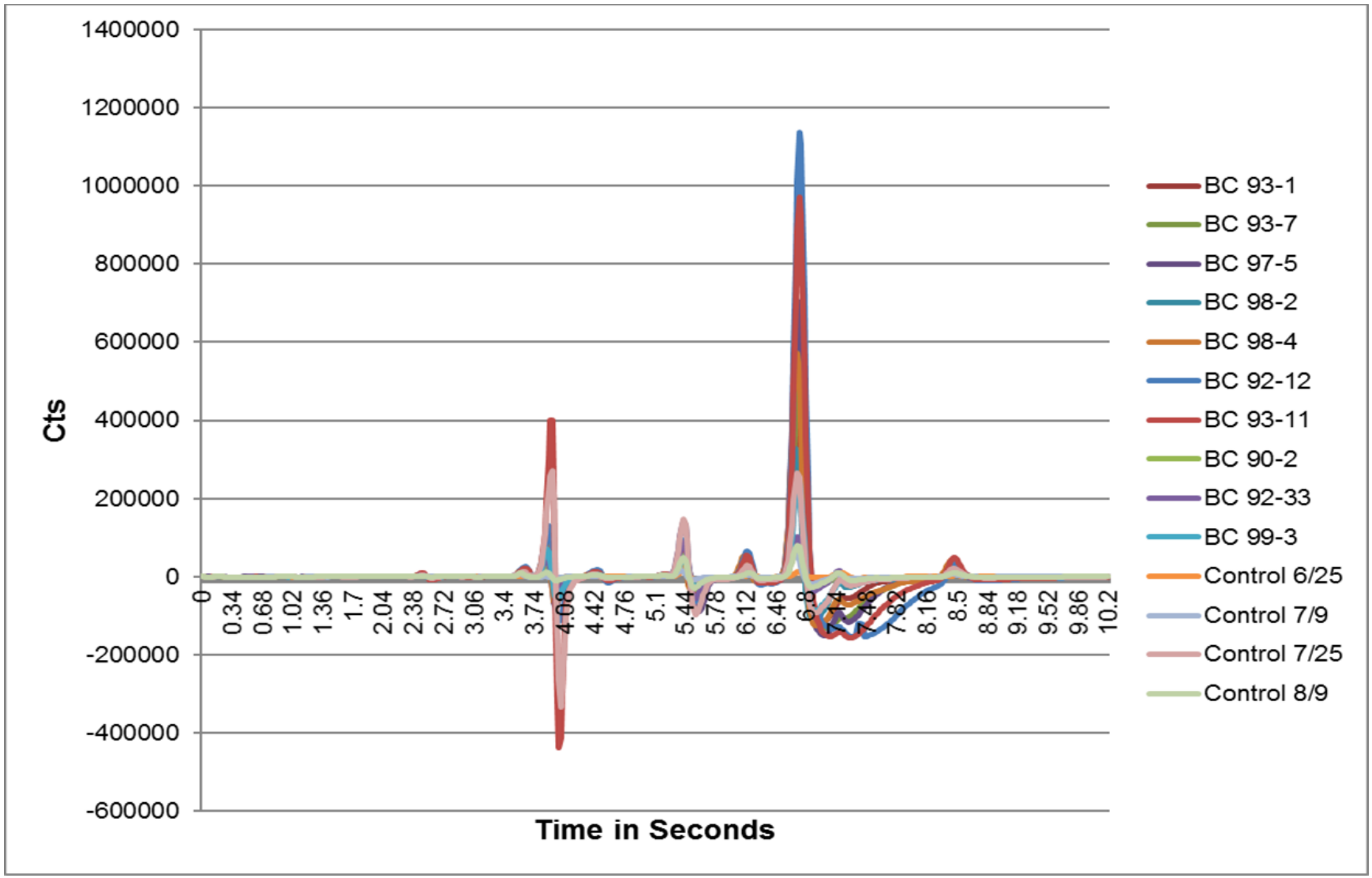

1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
-400000
-600000
Cts
0
0.34
0.68
1.02
1.36
1.7
2.04
2.38
2.72
3.06
3.4
3.74
4.08
4.42
4.76
5.1
5.44
5.78
6.12
6.46
6.8
7.14
7.48
7.82
8.16
8.5
8.84
9.18
9.52
9.86
10.2
Time in Seconds
BC 93-1
BC 93-7
BC 97-5
BC 98-2
BC 98-4
BC 92-12
BC 93-11
BC 90-2
BC 92-33
BC 99-3
Control 6/25
Control 7/9
Control 7/25
Control 8/9

Figure 4-3. Score plots of principal component analysis of headspace above *Burkholderia cepacia* (BC 98-4) infected onions and healthy control onions. Symbols “BC” represents infected onions and “C” represents healthy control onions from trial 1.

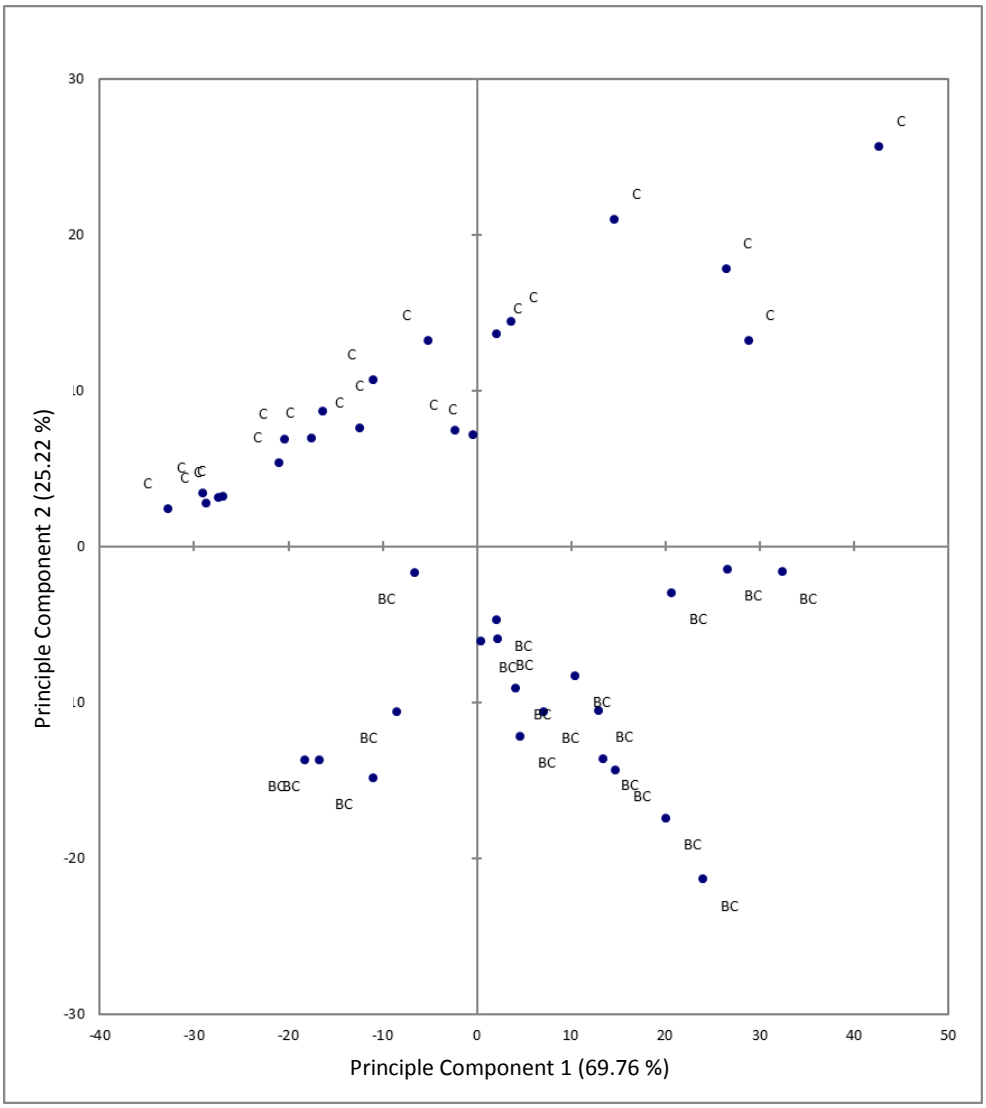


Figure 4-4. Score plots of principal component analysis of headspace above *Burkholderia cepacia* (BC 98-4) infected onions and healthy control onions. Symbols “BC” represents infected onions and “C” represents healthy control onions from trial 2.

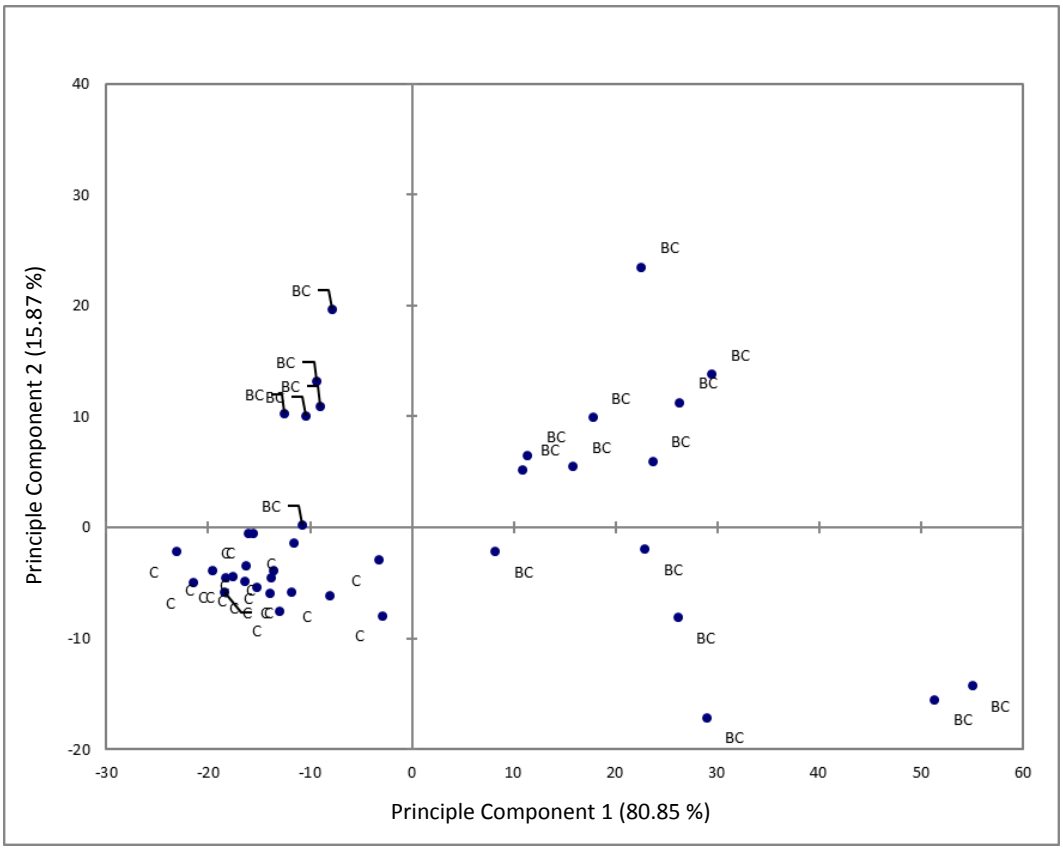


Figure 4-5. Score plots of principal component analysis of headspace above 10 different strains of *Burkholderia cepacia* infected onions and healthy control onions. Symbols “A,B, and D-K” represent infected onions and “C” represents healthy control onions from trial 1.

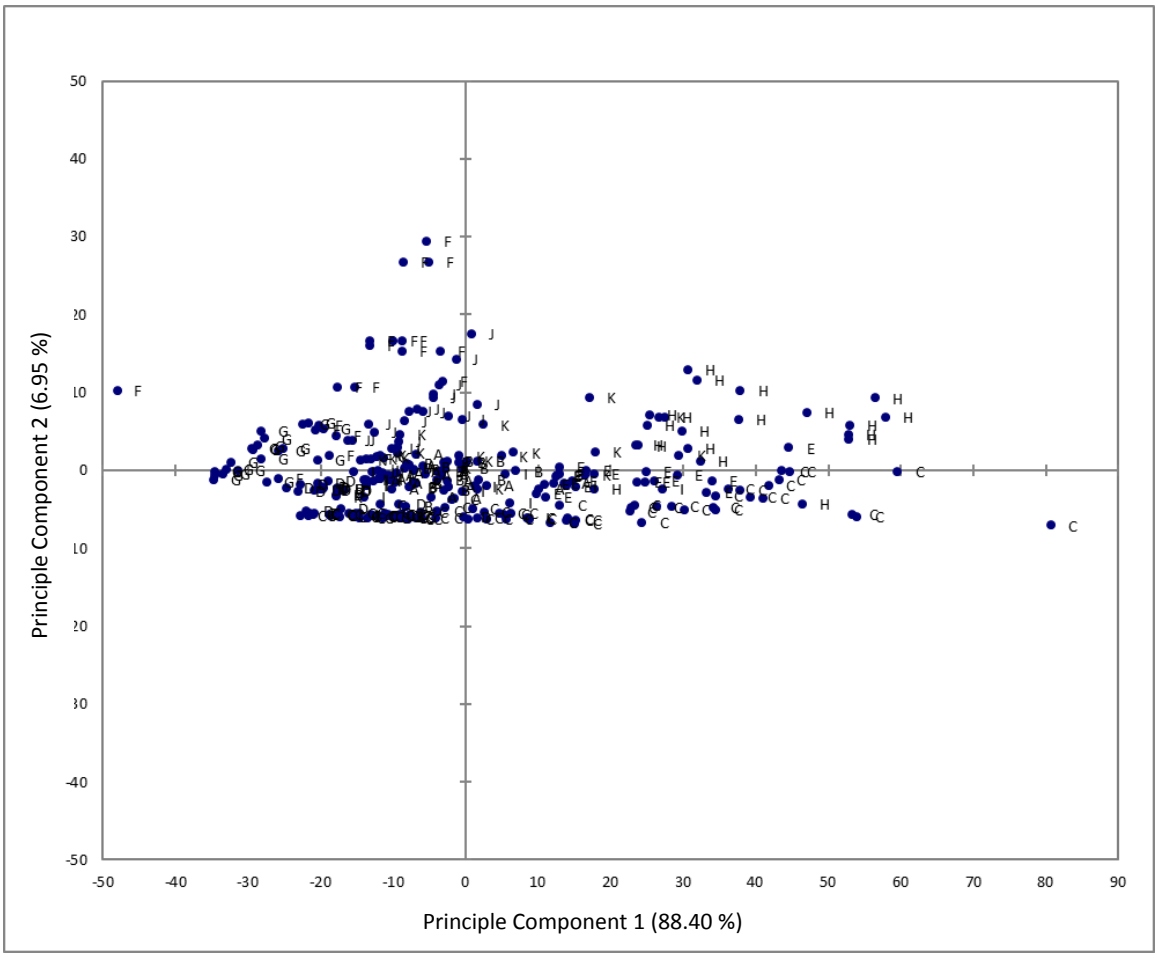
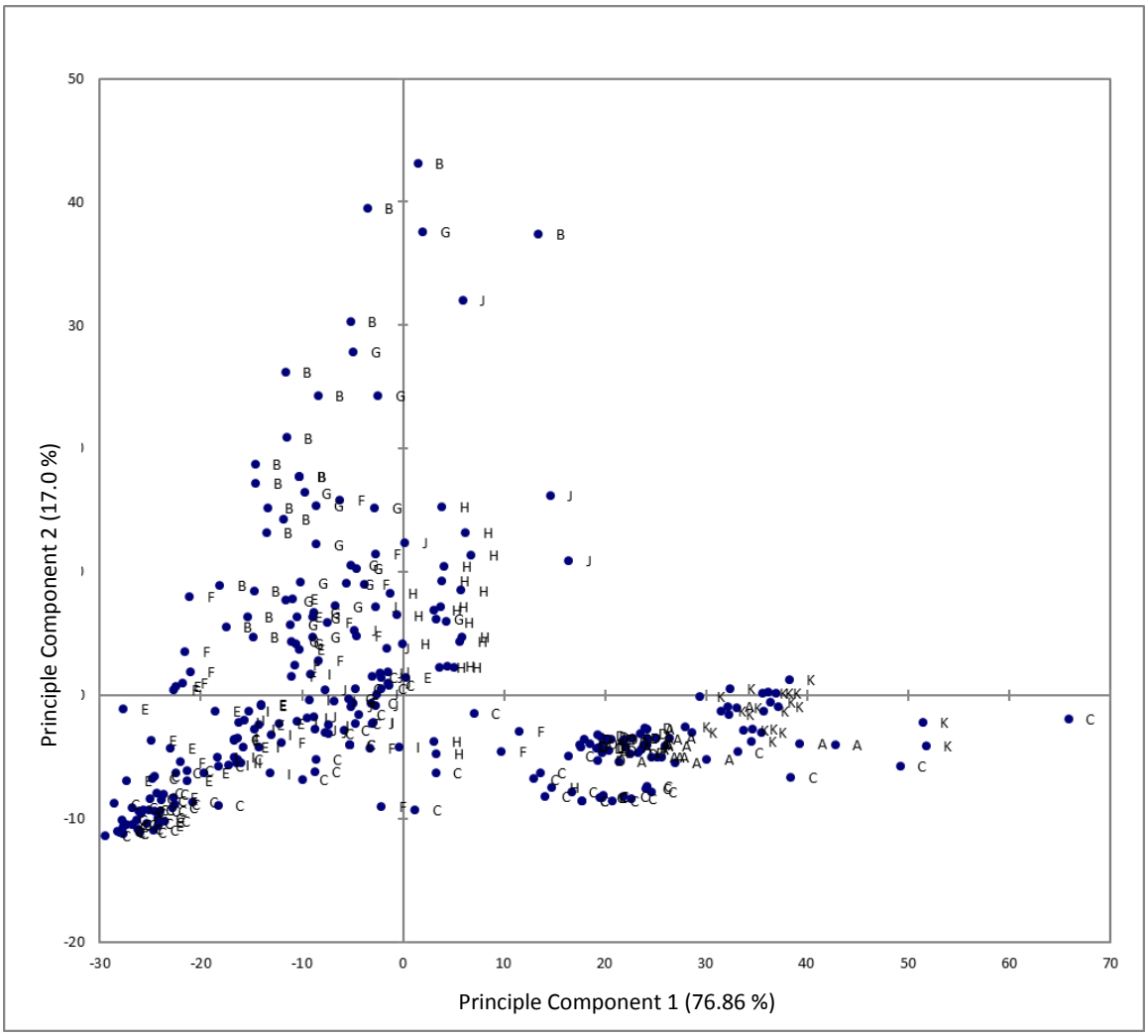


Figure 4-6. Score plots of principal component analysis of headspace above 10 different strains of *Burkholderia cepacia* infected onions and healthy control onions. Symbols “A,B, and D-K” represent infected onions and “C” represents healthy control onions from trial 2.



Chapter 5

CONCLUSION

Sour skin of onion is an ongoing problem for onion producers worldwide. New and innovative management strategies must be developed and implemented to effectively manage sour skin. The research presented in this study provides insights into innovative ways sour skin may be detected and managed more effectively in the future. In addition, new questions regarding onion production and disease management surfaced. These questions have opened the door for future research.

Throughout this study we have observed that there are reduced levels of sour skin in onions grown after a crop of pearl millet compared with sour skin levels in onions grown behind other crops. Significant differences in sour skin incidence in 2010 and marketable yield in 2011, combined with the overall trends of the data, indicated that pearl millet is affecting sour skin development in some manner, whether directly on the causal agent, *Burkholderia cepacia*, or the onion. These observations also support previous observations of Haudenshield and Lorbeer (2003), Haudenshield et al (2003) and Gitaitis (2005).

We also began to shed light on the mode of action by which pearl millet is potentially reducing disease. A direct interaction between pearl millet roots and

the bacteria may occur. An *in vitro* assay showed that pearl millet supported significantly lower *B. cepacia* populations than onion did in a grow-out assay in a closed container system. Also, spectrophotometric measurements were used to quantify the growth of *B. cepcia* strain Bc 98-4 in different concentrations of corn and pearl millet root exudates. Comparison of the corrected areas under the growth curve (AUGC) indicated that there was significantly less growth of Bc 98-4 in a 75% concentration of pearl millet root exudates than in a 75% concentration of corn root exudates. Since *B. cepacia* is a soil-borne pathogen, it can be concluded that root exudates affecting bacterial growth in a negative manner would have implications on the survival of the bacterium.

The exact interaction between micronutrients and sour skin incidence and severity is not completely understood, but results of this study indicate a potential relationship between mineral makeup in the soil and in plant tissues with disease development. Several multiple regression models that ranged in significance from $P = 0.0002$ to $P = 0.00002$ and with adjusted R^2 values ranging from 0.34 to 0.57 were developed from field studies in 2012 and 2014 and from a laboratory study in 2013. The models contained the following macro- and micronutrients in the majority of models: aluminum (Al), manganese (Mn), nitrogen (N) potassium (K), sulfur (S) and zinc (Zn). Although Mn and Zn occurred more frequently than the other nutrients and were in the majority of the models, only the Cu:Fe ratio occurred in every model. In 2012, the ratio of Cu:Fe alone was significantly correlated ($P = 0.004$) with sour skin incidence (%). It is noteworthy that the Cu:Fe ratio is also correlated with *Tomato spotted wilt virus*

severity in tobacco and was used to identify high risk and low risk locations in tobacco fields (Gitaitis et al. 2014). Furthermore the Cu:Fe ratio also is an independent variable in several models for bacterial leaf spot (BLS) of pepper, but does not appear to be as essential as the Zn:Fe ratio in that pathosystem (Dutta et al 2015). Although the nutrient models developed for tomato spotted wilt and BLS could be validated by quantifying the expression of *NPR1* and *PR1* plant disease resistance genes, the protocols to do so in onion have not yet been developed. As such, healthy onion bulbs containing a high Cu:Fe ratio and harvested from plots with low levels of sour skin and onion bulbs containing a low Cu:Fe ratio and harvested from plots with a higher levels of sour skin and coming from plots double-cropped with either corn, soybean or pearl millet were used for transcriptome analysis. Following analysis and annotation, at least 30 transcripts associated with plant disease resistance were significantly different in the different groups of bulbs analyzed. The most upregulated of these transcripts was associated with the *PR1* gene, which is known to be induced by salicylic acid and is involved in the systemic acquired resistance pathway (Durrant and Dong 2004). In addition, we concluded that 23 transcripts related to known plant disease defense genes were found only in onions grown after pearl millet and not in onions grown after corn or soybean. Although this evidence is preliminary, it can be hypothesized that the crop used to double-crop with onion could affect the soil environment in a manner that influences gene expression in subsequent onion crops. The relationships identified here warrant further research that could result in the development of risk prediction systems and eventually the

development of management strategies through the application of specific levels of micronutrients. Specifically, the models developed in this research could be evaluated in commercial fields by surveying soils in the Vidalia onion growing-region in Georgia. Prior to planting onions, locations of soil samples could be identified by global positioning system and soil samples analyzed for macro- and micronutrient concentrations. Values obtained would be inserted into the models and results used to identify sites of high risk and low risk for sour skin incidence or severity. Model validation could be achieved by monitoring the fields for percent sour skin observed. Then the models could be evaluated statistically by assessing the fit of predicted values with observed values. If models are validated, the ability to identify high risk and low risk areas based on soil analysis would be of great benefit to onion growers.

In addition to field studies being used to validate models, validation could be achieved through quantifying gene expression of important plant disease resistance defense genes. Although the protocols to do so in onion did not previously exist, primers for several PR genes in onion were recently developed (ElMorsi et al 2015). Their protocol needed adjustment, and now that modifications to the protocol have been made in our lab, the quantification of the expression of *PR1* in onion using qPCR is possible. Future experiments would include evaluating *PR1* expression in onions with different micronutrient profiles or in onions having come from areas previously planted to corn, pearl millet, peanut or soybean. Significant differences in *PR1* expression would indicate that these factors are affecting expression of plant disease resistance genes.

Also, it was concluded in this study that a number of volatile organic compounds (VOC) were associated with diseased onions and could be detected using zNose technology within certain limitations, namely volume of headspace above the infected onions. Use of zNose could significantly detect onions infected with *B. cepacia* under conditions when disease incidence was relatively high and the volume of the headspace was low, e.g., 2 l or 7.6 l. However, it was concluded that when the volume of headspace was larger (~ 11,000 l), zNose technology could not differentiate between healthy onions and onions with even advanced symptoms of sour skin. Another possible limitation to zNose technology that this research identified was that different strains of *B. cepacia* have different VOC signatures. In fact, there was no single compound identified in all 10 *B. cepacia* strains tested. It can be concluded that this technology was not sensitive enough to disentagle the subtle differences in compounds with similar retention times that are produced by different strains of *B. cepacia*.

GC-MS detected and differentiated the compounds that went undetected by the zNose. These results support conducting further research into the use of disease associated VOCs. Areas of study could include development of specific sensors in an eNose for the VOCs associated with onions with sour skin, development of sampling protocols that concentrate headspace samples, evaluation of the duration of the sampling period and development of protocols, possibly using robotics, for the collection of samples in immediate proximity to the onions in sealed storage rooms.

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