# EVALUATION OF RACE AND COPPER TOLERANT STRAINS OF *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA,* CAUSAL AGENT OF BACTERIAL LEAF SPOT OF BELL PEPPER IN GEORGIA

by

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(Under the Direction of David B. Langston Jr.)

ABSTRACT

Each year, Georgia pepper growers are confronted with the problem of bacterial leaf spot (BLS) caused by the bacterium *Xanthomonas axonopodis* pv. *vesicatoria* (XAV). The disease is devastating in hot, humid conditions and can cause millions of dollars in losses each year. Currently, management options include copper-based bactericides and growing pepper varieties with resistance to BLS. From 2007-2008, a survey was conducted to determine the frequency of copper (Cu) tolerance and distribution of BLS races in GA. Research was also conducted to evaluate two different Cu tolerance assays. Of 155 collected field strains, 89% were found to be Cu-tolerant on Cu-amended media. From 119 XAV strains, the majority were race 9 and 10, 36.1 and 50.4%, respectively. Strains grown in liquid Cu concentrations showed variable growth and demonstrated less growth than the control. No growth was observed at the highest concentration of Cu tested.

INDEX WORDS: Bacterial leaf spot, *Xanthomonas campestris* pv. *vesicatoria*, *Xanthomonas axonopodis* pv. *vesicatoria*, Race classification, Copper tolerance, Bell Peppers

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CHAPTER 1

# INTRODUCTION AND LITERATURE REVIEW

## Introduction

Bell peppers (*Capsicum annuum*) are an important vegetable crop grown in Georgia (GA). In 2008, GA reported a farm gate total of $105,300,645 for bell peppers, which accounted for 3.9% of all the vegetables grown (Boatright and McKissick, 2009). With bell pepper ranked as the third highest vegetable commodity in GA, it is important to understand the diseases that cause millions of dollars of loss each year. Bacterial leaf spot on pepper (caused by *Xanthomonas axonopodis* pv. *vesicatoria*) is one bacterial disease that infects many pepper types, including bell, hot, and specialty peppers. Bacterial leaf spot can cause millions of dollars in losses to farmers each year in yield and quality reductions and management costs. Understanding the disease and its distribution in peppers throughout Georgia is crucial for disease management.

## Literature review

Peppers are native to the tropical and subtropical Americas, including Mexico, Central America, and the northern region of South America. They are notable for their sweet or pungent flavor and are grown worldwide. The common bell pepper is the most widely cultivated and economically important species across the world (Rubatzky and Yamaguchi, 1999). Peppers are herbaceous, tropical perennials that are usually grown as annuals. Peppers vary in flower color, fruit size, shape, and color. They are frost sensitive, require warm temperatures, and have a 3-4

month growth period (Rubatzky and Yamaguchi, 1999). The ideal temperature for fruit development is 20-25°C and lower temperatures will limit flavor and color development (Rubatzky and Yamaguchi. 1999). Seed germinate in 6-10 days and flowers appear in 1-2 months (Rubatzky and Yamaguchi, 1999). Commercial peppers grown in GA are usually started as transplants and planted on raised, plastic-mulched beds. In GA, 56% of the total pepper acreage is grown in the spring (2007 GA Farm Gate, 2008).

BLS on peppers is caused by *Xanthomonas axonopodis* pv. *vesicatoria*. *X. a.* pv. *vesicatoria* is an aerobic, oxidase-negative, gram-negative, rod-shaped, phytopathogenic bacterium with a single polar flagellum (Kay et al., 2007). In culture, *X. a.* pv. *vesicatoria* produces a circular, yellow, butyrous colony on nutrient agar. The yellow color in colony formation is due to the pigment xanthomonadin. Xanthomonadins have been found to have little to no effect on pathogenesis but are important for epiphytic survival of the bacteria and protect the cells against damage from visible light (Poplawsky et al., 2000). Bacterial leaf spot was first observed in South Africa on tomato around 1914 (Doidge, 1921). Around the same time, Gardner and Kendrick (1921) discovered a similar disease in the United States. Sherbakoff described the disease on pepper (Sherbakoff, 1918). In the following years, there was debate about the name of the causal agent. In 1925, it was classified as *Pseudomonas vesicatoria* (Stevens, 1925), then changed to *Phytomonas vesicatoria* in 1930 (Bergey, 1930), and then to *Xanthomonas vesicatoria* in 1939 (Dowson, 1943; Hayward and Waterson, 1964). Eventually it was named *Xanthomonas campestris* pv. *vesicatoria* (Dye et al., 1980). However, controversy about the name continued and pathologists determined there were two groups of *X. c.* pv. *vesicatoria*, group A and group B (Bouzar et al., 1994). Group A and B were found to be distinct in that their total DNA was less than 50% homologous. In addition, they differed in the

utilization of carbon substrates, reaction to monoclonal antibodies, fatty acid composition, hypersensitive reaction on tomato differential cultivars, and amylolytic activity (Bouzar et al., 1994). *X. c.* pv. *vesicatoria* group A was either unable to, or weakly hydrolyzed starch (Bouzar et al., 1994), and was unofficially named *Xanthomonas axonopodis* pv. *vesicatoria* (Vauterin et al., 1995). Group B strains were found to hydrolyze starch, utilize pectate, and caused disease on tomato. This group was named *Xanthomonas vesicatoria* (Jones et al., 2004). The two groups are considered to be closely related organisms. The pepper strains are considered to be in group A (Jones et al., 1998). Group A strains have been further debated to be re-classified into their own species and are currently referred to as *Xanthomonas euvesicatoria* (Jones et al., 2004). The *X. euvesicatoria* species designation was given to only the original strains that were weakly amylolytic and originally identified by Doidge (Jones et al., 2004). Further classification in 2004 was given to the xanthomonads and two more groups were formed. Group C xanthomonads were classified as *Xanthomonas perforans* and were closely related to the group A strains, yet differed in DNA homology by less than 70% (Jones et al., 2004). Group D strains were found to be quite different from other xanthomonads isolated from tomato plants and were classified as *Xanthomonas gardneri* (Jones et al., 2004). *Xanthomonas euvesicatoria* has weak amylolytic and pectolytic activity, a distinct pattern of reaction to a specific panel of monoclonal antibodies, a distinct sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profile, utilizes *cis*-aconitic acid, an intermediate molecule in the Kreb's cycle, and has a specific 32-kDa protein (Jones et al., 2004). *Xanthomonas vesicatoria* strongly digests starch and pectic substrates, has a different reaction to the same monoclonal antibodies, does not utilize *cis*-aconitate, and has a 25- to 27-kDa protein (Jones et al., 2004). *Xanthomonas perforans* is usually isolated from tomato plants, strongly amylolytic and pectolytic, has a distinct pattern of

reaction to the same panel of monoclonal antibodies, a distinct SDS-PAGE profile, utilizes all carbon sources tested except *cis*-aconitic acid and glycogen, and has a 25- to 27-kDa protein (Jones et al., 2004). The last group, *Xanthomonas gardneri*, is usually isolated from tomato, weakly amylolytic and pectolytic, has yet another distinct pattern of reaction to the monoclonal antibodies tested, a distinct SDS-PAGE profile, and did not utilize any of the carbon sources tested (Jones et al., 2004). All four species listed above also differ in DNA fingerprints from Rep-PCR experiments. For the remainder of the paper, the causal organism of BLS on pepper will be referred to as *Xanthomonas axonopodis* pv. *vesicatoria* since it is a recent name given to the organism and still widely accepted by the scientific community. Furthermore, DNA verification was never completed in this research to determine if the strains in this study are *X. euvesicatoria* or another *Xanthomonas* species.

*X. a.* pv. *vesicatoria* strains have some unique characteristics that differentiate them into species and pathovars. As previously mentioned, group A *xanthomonads* were found to hydrolyze starch and degrade pectate weakly or not at all (Jones et al., 1998). Group B *xanthomonads* have strong amylolytic activity. The use of starch and pectate is used as a factor to distinguish phenotypic variation. Another factor that separates the two groups can be found by looking at expressed heat-stable proteins. Group A produced a 32-35 kDa protein, or α protein, and group B-D produced a 25-27 kDa protein, named β protein (Bouzar et al., 1994). These heat-stable proteins can withstand high temperatures, up to 121°C, without denaturation and are detectable (Bouzar et al., 1994). Bouzar *et al.* (1994) found that only one carbon substrate, *cis*-aconitate, could be used to distinguish the *Xanthomonad* groups. Group A oxidized 97% of the substrate and group B oxidized none (Bouzar et al., 1994). Fatty-acid analysis is another method for distinguishing bacterial strains. Bouzar et al. (1994) found that

one fatty-acid molecule, C15:0 ante-iso, was different between the two groups. Group A demonstrated a significantly lower amount of C15:0 ante-iso present than group B strains (Bouzar et al., 1994). DNA-DNA hybridization studies were performed to further separate the *Xanthomonas* groups and it was found that strains from group A and group B were less than 50% homologous (Stall et al., 1994). Strains analyzed within each group were found to be more than 70% similar (Stall et al., 1994).

The use of pulsed field gel electrophoresis (PFGE) has been used to genetically distinguish *X. a.* pv. *vesicatoria* strains by their genetic fingerprints. The PFGE technique is similar to the standard gel electrophoresis technique used in other DNA separation procedures except that with PFGE, the voltage is periodically switched among different directions. The advantage to this procedure is that it allows for better separation of small and large DNA pieces. Jones and Stall (1998) utilized this procedure to further analyze and genetically separate the *Xanthomonas* phenotypic groups A and B. Results from this study showed that two distinct clusters were formed. In another study (Roberts, P. D., Jones, J. B., and Bouzar, H., unpublished), groups A thru D were analyzed and four distinct clusters were formed. PFGE has also been used in determining the effects that the pepper *Bs2* gene has on the evolution of the *X. a.* pv. *vesicatoria AvrBs2* gene (Gassmann et al., 2000), the characterization of *X. a.* pv. *vesicatoria* strains in Brazil (Quezado-Duval et al., 2004), and the verification of *X. a.* pv. *vesicatoria* chromosomal DNA transfer of copper resistance genes to a recipient strain (Basim et al., 1999).

The host range of *X. a.* pv. *vesicatoria* is primarily in the family Solanaceae, which includes tomato (*Lycopersicon esculentum*), bell pepper (*Capsicum annuum*), chili pepper (*Capsicum rutescens*) and many other plant species (Jones et al., 1998). Whether all the

solanaceaous plants are hosts to the four groups of *X. a.* pv. *vesicatoria* is unknown and the full extent of the host range is unknown. Symptoms on host plants appear on leaves, stems, and fruit. Symptoms include leaf spots and lesions, wilting, and premature defoliation (Fig. 1.1). Fruit lesions are less common compared to leaf lesions, but can still be devastating. Infected fruit is usually non-marketable due to poor quality (Ritchie, 2000). On leaves, symptoms begin as small yellowish, green lesions and progress to darker, water-soaked lesions on older leaves (Ritchie, 2000). A chlorotic halo with water-soaked margins forms around the leaf lesion area. The chlorotic haloes and water-soaking are caused by bacterial toxins and enzymes acting on plant cells along the perimeter of the infection (Schumann and D'Arcy, 2006; Agrios, 2005). Moisture also plays a large role in the formation of leaf lesions. Many lesions occur near the hydathodes on the outer edge of the leaves where moisture is released and lesions are often larger and more advanced with water saturated conditions (Ritchie, 2000). Fruit lesions begin as pale green, water-soaked spots and are usually raised, rough and serve as an infection court for secondary pathogens (Ritchie, 2000).

*X. a.* pv. *vesicatoria* reproduces faster in higher temperatures (24-30 °C), higher humidity, and higher amounts of free standing moisture (Jones, 1991). The organism can overwinter on infected plant debris or epiphytically on host volunteers as well as within seed (Jones et al., 1986). Bacterial dissemination occurs by wind, rain or irrigation droplets, aerosols, and mechanically during handling. *X. a.* pv. *vesicatoria* enters the host through natural openings such as stomata and hydathodes and through wounds (Jones, 1991). *X. a.* pv. *vesicatoria* can survive a few days to a week on bare soil without a host plant; therefore it is important that the bacteria infect live plant material or plant debris to survive (Ritchie, 2000). Studies by Leite *et al*. (1995) have shown that *X. a.* pv. *vesicatoria* can be found on seed internally and externally.

External seed infections can transmit bacteria to growing cotyledons when the *X. a.* pv. *vesicatoria* cells contact the seed coat (Ritchie, 2000). Young, infected seedlings will exhibit symptoms after emerging from the soil and in optimal environmental conditions (Ritchie, 2000).

Many gram-negative bacteria use a type III secretion system (TTSS) to deliver various proteins into host cells during infection. The TTSS is a syringe-like structure consisting of inner and outer membrane rings and a protruding filament, or *hrp* pilus, which infiltrates host cells (Tang et al., 2006). The *hrp* pilus is a channel that translocates type III effectors inside host cells to increase the virulence of the pathogen. The TTSS is encoded by 22 hypersensitive response and pathogenicity (*hrp*) genes which are clustered together on numerous operons on a chromosome or plasmid (Koebnik et al., 2006). The *hrp* gene cluster is often flanked by other virulence related genes, type III effector genes (Gurlebeck et al., 2006), and collectively they form the pathogenicity island (Arnold et al., 2003). There are 11 proteins that are highly conserved in many plant, and even animal bacterial pathogens, and make up the core of the TTS apparatus called *hrc* (*hrp* conserved) genes (Gurlebeck et al., 2006). Regulation of the *hrp* operon is controlled by a complex regulatory system and is triggered by environmental factors including temperature, soil nutrition, osmolarity, and pH (Tang et al., 2006). *Xanthomonas* sp. *hrp* genes are regulated by an AraC-like activator, HrpX, and HrpG (Koebnik et al., 2006). HrpX is a downstream regulator that binds to regulated genes that have a conserved cis-regulatory element called the plant-inducible promoter (PIP) box (Koebnik et al., 2006). However many type III effectors in *X. a.* pv. *vesicatoria* lack a PIP box which indicates that genes without the PIP box region can still be regulated by HrpX, but indirectly (Tang et al., 2006). HrpG is an upstream regulator of effector genes and it is negatively regulated by PhcA, a LysR family transcriptional regulator (Tang et al., 2006). PhcA coordinates expression of other

*X. a.* pv. *vesicatoria* virulence factors which includes exopolysaccharides, various plant cell-wall-degrading enzymes, quorum sensing, and bacterial motility (Tang et al., 2006). Bacteria need mechanisms to negatively regulate gene expression so that valuable energy is not wasted on proteins not utilized in infection.

One important function of the TTSS is the translocation of effector proteins. Effector proteins are molecules (such as virulence and avirulence factors, toxins, and elicitors) that manipulate host cell structure and function thus improving pathogen infection and trigger a defense response (Kamoun, 2007). Many secreted effectors contain a TTS-chaperone binding site so that TTS chaperones can attach to stabilize effectors, keeping them partially unfolded during movement through the *hrp* pilus, and connect them to the TTSS for efficient secretion (Gurlebeck et al., 2006). Chaperones, like HpaB in *X. a.* pv. *vesicatoria*, have also been found to control the exiting of proteins through the TTSS (Buttner et al., 2004). Many effector proteins are secreted by the TTSS which includes avirulence proteins (AvrBs1, AvrBs2, AvrBs3, and AvrBs4), XopC (a *Xanthomonas* specific effector that is believed to be a transposase and/or cointegrate resolution protein), XopD (a C48 SUMO cysteine protease), XopJ (a SUMO peptidase and acetyltranferase that localizes with the plant cell membrane and vesicle-like structures and co-localizes with Golgi marker proteins to disrupt or inhibit plant defense protein secretion) (Bartetzko et al., 2009), XopQ (a putative inosine-uridine nucleoside N-ribohydrolase), Ecf (an early chlorosis factor), and many other proteins with unknown functions (Gurlebeck et al., 2006).

When plant resistance gene products detect pathogen elicitor molecules, they activate the hypersensitive response (HR), or programmed cell death, to defend itself against the spread of bacteria or further pathogen attack. Programmed cell death occurs in host cells via reactive

oxygen molecules, such as hydrogen peroxide, and is negatively regulated in neighboring cells by jasmonic acid (Overmyer et al., 2003). Currently two theories are used to describe the interaction between the pathogen and the host. The first theory is the gene-for-gene model. In this theory, the pathogen's avirulence protein interacts directly with the host's resistance gene protein (Flor, 1971). In the second theory, the guard hypothesis, the pathogen's avirulence protein has another function and interacts with another host protein, the guardee, to help increase the fitness of the pathogen. During this interaction, the host's resistance gene protein, the guard, recognizes the interaction and signals the HR to surrounding cells (van der Biezen and Jones, 1998). In both cases, the pathogen avirulence gene and host resistance gene are needed to initiate the HR. Without both present, infection occurs. In many cases the avirulence proteins are thought to be more beneficial to the plant rather than the bacteria because those proteins help contribute to the activation of the plant's defense system. However, the avirulence proteins must have an important function in pathogenesis, infection, pathogen spread, or general fitness or those genes would be discarded through genetic evolution.

Bacterial avirulence genes encode for hydrophilic proteins that are used in the infection process. These proteins elicit or fail to elicit an HR in host plants when detected. Some bacterial genes are conserved among species and others can have a non-functional or recessive form in certain races (Keen, 1990). Avirulence proteins, like AvrBs2, also function to increase the virulence and pathogenicity of the bacterial pathogen (Gurlebeck et al., 2006). *X. a.* pv. *vesicatoria* has four major avirulence proteins that contribute to a pathogenic reaction or induce HR. *AvrBs1* interacts with the pepper resistant gene, *Bs1*, to form a HR on leaves. The function of AvrBs1 is still unknown but it is thought to contribute to pathogen fitness in the field and is located on *X. a.* pv. *vesicatoria*'s largest plasmid (Gurlebeck et al., 2006). *AvrBs1* has two open

reading frames (ORFs) with the second being the most important for activity (Ronald and Staskawicz, 1988). *AvrBs2* which is recognized by *Bs2* in resistant pepper plants is a highly conserved avirulence gene (Wichmann et al., 2005). It is found in almost all strains and is located in chromosomal DNA (Wichmann et al., 2005). These characteristics are quite different from other similar avirulence genes as most are strain specific and found on bacterial plasmids. *AvrBs2* is thought to be highly conserved due to its importance in pathogen fitness and pathogenicity (Wichmann et al., 2005). *AvrBs1* and *AvrBs2* work together to provide the pathogen with improved fitness during infection. With *AvrBs2* being a conserved gene, it is one of the most targeted avirulence genes for developing resistant pepper lines. This avirulence gene has homology with many other bacteria enzymes that synthesize and hydrolyze phosphodiester bonds (Gurlebeck et al., 2006). *AvrBs3* interacts with the pepper resistance gene *Bs3* to cause an HR. It is different from the other avirulence gene as it manipulates the host cell transcriptome directly instead of through enzymatic proteases (Gurlebeck et al., 2006) and it is expressed independent of the *hrp* gene cluster (Knoop et al., 1991). *AvrBs3* is thought to induce hypertrophy in susceptible plants as well as to promote bacterial spread (Gurlebeck et al., 2006; Marois et al., 2002). *AvrBs3* has a specific domain that interacts with host importin α. Importin α is part of the nuclear import machinery that interacts with importin β to mediate the import of the entire protein complex into the plant cell nucleus (Gurlebeck et al., 2006). Once in the nucleus, the AvrBs3 protein binds with the host DNA or in another complex to modify the host cell transcriptome to cause hypertrophy in susceptible plants or induce HR in resistant plants (Marois et al., 2002). Due to *AvrBs3*'s function in the infection process, the *Bs3* resistance gene is also used heavily in commercial plants to reduce infection. *AvrBs4* interacts with the *Bs4* resistance gene. *AvrBs4* is 97% similar in genetic structure to *AvrBs3* (Schornack et al., 2003).

The use of *Bs4* in resistant peppers can be found in a hot type, *Capsicum* PI #504809 and tomato cultivars. Currently, seed companies and breeders have developed and distributed a commercial bell pepper cultivar that contains the *Bs4* resistance gene (Langston, personal communication).

Race is a term use to classify a subgroup of bacterial strains within a species according to the pattern of HR that they cause (Table 1.1) or the specific cultivars a pathogen can infect within a host (Agrios, 2005). To date, 11 races of *X. a.* pv. *vesicatoria* recovered from peppers have been characterized (Stall et al., 2009). Avirulence genes in *X. a.* pv. *vesicatoria* races can mutate by inactivation due to the insertion of a transposable element, the loss of a plasmid, deletions occurring in repeat or unimportant genetic sequences, and by other processes (Kousik and Ritchie, 1996). In 1996, there were only seven *X. a.* pv. *vesicatoria* races reported (Kousik and Ritchie, 1996) compared to the current eleven. Even conserved avirulence genes, such as *AvrBs2*, can have a mutation in a single or numerous base pairs that avoids plant recognition but maintain virulence function (Gassmann et al., 2000).

Using resistance genes (R-genes) in pepper plants has been an effective method to control BLS in the past and still has an impact on the pathogen each year. However, the development of new races and the rate of their development has led to concern for the longevity of the currently used resistance genes. Single resistance genes in the host confers resistance to multiple races. The method of using single or even multiple resistance genes in a host plant that confer complete resistance to a pathogen's attack is a strategy called vertical resistance. Vertical resistance targets specific pathogens or specific pathogen races (Agrios, 2005). With the current system, plants are either fully resistant to a pathogen's attack or have no resistance. One reason that we have relied on vertical resistance for so long is because it is easy to manipulate in breeding programs and to incorporate the needed resistance genes into host crops (Agrios, 2005). Using

vertical resistance can be quite effective until the resistance begins to break down as a result of new races. Current work at the University of Florida and with vegetable seed companies is being conducted to develop horizontal (or partial) resistance to *X. a.* pv. *vesicatoria* (Pernezny et al., 2008). Horizontal resistance is a method that confers incomplete but more durable resistance to a pathogen's attack (Agrios, 2005). This form of resistance utilizes multiple recessive genes and can confer resistance to all races of *X. a.* pv. *vesicatoria* and remain resistant for a longer time period (Pernezny et al., 2008).

Suppressing disease in the field and preventing disease by using disease free seed and transplants is a difficult task. Research has shown that *X. a.* pv. *vesicatoria* populations can survive epiphytically on leaves and inside buds of hosts adjacent to pepper fields (Pernezny and Collins, 1997). These epiphytic bacteria can colonize on the outer surface of the host plants and be protected from the harsher environment and UV light in protective, moist pockets like the flower and buds (Pernezny and Collins, 1997). Therefore, even if seed is planted that is not infected with *X. a.* pv. *vesicatoria*, control and prevention is still hard to accomplish because of resident sources of inoculum. Due to unknown sources of inoculum, effective management strategies outside of using host resistance for the control of BLS can be difficult to employ. *X. a.* pv. *vesicatoria* can only survive for a short time without a host in the soil and therefore crop rotation can also be effective (Ritchie, 2000). Heavy metal-based bactericides formulated with copper, zinc, and manganese are also used to reduce and prevent bacterial populations from increasing. Other antibiotic chemicals, plant defense activators, and bacteriophages are also being researched to prevent disease.

Copper-based compounds comprise the main chemical group used for preventive control. Copper is an essential trace element used by many bacteria (Bai et al., 2007). Copper is used in

bacterial cells in structural composition and functions of enzymes, such as cytochrome oxidase, copper-zinc superoxide dismutase, amine oxidase, and ATPase (Bai et al., 2007). Copper also functions to help with electron transport and oxidation-reduction reactions (Cervantes and Corona, 1994). However at higher concentrations, copper molecules can become toxic and disrupt membrane-bound copper that catalyzes the formation of hydroperoxide free radicals, damage lipids, damage proteins, and even damage DNA or other important biomolecules (Bai et al., 2007). Due to copper's dual role as a necessity and a harmful molecule, it must be regulated and easily transported in and out of the cell. Certain populations of bacteria have also developed and/or acquired plasmids with specialized genes to help them tolerate high levels of free copper molecules. In *X. a.* pv. *vesicatoria*, six copper genes were identified. These include *copL*, *copA*, *copB*, *copM*, *copG*, and *copF* (Voloudakis et al., 2005). *Pseudomonas syringae* has shown to have more *cop* genes that have not yet been discovered in *X. a.* pv. *vesicatoria*. These include *copC*, *copD*, *copR*, *copS*, *copY*, and *copZ* (Cervantes and Corona, 1994). *P. syringae* CopA has been found in to be a periplasmic protein that binds to multiple copper molecules and CopB functions as an outer membrane protein to bind to copper ions (Cooksey, 1993). In this mechanism described by Cooksey, the bacteria utilize the CopA, CopB, CopC, and CopD proteins. CopB is an outer-membrane protein that binds to free copper ions and imports them into the periplasm. From here, CopC transports copper ions from the outer-membrane to the inner-membrane to CopD. CopD is the inner-membrane protein that receives copper ions from CopC and imports them directly into the cytoplasm. Meanwhile, CopB continues to import free copper ions that accumulate in the periplasmic space. CopC and CopA are abundant proteins. CopA is responsible for collecting and holding free copper ions not transported by the CopC protein. The amount of free ionic copper that CopA, a 72 kDa protein, can bind is unknown

(Cooksey, 1993). It is thought that this periplasmic protein can hold approximately 11 copper atoms per polypeptide chain versus the one copper atom per polypeptide in the 12 kDa CopC protein (Cooksey, 1993). Considering that copper is present in all bacteria, they require copper genes that function in copper uptake, intracellular storage/transport, export, and regulation (Brown et al., 1992). These functions may be controlled by multiple or just a few genes.

The full mechanism of copper resistance in *X. a.* pv. *vesicatoria* is still unknown. Some possible theories include pathogen proteins that help with the export of free copper ions out of the cell, proteins that bind to extracellular copper ions and prevent them from entering the cell while the bacteria recycles intracellular copper for its use (Cooksey, 1993), and binding of the proteins to free copper ions to protect the rest of the bacterial cell (Cervantes and Corona, 1994). It is believed that another possible method for resistance occurs via translocation pumps on the cellular membrane that require ATP to function and keep a safe level of copper ions inside the membrane (Cervantes and Corona, 1994). Most of the copper tolerance genes are plasmid borne and inducible, like pXV10A (Bender et al., 1990). These copper resistance genes are capable of being transmitted rapidly from one strain to another.

Many copper-based bactericides are used for peppers and other crops to control not only BLS but other bacterial and fungal diseases. Some of these include cupric hydroxide, Bordeaux mixture, and cupric sulfate. Due to the exposure to many copper-based bactericides, strains that have developed tolerance can be 10 to 80 times more tolerant to copper sprays than normal bacterial populations (Menkissoglu and Lindow, 1991). Research has also shown that copper salts can be solubilized by rain, dew, or water droplets on the leaf surface and that a majority of the copper ions in the solution are complexed with organic compounds on the leaf (Andrew et al., 1977). More interestingly, only approximately 0.1% of the total soluble copper in a cupric

hydroxide solution on a leaf surface exists as free, toxic $Cu^{2+}$ ions (Menkissoglu and Lindow, 1991). Also, dosage of copper-based bactericides seems to have minimal effect on the amount of free $Cu^{2+}$ ions present on a leaf surface and that the amount of ions depends largely on the equilibrium constant of the complexes and the leaf surface pH (Menkissoglu and Lindow, 1991).

Often copper tolerance is tested by transferring bacterial strains to a copper amended media and observing growth after incubation. Although it is an accepted test (Pernezny et al., 2008; Stall et al., 1986), variability within and across strains has been observed. Pernezny *et al.* (2008) found that different types of media used in copper tolerance testing can have variable effects on the results and growth of tolerant strains. The effectiveness of screening strains on a plate-based method can differ from one experiment to the next and results can depend on the carbon and copper source used in the medium (Pernezny et al., 2008). Other reported methods include viability testing after direct exposure to different concentrations of liquid copper solutions (Marco and Stall, 1983). Due to the variability observed in some plate tests, copper tolerance may be more quantitative than qualitative and may depend largely on the nutrients available.

## Literature cited


Agrios, G. N. 2005. *Plant Pathology*. 5th Edition. Elsevier Academic Press, Burlington, USA.

Andrew, R. W., Biesinger, K. E., and Glass, G. E. 1977. Effects of inorganic complexation on the toxicity of copper to Daphnia magna. Water Research 11:309-315.

Arnold, D. L., Pitman, A., and Jackson, R. W. 2003. Pathogenicity and other genomic islands in plant pathogenic bacteria. Mol. Plant Pathol. 4:407-420.

Bai, W., Zhao, K., and Asami, K. 2007. Effects of copper on dielectric properties of *E. coli* cells. Coll. Surf. B. Bioinfo. 58:105-115.

Bartetzko, V., Sonnewald, S., Vogel, F., Hartner, K., Stadler, R., Hammes, U. Z., and Bornke, F. 2009. The *Xanthomonas campestris* pv. *vesicatoria* type III effector protein XopJ inhibits protein secretion: Evidence for interference with cell wall-associated defense responses. Mol. Plant Microbe Interact 22(6):655-664.

Bashan, Y., Okon, Y., and Henis, Y. 1982. Long-term survival of *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* in tomato and pepper seeds. Eco. Epidemiol. 72(9):1143-1144.

Basim, H., Stall, R. E., Minsavage, G. V., and Jones, J. B. 1999. Chromosomal gene transfer by conjugation in the plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*. Phytopathology 89:1044-1049.

Bender, C. L., Malvick, D. K., Conway, K. E., George, S., and Pratt, P. 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Ap. Env. Microbiol. 56(1):170-175.

Bergey, D. H. 1930. *Bergey's Manual of Determinative Bacteriology*. 3rd Edition. Williams and Wilkins Co., Baltimore, USA.

Boatright, S. R. and McKissick, J. C. 2009. 2008 Georgia Farm Gate Vegetable Report. The University of Georgia, Center for Agribusiness and Economic Development. No. AR-09-02.

Bouzar, H., Jones, J. B., Minsavage, G. V., Stall, R. E., and Scott, J. W. 1994. Proteins unique to phenotypically distinct groups of *Xanthomonas campestris* pv. *vesicatoria* revealed by silver staining. Phytopathology 84:39-44.

Bouzar, H., Jones, J. B., Stall, R. E., Hodge, N. C., Minsavage, G. V., Benedict, A. A., and Alvarez, A. M. 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. Amer. Phytopathol. Soc. 84(7):663-671.

Brown, N. L., Rouch, D. A., and Lee, B. T. 1992. Copper resistance determinants in bacteria. Plasmid 27:41-51.

Buttner, D., Gurlebeck, D., and Noel, L. D., and Bonas, U. 2004. HpaB from *Xanthomonas campestris* pv. *vesicatoria* acts as an exit control protein in type III-dependent protein secretion. Mol. Microbiol. 54:755-768.

Cervantes, C., and Gutierrez-Corona, F. 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev. 14:121-138.

Cooksey, D. A. 1993. Copper uptake and resistance in bacteria. Mol. Microbiol. 7(1):1-5.

Doidge, E. M. 1921. A tomato canker. Ann. Appl. Biol. 7:407-430.

Dowson, W. J. 1943. On the generic names *Pseudomonas*, *Xanthomonas*, and *Bacterium* for certain bacterial plant pathogens. Trans. British Mycol. Soc. 26:4-14.

Dye, D. W., Bradbury, J. F., Dickey, R. S., Goto, M., Hayward, A. C., et al. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Pathol. 59:153-168.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 9:275-296.

Gardner, M. W. and Kendrick, J. B. 1921. Bacterial spot of tomato. J. Agric. Res. 21:123-156.

Gassmann, W., Dahlbeck, D., Chesnokova, O., Minsavage, G. V., Jones, J. B., and Staskawicz, B. J. 2000. Molecular evolution of virulence in natural filed strains of *Xanthomonas campestris* pv. *vesicatoria*. J. Bacteriol. 182(24):7053-7059.

Gurlebeck, D., Thieme, F., and Bonas, U. 2006. Type III effector proteins from the plant pathogen *Xanthomonas* and their role in the interaction with the host plant. J. Plant Physiol. 163:233-255.

Hayward, A. C., and Waterson, J. M. 1964. *Xanthomonas vesicatoria*. CMI Descriptions of Pathogenic Fungi and Bacteria. No. 20.

Jones, J. B. 1991. Bacterial Spot. In: Compendium of Tomato Diseases. Eds: J.B. Jones et al., APS press. Page 27.

Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst. Appl. Microbiol. 27(6):755-62.

Jones, J. B., Pohronezny, K. L., Stall, R. E., and Jones, J. B. 1986. Survival of *Xanthomonas campestris* pv. *vesicatoria* in Florida on tomato crop residues, weeds, seeds and volunteer tomato plants. Phytopathology 76:430-434.

Jones, J. B., Stall, R. E., and Bouzar, H. 1998. Diversity among *Xanthomonads* pathogenic on pepper and tomato. Annu. Rev. Phytopathol. 36:41-58.

Kamoun, S. 2007. Groovy times: filamentous pathogen effectors revealed. Current Opinion in Plant Biology 10:358-365.

Kay, S., Hahn, S., Marois, E., Hause, G., and Bonas, U. A bacterial effector acts as a plant transcription factor and induces a cell size regulator. 2007. Science 318:648-651.

Keen, N. T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Annu. Rev. Genet. 24:447-463.

Knoop, V., Staskawicz, B., and Bonas, U. 1991. Expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under control of the *hrp* genes and is independent of plant factors. J. Bacteriol. 173(22):7142-7150.

Koebnik, R., Kruger, A., Thieme, F., Urban, A., and Bonas, U. 2006. Specific binding of the *Xanthomonas campestris* pv. *vesicatoria* AraC-Type transcriptional activator HrpX to plant-inducible promoter boxes. J. Bacteriol. 188(21):7652-7660.

Kousik, C. S. and Ritchie, D. F. 1996. Race shift in *Xanthomonas campestris* pv. *vesicatoria* within a season in field grown pepper. Phytopathology 86:952-958.

Leite, R. P., Jones, J. B., Somodi, G. C., Minsavage, G. V., and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Disease 79(9):917-922.

Marco, G. M. and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Disease 67:779-781.

Marois, E., Van den Ackerveken, G., and Bonas, U. 2002. The *Xanthomonas* type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. MPMI 15:637-646.

Menkissoglu, O. and Lindow, S. E. 1991. Chemical forms of copper on leaves in relation to the bactericidal activity of cupric hydroxide deposits on plants. Phytopathology 81:1263-1270.

Momol, T., Jones, J., Olson, S., Obradovic, A., Balogh, B., and King, P. 2002. Integrated management of bacterial spot on tomato in Flordia. Fact sheet PP110. University of Florida. http://edis.ifas.ufl.edu

Overmyer, K., Brosche, M., and Kangasjarvi, J. 2003. Reactive oxygen species and hormonal control of cell death. Trends in Plant Science 8(7):335-342.

Pernezny, K. and Collins, J. 1997. Epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on pepper: Relationships to host-plant resistance and exposure to copper sprays. Plant Disease 81:791-794.

Pernezny, K., Jones, J. B., Nagata, R. T., and Havranek, N. 2008. Host-plant resistance and management of bacterial spot of pepper. University of Florida, Publication. #PP255.

Pernezny, K., Nagata, R., Havranek, N., and Sanchez, J. 2008. Comparison of two culture media for determination of the copper resistance of *Xanthomonas* strains and their usefulness for the prediction of control with copper bactericides. Crop Protection 27:256-262.

Poplawsky, A. R., Urban, S. C., and Chun, W. 2000. Biological role of xanthomonadin pigments in *Xanthomonas campestris* pv. *campestris*. App. Environ. Microbiology 66(12):5123-5127.

Quezado-Duval, A. M., Leite, R. P., Truffi, D., and Camargo, L. E. 2004. Outbreaks of bacterial spot caused by *Xanthomonas gardneri* on processing tomato in central-west Brazil. Plant Dis. 88:157-161.

Ritchie, D. F. 2000. Bacterial spot of pepper and tomato. The Plant Health Instructor. DOI: 10.1094/PHI-I-2000-1027-01

Ronald, P. C. and Staskawicz, B. J. 1988. The Avirulence gene *avrBs1* from *Xanthomonas campestris* pv. *vesicatoria* encodes a 50-kD protein. MPMI 1(5):191-198.

Rubatzky, V. E. and Yamaguchi, M. 1999. World Vegetables: Principles, Production, and Nutritive Values. 2nd Eds., Aspen Publishers, Inc. Pages 553-562.

Sahin, F. and Miller, S. A. 1998. Resistance in *Capsicum pubescens* to *Xanthomonas campestris* pv. *vesicatoria* pepper race 6. Plant Disease 82(7):794-799.

Schornack, S., Ballvora, A., Gurlebeck, D., Peart, J., Ganal, M., Baker, B.J., Bonas, U., Lahaye, T. 2003. The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. Plant Journal 37(1) 46-60.

Schumann, G. L. and D'Arcy, C. J. 2006. Essential Plant Pathology. APS Press; St. Paul, Minnesota. Pages 52-53.

Sherbakoff, C. D. 1918. Report of the associate plant pathologist. Fla. Agric. Exp. Stn. Rep. 1916-1917:66R-86R.

Stall, R. E., Beaulieu, C., Egel, D., Hodge, N. C., Leite, R. P., et al. 1994. Two genetically diverse groups of strains are included in *Xanthomonas campestris* pv. *vesicatoria*. Int. J. Syst. Bacteriol. 44:47-53.

Stall, R. E., Jones, J. B., and Minsavage, G. V. 2009. Durability of resistance in tomato and pepper to xanthomonads causing bacterial spot. Annu. Rev. Phytopathol. 47:265-284.

Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 76:240-243.

Stevens, F. L. 1925. *Plant Disease Fungi*. MacMillan Co., New York, USA.

Tang, X., Xiao, Y., and Zhou, J. M. 2006. Regulation of the type III secretion system in phytopathogenic bacteria. MPMI 19(11):1159-1166.

Thieme, F., Koebnik, R., Bekel, T., Berger, C., Boch, J., Buttner, D., Caldana, C., Gaigalat, L., Goesmann, A., Kay, S., Kirchner, O., Lanz, C., Linke, B., McHardy, A.C., Meyer, F., Mittenhuber, G., Nies, D.H., Niesbach-Klsgen, U., Patschkowski, T., Ruckert, C., Rupp, O., Schneiker, S., Schuster, S.C., Vorholter, F.-J., Weber, E., Phler, A., Bonas, U., Bartels, D., and Kaiser, O. 2005. Insights into genome plasticity and pathogenicity of the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* revealed by the complete genome sequence. J. Bacteriol. 187:7254-7266.

Van Der Biezen, E. and Jones, J. D. G. 1998. Plant disease-resistance proteins and the gene-for-gene concept. Trends in Biochem. Sci. 23(12):454-456.

Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. 45:472-489.

Voloudakis, A. E., Reignier, T. M., and Cooksey, D. A. 2005. Regulation of resistance to copper in *Xanthomonas axonopodis* pv. *vesicatoria*. Ap. Env. Microbiol. 71(2):782-789.

Weber, E., Ojanen-Reuhs, T., Huguet, E., Hause, G., Romantschuk, M., Korhonen, T. K., Bonas, U., and Koebnik, R. 2004. The type III-Dependent Hrp pilus is required for productive interaction of *Xanthomonas campestris* pv. *vesicatoria* with pepper host plants. J. Bacteriol. 187(7):2458-2468.

Wichmann, G., Ritchie, D., Kousik, C. S., and Bergelson, J. 2005. Reduced genetic variation occurs among genes of the highly clonal plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*, including the effector gene *avrBs2*. Appl. Environ. Microbiol. 71(5):2418-2432.

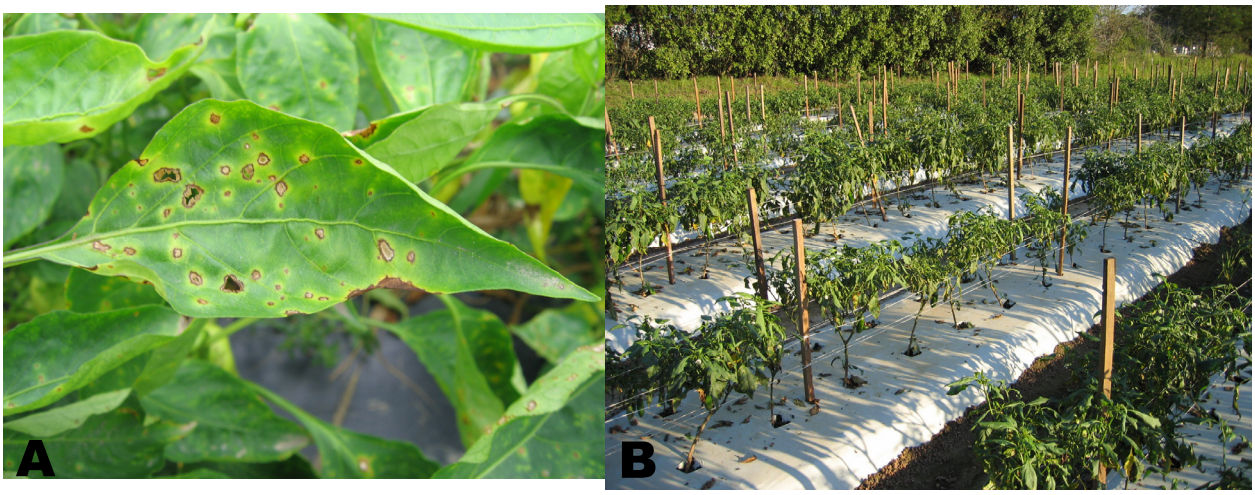


**Fig. 1.1 – Typical symptoms of Bacterial Leaf Spot (BLS) caused by *Xanthomonas axonopodis* pv. *vesicatoria*.** A) Common leaf spots with necrotic center and yellow halo on infected pepper cultivar, Aristotle. B) Devastating field infection with severe defoliation of lower leaves.

**Table 1.1 – Pepper race classification of susceptible and hypersensitive response reactions.** A description of races defined by the hypersensitive response (HR) induced on each pepper host containing the corresponding resistance genes. Typical pepper cultivars with no resistance genes, such as Early California Wonder, are used as a positive control to ensure infection occurs with the inoculated bacterial strain. Table taken from Stall *et al.* 2009.

| | Pepper Resistance Gene | | | | |
|---|---|---|---|---|---|
| Race | None | *Bsr1* | *Bsr2* | *Bsr3* | *Bsr4* |
| 0 | **S** | **HR** | **HR** | **HR** | **HR** |
| 1 | **S** | **S** | **HR** | **HR** | **HR** |
| 2 | **S** | **HR** | **HR** | **S** | **S** |
| 3 | **S** | **S** | **HR** | **S** | **HR** |
| 4 | **S** | **S** | **S** | **HR** | **HR** |
| 5 | **S** | **HR** | **S** | **S** | **S** |
| 6 | **S** | **S** | **S** | **S** | **HR** |
| 7 | **S** | **S** | **HR** | **HR** | **S** |
| 8 | **S** | **S** | **HR** | **S** | **S** |
| 9 | **S** | **S** | **S** | **HR** | **S** |
| 10 | **S** | **S** | **S** | **S** | **S** |

S (susceptible)
HR (Hypersensitive Response)

# CHAPTER 2

# EVALUATION OF FIELD-COLLECTED *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA* STRAINS, ON BELL PEPPER, WITH RESPECT TO RACE DETERMINATION AND SENSITIVY TO COPPER IN SOUTHERN GEORGIA

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**Abstract** A survey of *Xanthomonas axonopodis* pv. *vesicatoria* strains was conducted in southern Georgia in 2007-2008. Sample sites were selected based on the presence of bacterial leaf spot of pepper. The objective of the survey was to identify the distribution of *X. a.* pv. *vesicatoria* races across the pepper growing region of Georgia. Strains were also tested for their tolerance to copper. Of 119 pathogenic strains race-typed, 1.7, 4.2, 3.4, 1.7, 2.5, 36.1, and 50.4% were races 3, 4, 6, 7, 8, 9, and 10, respectively. The entire collection of *X. a.* pv. *vesicatoria* strains were assayed on copper-amended nutrient agar containing 200ppm of $CuSO_4 \cdot 5H_2O$. Out of 155 strains, 89% were copper tolerant as they displayed growth on copper amended media. These data indicate that race 10 is the most predominant race of *X. a.* pv. *vesicatoria* on bell pepper in Georgia and is able to infect varieties with all four resistance genes commercially available. The use of copper on *X. a.* pv. *vesicatoria* has been found to be effective on 11% of the tested population and not a reliable alternative to pepper resistance genes.

**Key Words** *Xanthomonas axonopodis* pv. *vesicatoria*, *Xanthomonas campestris* pv. *vesicatoria*, Bacterial leaf spot, pepper, race, copper tolerance

## Introduction

Bacterial leaf spot (BLS) of bell pepper is a devastating disease caused by *Xanthomonas axonopodis* pv. *vesicatoria* (17, 41) (also referred to as *Xanthomonas campestris* pv. *vesicatoria*) that causes millions of dollars in losses each year in Georgia (Langston, personal communication). When inoculum and environmental conditions are optimal for disease development, this disease can be destructive in both the greenhouse and field (33). BLS can have detrimental effects on fruit quality, fruit yield, photosynthetic rates and plant growth, and cause severe defoliation (32).

In the southeastern region of the US, specifically Florida, North Carolina, and Georgia, management of BLS relies heavily on host resistance (27) and copper-based bactericides (16, 23, 25, 30). Other management strategies include using disease-free seed and transplants, crop rotation with non-host plants, sanitation practices, and application of antibiotics, including streptomycin (Firewall, Cerexagri-Nisso LLC, U.S.A.). Several field studies have shown that host resistance and copper applications may be losing effectiveness (25, 29).

Three groups and eleven races of *X. a.* pv. *vesicatoria* can be described using tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*) cultivar Early California Wonder (ECW), and three near-isogenic lines of ECW that each carry a single resistance gene, *Bs1* (ECW-10R), *Bs2* (ECW-20R), *Bs3* (ECW-30R), and one resistance gene in a breeding pepper cultivar, *Bs4* (PI 504809) (27, 30). The strains that are only pathogenic on tomato are in the tomato group (XcvT) and will not be discussed further in this study (27). This research focuses on the pepper group (XcvP) which causes disease only on pepper and the pepper-tomato group (XcvPT) which is pathogenic on both hosts (27). Strains can be further categorized by races according to reactions they induce on differential pepper lines.

Until recently, use of copper bactericides along with host resistance has been a stable and effective method to control bacterial leaf spot (23). Cultivars that are resistant to *X. a.* pv. *vesicatoria* induce a hypersensitive response (HR) indicated by an area of confluent necrosis when leaves are inoculated with bacterial concentrations of 1 x $10^8$ colony forming units (CFU) per milliliter (27). Strains that carry the avirulence gene that corresponds with host resistance genes (*avrBs2* and *Bs2*) will induce the HR which inhibits bacterial colonization and prevents symptom expression (37). *X. a.* pv. *vesicatoria* frequently mutates to overcome resistance genes (13, 27, 33). Bacterial avirulence genes can be inactivated due to the insertion of transposable elements, the loss of plasmids carrying the avirulence genes, and other genetic mutations (21) which results in the breakdown of host resistance (22).

Strains of *X. a.* pv. *vesicatoria* have varying levels of tolerance to copper-based bactericides (29). Several factors may be responsible for this such as poor spray coverage, improper timing of copper sprays, the presence of tolerant strains, or high disease pressure (26).

The objectives of this study were to determine the frequency and distribution of races of strains of *X. a.* pv. *vesicatoria* collected from the pepper growing areas of Georgia and to determine the frequency of copper tolerance among these strains.

## Materials and Methods

**Isolation of strains.** From June of 2007 thru September of 2008, 155 pepper samples with BLS symptoms were collected from both fields and greenhouse epidemics across southern Georgia. All samples were collected from commercial plantings of hot, specialty, and bell pepper cultivars. During this two year study, the survey area covered Berrien, Colquitt, Lowndes, Tift, and Sumter counties (Table 2.1). Three of the sample areas, namely Colquitt,

Lowndes, and Tift counties, were rated in the top five counties for commercial bell pepper acreage in 2008 according to the Georgia Farm Gate Report (7). Thirteen different commercial pepper types and cultivars (Jalapeno, Habanero, Plato, Revolution, Aquiles, Chapala X3R, Naza, Cubanelle, Early California Wonder, Jalafuego, Tula, Aristotle, and experimental variety 0287) were sampled for BLS. Each sample consisted of a single, symptomatic leaf detached from a region of a plant displaying BLS symptoms. Samples were collected from fields with BLS outbreaks by sampling plants at the four corners of the field and one in the center of the field. If the field displaying BLS symptoms was approximately 1.6 Ha or larger, collection in the center of the area was increased to two or three samples. Leaf samples were placed in clear plastic bags, labeled, and transported in a cooler to the laboratory. Bacterial strains were isolated from diseased leaves by removing a section of infected tissue from the outer region of a leaf spot with a scalpel and macerating the tissue in 100 μL of sterile, tap water. The scalpel, forceps, and inoculating needle used in the isolation were surface-sterilized by dipping the tool in 90% alcohol and flamed with an alcohol burner between individual samples. The tissue sample remained in the water for approximately 1 min before a quad-loop (Globe Scientific, INC., U.S.A.) was used to remove 10 ul of the suspension. The loopful of the suspension was streaked for isolation onto a nutrient-rich (NA) medium (Difco Laboratories, Detroit, MI) and yeast dextrose carbonate (YDC) medium (34) and incubated for 24 to 48 hours at 28°C. After incubation, single yellow, circular, butyrous, shiny colonies were selected from each streak and were re-streaked until pure cultures were obtained. Plates were incubated at 28°C for 2 to 5 days. Once in a pure state, colonies were suspended in 15% glycerol and stored at -80°C.

**Identification of strains.** All bacterial strains were identified by colony characteristics on NA and YDC. An ELISA based test kit (Product code 1099, Adgen Phytodiagnostics:

Neogen Europe Ltd., Europe) specific for *X. a.* pv. *vesicatoria* was used to verify strain identity according to manufacture's instructions. All positive strains were tested for pathogenicity on a susceptible pepper cultivar (ECW). A small sub-sample (n=62 strains) was selected from the total collection and tested for Gram reaction and oxidase activity. Gram reactions were determined using the 3% potassium hydroxide string test (34). Oxidase reactivity was tested on a chemical based test kit (BBL BD Dryslide Oxidase) according to manufactures instructions.

**Race determination.** Races were defined by the HR or susceptible reaction on the differential pepper cultivars described earlier (9, 10, 19, 20, 31). All strains were removed from -80°C storage, were streaked on NA and incubated at 28°C for 2-4 days. Colonies from the plates were suspended in 2 ml of sterile, tap water. The suspension was then adjusted to 1 x $10^8$ CFU/ml using a spectrophotometer ($A_{600nm}$ = 0.1) (33). Each suspension of each strain was infiltrated into the intercellular spaces of fully expanded pepper leaves using a 3 ml syringe pressed against the abaxial leaf surface until an area of approximately 1.5 $cm^2$ became visibly water-soaked. Each infiltration zone was labeled with permanent, black ink to identify the strain inoculated. The plants were incubated under greenhouse conditions for 2 weeks. HR reactions were observed and recorded 24 to 48 hours after inoculation. Disease reactions were recorded beginning 7 days after inoculation.

**Copper tolerance.** Strains were removed from storage and streaked onto NA medium and incubated at 28°C for 2 days. Cell suspensions of 1 x $10^8$ CFU/ml ($A_{600nm}$ = 0.1) were prepared for each sample and 25µl (for a total of 1 x $10^5$ CFU/drop) was spotted onto NA and NA amended with 200 ppm $CuSO_4 \cdot 5H_20$ (5 g of peptone, 3 g of beef extract, 15 g of agar, and 0.20 g of $CuSO_4 \cdot 5H_20$ in 1 L of distilled water) (29, 30, 39). After transfers, plates were incubated for 2 days at 28°C. Visual inspection of each spot was conducted two days after

inoculation and the size and growth characteristics of each colony were recorded. Strains were categorized as tolerant, intermediate, or sensitive to copper (Fig. 2.1). The tolerant strains exhibited full, confluent growth on the amended media while the intermediate strains were classified by uneven, variable colony growth within the inoculated area. Sensitive strains exhibited no growth.

## Results

**Pathogen identification.** All strains were found to be *X. a.* pv. *vesicatoria* by the procedures listed above. However, only ELISA-positive strains were used in this study. All 52 sub-sample strains tested were gram-negative and oxidase-negative. Strains were also grown on NA and YDC for identification. Colonies on NA were yellow, circular, butyrous, and shiny while the colonies on YDC were round, convex, bright yellow, and mucoid. Strains used for race typing were tested for a disease reaction on ECW. All strains causing a disease reaction on ECW were race-typed.

**Race distribution.** A total of 119 strains were race-typed on ECW and near-isogenic lines with resistance genes, as described earlier (Table 2.1). Pepper races 3, 4, 6, 7, 8, 9, and 10 were detected in southern Georgia from 2007 thru 2008. Sixty strains (50.4%) were race 10, 43 (36.1%) were race 9, three (2.5%) were race 8, two (1.7%) were race 7, four (3.4%) were race 6, five (4.2%) were race 4, and two (1.7%) were race 3 (Table 2.1). All the pepper races listed above were detected in 2007. Also in 2007, 10 strains of race 10 were found in fields across Tift County on the pepper cultivar Plato (Table 2.1). This was the highest occurrence of race 10 on any of the sampled pepper cultivars or types. The majority of strains isolated in 2007 were race 10. In 2008, 26 samples were collected and all races previously detected in 2007 were found with the exception of races 3, 7, and 8. The majority of strains collected in 2008 were race 9 and

had been isolated from an unknown cultivar(s) from unknown origin(s). Strains of only one race on specific pepper cultivars or types, namely race 3 (1 strain), race 10 (2 strains), race 10 (2 strains), and race 10 (5 strains), were recovered from ECW, Cubanelle, Revolution, and Var. 0287, respectively. The largest number of strains collected were from Colquitt (n=46) and Tift (n=41) counties (Table 2.1).

**Sensitivity to copper.** Out of 155 strains tested for copper tolerance, 138 strains (89%) were copper tolerant (Table 2.1). One hundred and twenty-three strains (79.4%) were classified as tolerant, 15 (9.7%) were classified as intermediate, and 17 (11%) were classified as sensitive (Table 2.1). The majority of the copper-tolerant strains were isolated from the Plato pepper cultivar from Tift County. Berrien County was the only county from which only copper sensitive strains were recovered in 2007.

## Discussion

Seven of the 11 known races were found across Georgia. No counties were found to have only one race present, however four pepper cultivars or types were found to have only one race present. Once *X. a.* pv. *vesicatoria* strains are introduced into an area by means of seed or transplants, it seems likely that these populations may still be present and overwinter or live epiphytically on other hosts until a pepper host returns (28). This may explain why multiple races are found in most Georgia Counties. To date, copper resistance in *X. a.* pv. *vesicatoria* has been reported in California (11), Florida (14), Oklahoma (6), Ohio (32), North Carolina (30), Mexico (1), and Australia (26). This research indicates that copper tolerant *X. a.* pv. *vesicatoria* strains can also be found in Georgia. Ritchie and Dittapongpitch (1991) stated that it is not unexpected to find different strains with different tolerance levels to copper in a field survey. Various races and multiple strains with varying degrees of copper tolerance found in this study

may be associated with the type of copper-based bactericide utilized, the timing, and the frequency of use on transplants and commercial fields. Therefore, it seems possible that contaminated seed and transplants can add to the current diversity of *X. a.* pv. *vesicatoria* populations in the field and be a possible source of inoculum.

Other studies have indicated that *X. a.* pv. *vesicatoria* populations are capable of shifting their predominant race type during a growing season (21). Kousik and Ritchie (1996) found that when the *X. a.* pv. *vesicatoria* strain, Xcv33$^{rif}$ (race 1), was tested on ECW-30R plants in the field, it was incompatible and caused an HR on the pepper cultivar at the beginning of the season. After 10 weeks in the field, all strains were found to be race 3 and caused disease on ECW-30R (21). It is possible that race shifts occurred in the populations tested in this survey. This study demonstrated that multiple races can be found in close proximity to one another within the same field. At some collection sites, multiple pepper races were found within the same field and from the same pepper cultivar or type. Some of these strains may have shifted from their original race to race 10.

In 2007 and 2008, the majority of the *X. a.* pv. *vesicatoria* strains tested were races 10, 9, 4, 6, 3, and 7 and 3, respectively. Pepper race 10 is devastating to the current commercial pepper industry (38). Pepper race 6 (P6) (31) and pepper race 10 (P10) differ only in their reaction in plants containing *Bs4*, currently found in pepper PI 504809. P10 is capable of causing disease on pepper cultivars with the *Bs4* gene but P6 induces an HR. Both P6 and P10 are capable of causing disease on all three of the currently used resistance genes in commercial pepper cultivars. If the majority of the *X. a.* pv. *vesicatoria* strains in Georgia are P10, than the usefulness of incorporating the *Bs4* resistance gene into commercial bell pepper cultivars may be limited. Until 2009, there had not been a commercial pepper cultivar carrying the *Bs4* gene,

which makes explaining the high frequency of P10 in Georgia difficult. Strain populations would be expected to overcome only those resistance genes in host pepper cultivars they infect. Strains that have overcome the *Bs4* resistance gene may have been influenced by resistant tomato cultivars (3). *X. a.* pv. *vesicatoria* strains in the tomato group may have mutated the AvrBs4 avirulence protein and translocated the gene on a plasmid to the pepper strains. This avirulence protein may contribute to virulence of the pepper strains. It is also possible that new races were introduced into the environment from commercial pepper seeds from fields used in breeding new pepper cultivars that carry the *Bs4* gene. Gurlebeck *et al.* (2009) recently reported the *AvrBs4* gene to encode for a large catalase, crystal in peroxisomes and suggests that this avirulence protein plays an important role in suppression of plant defense responses by accumulating catalase. Catalase has been found to detoxify hydrogen peroxide which is used by host plants as a signal molecule for pathogen attack and triggering plant defense mechanisms (15). Because of the virulence function and suppression of host plant defenses with the *AvrBs4* gene, P10 strains may be more aggressive on peppers. This indicates that race 10 strains would be a more successful pathogen on pepper plants and in higher quantities.

Pepper production and the pepper acreage in a given area seem to have an impact on race number and distribution. These data demonstrate that counties with more pepper acreage had more races and a greater distribution of *X. a.* pv. *vesicatoria* races. In two of the five counties sampled, Colquitt and Tift, five and four different peppers races were found, respectively. These two counties also are in the top five counties in Georgia for production bell pepper acreage. These two counties also had the highest diversity of pepper cultivars and types. It appears that there may be a link between pepper cultivar diversity and the number of different races among strains. If the disease's primary inoculum source is thought to be via seed, then an area with a

higher number of pepper cultivars from various seed companies and genetic diversity would expect to have a higher diversity in races collected.

Copper tolerance was also evaluated from collected field strains. In a total of 155 strains tested on copper amended media, 89% were found to have some measure of copper tolerance. Strains were also grouped according to their level of tolerance to the copper. A similar study conducted at the University of Georgia Tifton Campus in 2004 thru 2006, found that of 63 strains collected, 90.5% were tolerant to copper when colonies were streaked onto cupric sulfate amended NA media (Langston and Sanders, data not published). In both studies, similar frequencies of copper-tolerant strains were found. In the previous study, the sample size was smaller and strains were isolated in pure culture and streaked onto copper amended media. In this study, the sample size was more than double that of the previous study and cell suspensions of 1 x $10^5$ CFU/drop were spotted onto NA amended with 200 ppm $CuSO_4 \cdot 5H_20$.

In both studies, the majority of the strains collected were found to be tolerant to copper. Tolerant strains have been reported in many other closely related bacteria from multiple states (2, 5, 12, 35, 40) as well as *X. a.* pv. *vesicatoria* in past studies (25, 26, 30). Only Berrien County had four copper-sensitive strains alone in commercial pepper fields (Table 2.1). The pepper cultivars sampled in that county were not unique to that county and were sampled in other counties. Since copper tolerance is plasmid borne (43), it is likely that the plasmids required were not present in the *X. a.* pv. *vesicatoria* strains from Berrien County. It also should be considered that the sample size from Berrien County was not large enough to draw conclusions that are accurate to actual population numbers. Tolerant strains may be present in this county but undetected.

Results from this research indicate that *X. a.* pv. *vesicatoria* is a pathogen that has outpaced our ability to use copper for effective disease management. The high frequency of copper tolerant strains coupled with the occurrence of strains that can overcome all four known resistance genes in bell pepper is disturbing, and may predict more damaging losses to pepper growers in Georgia. Fifty *X. a.* pv. *vesicatoria* strains were found to be copper-tolerant and race 10. Past control recommendations have included a mancozeb (manganese ethylenebis dithiocarbamate) tank-mixed with copper-based bactericides in order to suppress copper tolerant field strains (35). To date, those recommendations are still being used, but the effectiveness of even the tank mixtures has appeared to decline in southeastern Georgia. Novel remedial controls need to be integrated into BLS-management systems currently using copper bactericides, mancozeb tank mixtures, and resistant cultivars to slow the development of new races and bactericide-tolerant bacteria. Also, future testing needs to be conducted on the effect of mancozeb mixtures and if mancozeb tolerant strains exist. Pepper varieties utilizing multiple resistance genes and different forms of resistance (horizontal and vertical), should also be utilized and would provide a more durable pepper cultivar than cultivars carrying the *Bs1*, *Bs2*, *Bs3*, and *Bs4* resistance genes.

## Literature cited


1 Adaskaveg, J. E. and Hine, R. B. 1985. Copper tolerance and zinc sensitivity of Mexican strains of *Xanthomonas campestris* pv. *vesicatoria,* causal agent of bacterial spot of pepper. Plant Disease 69:993-996.

2 Anderson, G. L., and Lindow, S. E. 1986. Occurrence and control of copper tolerant strains of *Pseudomonas syringae* on almond and citrus in California. Phytopathology 76:1118.

3 Ballvora, A., Pierre, M., van der Ackerveken, G., Schornack, S., Rossier, O., Ganal, M., Lahave, T., and Bonas, U. 2001. Genetic mapping and functional analysis of the tomato *bs4* locus governing recognition of the *Xanthomonas campestris* pv. *vesicatoria* AvrBs4 protein. Mol. Plant Microbe Interact. 14(5):629-638.

4 Bashan, Y. and Okon, Y. 1986. Internal and external infections of fruits and seeds of pepper by *Xanthomonas campestris* pv. *vesicatoria*. Can. J. Bot. 64(12):2865-2871.

5 Bender, C. L. and Cooksey, D. A. 1986. Indigenous plasmids in *Pseudomonas syringae* pv. *tomato*: conjugative transfer and role in copper resistance. J. Bacteriol. 165:534-541.

6 Bender, C. L., Malvick, D. K., Conway, K. E., George, S., and Pratt, P. 1990. Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. 56:170-175.

7 Boatright, S. R. and McKissick, J. C. 2009. 2008 Georgia Farm Gate Vegetable Report. The University of Georgia, Center for Agribusiness and Economic Development. No. AR-09-02.

8 Boelema, B. H. 1985. Dose-response relationships between phytopathogenic bacteria and their hosts. Hort. Res. Inst. 91(4):189-202.

9 Bouzar, H., Jones, J. B., Stall, R. E., Hodge, N. C., Minsavage, G. V., Benedict, A. A., and Alvarez, A. M. 1994. Physiological, chemical, serological, and pathogenic analyses of a worldwide collection of *Xanthomonas campestris* pv. *vesicatoria* strains. Amer. Phytopathol. Soc. 84(7):663-671.

10 Cook, A. A. 1973. Characterization of hypersensitivity in *Capsicum annuum* induced by the tomato strain of *Xanthomonas vesicatoria*. Phytopathology 63:915-918.

11 Cooksey, D. A., Azad, H. R., Cha, J. S., and Lim, C. K. 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. 56:431-435.

12 Gadd, G. M. and Griffiths, A. J. 1978. Microorganisms and heavy metal toxicity. Microb. Ecol. 34:303-317.

13 Gassmann, W., Dahlbeck, D., Chesnokova, O., Minsavage, G. V., Jones, J. B., and Staskawicz, B. J. 2000. Molecular evolution of virulence in natural field strains of *Xanthomonas campestris* pv. *vesicatoria*. J. of Bacteriology 182(24):7053-7059.

14 Gitaitis, R. D., McInnes, T. B., and Jones, J. B. 1985. A survey of Georgia and Florida for the presence of copper tolerant phytopathogenic bacteria.

15 Gurlebeck, D., Jahn, S., Gurlebeck, N., Szczesny, R., Szurek, B., Hahn, S., Hause, G., and Bonas, U. 2009. Visualization of novel virulence activities of the Xanthomonas type III effectors AvrBs1, AvrBs3, and AvrBs4. Mol. Plant Pathology 10(2):175-188.

16 Jones, J. B., Jones, J. P., and Woltz, S. S. 1993. The effect of selected copper bactericides on population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets. Pro. of the Fl. St. Hort. Soc. 106:160-163.

17 Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E., and Schaad, N. W. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Sys. Appl. Microbiol. 27:755-762.

18 Jones, J. B., Pohronezny, K. L., Stall, R. E., and Jones, J. B. 1986. Survival of *Xanthomonas campestris* pv. *vesicatoria* in Florida on tomato crop residues, weeds, seeds and volunteer tomato plants. Phytopathology 76:430-434.

19 Jones, J. B. and Scott, J. W. 1986. Hypersensitive response in tomato to *Xanthomonas campestris* pv. *vesicatoria*. Plant Disease 70:337-339.

20 Kousik, C. S. and Ritchie, D. F. 1995. Isolation of pepper races 4 and 5 of *Xanthomonas campestris* pv. *vesicatoria* from diseased peppers in southeastern U.S. fields. Plant Disease 79:540.

21 Kousik, C. S. and Ritchie, D. F. 1996. Race shift in *Xanthomonas campestris* pv. *vesicatoria* within a season in field-grown pepper. Phytopathology 86:952-958.

22 Kousik, C. S. and Ritchie, D. F. 1998. Response of bell pepper cultivars to bacterial spot pathogen races that individually overcome major resistance genes. Plant Disease 82:181-186.

23 Kousik, C. S., Sanders, D. C., and Ritchie, D. F. 1996. Mixed genotypes combined with copper sprays to manage bacterial spot of bell peppers. Phytopathology 86:502-508.

24 Leite, R. P., Jones, J. B., Somodi, G. C., Minsavage, G. V., and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Disease 79(9):917-922.

25 Marco, G. M. and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Disease 67:779-781.

26 Martin, H. L. and Hamilton, V. A. 2004. Copper tolerance in Australian populations of *Xanthomonas campestris* pv. *vesicatoria* contributes to poor field control of bacterial spot of pepper. Plant Disease 88:921-924.

27 Minsavage, G. V., Dahlbeck, D., Whalen, M. C., Kearney, B., Bonas, U., Staskawicz, B. J., and Stall, R. E. 1990. Gene-for-gene relationships specifying disease resistance in *Xanthomonas campestris* pv. *vesicatoria* – pepper interactions. Mol. Plant Micro. Inter. 3(1):41-47.

28 Pernezny, K. and Collins, J. 1997. Epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on pepper: relationships to host-plant resistance and exposure to copper sprays. Plant Disease 81:791-794.

29 Pernezny, K., Nagata, R., Havranek, N., and Sanchez, J. 2008. Comparison of two culture media for determination of the copper resistance of *Xanthomonas* strains and their usefulness for the prediction of control with copper bactericides. Crop Protection 27:256-262.

30 Ritchie, D. F. and Dittapongpitch, V. 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. Plant Disease 75:733-736.

31 Sahin, F. and Miller, S. A. 1995. First report of pepper race 6 of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Disease 79:1188.

32 Sahin, F. and Miller, S. A. 1996. Characterization of Ohio strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Disease 80:773-778.

33 Sahin, F. and Miller, S. A. 1998. Resistance in *Capsicum pubescens* to *Xanthomonas campestris* pv. *vesicatoria* pepper race 6. Plant Disease 82:794-799.

34 Schaad, N. W., Jones, J. B., and Chun, W. (Eds.) 2001. Laboratory guide for identification of plant pathogenic bacteria. Third Edition. APS Press.

35 Scheck, H. J. and Pscheidt, J. W. 1998. Effect of copper bactericides on copper-resistant and –sensitive strains of *Pseudomonas syringae* pv. *syringae*. Plant Disease 82:397-406.

36 Shukla, A and Gupta, S. K. 2004. Management of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) through chemicals. J. Myco. Plant Pathol. 34(1):88-90.

37 Stall, R. E. and Cook, A. A. 1966. Multiplication of *Xanthomonas vesicatoria* and lesion development in resistant and susceptible pepper. Phytopathology 56:1152-1154.

38 Stall, R. E., Jones, J. B., and Minsavage, G. V. 2009. Durability of resistance in tomato and pepper to xanthomonads causing bacterial spot. Annu. Rev. Phytopathol. 47:265-284.

39 Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 76:240-243.

40 Sundin, G. W., Jones, A. L., and Fulbright, D. W. 1989. Copper resistance in *Pseudomonas syringae* pv. *syringae* from cherry orchards and its associated transfer *in vitro* and *in planta* with a plasmid. Phytopathology 79:861-865.

41 Vauterin, L., Hoste, B., Kersters, K., and Swings, J. 1995. Reclassification of *Xanthomonas*. Int. J. Sys. Bacteriol. 45:472-489.

42 Vauterin, L., Rademaker, J., and Swings, J. 2000. Synopsis on the taxonomy of the genus *Xanthomonas*. Phytopathology 90:677-682.

43 Voloudakis, A. E., Bender, C. L., and Cooksey, D. A. 1993. Similarity between copper resistance genes from *Xanthomonas campestris* and *Pseudomonas syringae*. App. Environ. Microbiol. 59(5):1627-1634.

**Table 2.1 - *Xanthomons axonopodis* pv. *vesicatoria* strains collected from outbreaks in commercial pepper fields and greenhouses in Georgia and the distribution of races and copper tolerance from 2007 and 2008.**

| | | | | | Copper tolerance[†] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Tolerant | Intermediate | Sensitive | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
| LTF 2* | 07/07/07 | Bell | Tift | 10 | | | 1 | S | + | + | + |
| 1 | 07/07/07 | ECW | Tift | 3 | | | 1 | S | + | + | + |
| 2A | 07/17/07 | Jalapeno | Berrien | 8 | | | 1 | S | + | | |
| 2B | 07/17/07 | Jalapeno | Berrien | 8 | | | 1 | S | + | + | + |
| 2C | 07/17/07 | Jalapeno | Berrien | 8 | | | 1 | S | + | + | + |
| 4B | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | + | + |
| 4C | 07/25/07 | Sweet | Colquitt | 9 | 1 | | | S | + | + | + |
| 4D | 07/25/07 | Sweet | Colquitt | 9 | 1 | | | S | + | | |
| 4E | 07/25/07 | Sweet | Colquitt | 9 | 1 | | | S | + | + | + |
| 4F | 07/25/07 | Sweet | Colquitt | 6 | | 1 | | S | + | | |
| 4G | 07/25/07 | Sweet | Colquitt | 10 | | | 1 | S | + | | |
| 4H | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | | |
| 4I | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | | |
| 4J | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | | |
| 5A | 07/25/07 | Hot | Colquitt | 10 | 1 | | | S | + | + | + |
| 5B | 07/25/07 | Hot | Colquitt | 10 | | 1 | | S | + | + | + |
| 5C | 07/25/07 | Hot | Colquitt | 4 | 1 | | | S | + | | |
| 5D | 07/25/07 | Hot | Colquitt | 9 | 1 | | | S | + | | |
| 5E | 07/25/07 | Hot | Colquitt | 6 | 1 | | | S | + | | |
| 5F | 07/25/07 | Hot | Colquitt | 7 | | | 1 | S | + | + | + |
| 5G* | 07/25/07 | Hot | Colquitt | 9 | | 1 | | S | + | + | + |
| 5H | 07/25/07 | Hot | Colquitt | 4 | | | 1 | S | + | | |
| 5I | 07/25/07 | Hot | Colquitt | 7 | | | 1 | S | + | + | + |
| 6A | 07/25/07 | Habanero | Colquitt | 10 | 1 | | | S | + | + | + |
| 6B | 07/25/07 | Habanero | Colquitt | 10 | 1 | | | S | + | | |
| 6C | 07/25/07 | Habanero | Colquitt | 9 | 1 | | | S | + | + | + |
| 6E | 07/25/07 | Habanero | Colquitt | 10 | 1 | | | S | + | | |
| 7C | 07/25/07 | Sweet | Colquitt | 9 | 1 | | | S | + | | |

**Table 2.1** *(continued from preceding page)*

| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Copper tolerance† | | | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tolerant | Intermediate | Sensitive | | | | |
| 7E | 07/25/07 | Sweet | Colquitt | 9 | 1 | | | S | + | | |
| 7F | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | | |
| 7H | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | + | + |
| 7I | 07/25/07 | Sweet | Colquitt | 10 | 1 | | | S | + | + | + |
| 8 | 07/17/07 | Bell | Berrien | 3 | | | 1 | S | + | + | + |
| 12A* | 09/04/07 | Bell | Colquitt | 6 | 1 | | | S | + | + | + |
| 12B | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 12C | 09/04/07 | Bell | Colquitt | - | 1 | | | - | + | | |
| 12D | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 12F | 09/04/07 | Bell | Colquitt | - | 1 | | | - | + | | |
| 12G | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 12H | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 12I | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 12J | 09/04/07 | Bell | Colquitt | 10 | 1 | | | S | + | | |
| 13A | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | + | + |
| 13B | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13C | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13D | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13E | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13F | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13G | 09/18/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 13H | 09/18/07 | Plato | Tift | - | 1 | | | - | + | + | + |
| 13I | 09/18/07 | Plato | Tift | 9 | 1 | | | S | + | | |
| 13J | 09/18/07 | Plato | Tift | - | 1 | | | - | + | | |
| 14A | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | + | + |
| 14B | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | + | + |
| 14C | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | + | + |
| 14D | 09/18/07 | Hot | Colquitt | - | 1 | | | - | + | | |
| 14E | 09/18/07 | Hot | Colquitt | - | 1 | | | - | + | | |
| 14F | 09/18/07 | Hot | Colquitt | - | 1 | | | - | + | | |

**Table 2.1** *(continued from preceding page)*

| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Copper tolerance† | | | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tolerant | Intermediate | Sensitive | | | | |
| 14G | 09/18/07 | Hot | Colquitt | - | 1 | | | - | + | | |
| 14H | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | | |
| 14I | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | | |
| 14J | 09/18/07 | Hot | Colquitt | 9 | 1 | | | S | + | | |
| 15A | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15B | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15C | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15D | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15E | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15F | 09/19/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 15G* | 09/19/07 | Plato | Tift | 10 | 1 | | | S | + | + | + |
| 15H | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15I | 09/19/07 | Plato | Tift | - | 1 | | | - | + | | |
| 15J | 09/19/07 | Plato | Tift | 10 | 1 | | | S | + | | |
| 16A | 09/27/07 | Revolution | Lowndes | 10 | 1 | | | S | + | + | + |
| 16B | 09/27/07 | Revolution | Lowndes | 10 | | 1 | | S | + | + | + |
| 16F | 09/27/07 | Revolution | Lowndes | - | | 1 | | - | + | | |
| 16H | 09/27/07 | Revolution | Lowndes | - | | | 1 | - | + | + | + |
| 17A | 10/09/07 | Tula | Tift | 9 | | | 1 | S | + | | |
| 17B* | 10/09/07 | Tula | Tift | - | | 1 | | - | + | + | + |
| 17C | 10/09/07 | Tula | Tift | 10 | | 1 | | S | + | | |
| 17D | 10/09/07 | Tula | Tift | 10 | 1 | | | S | + | + | + |
| 17E | 10/09/07 | Tula | Tift | - | 1 | | | - | + | | |
| 17F | 10/09/07 | Tula | Tift | 10 | 1 | | | S | + | + | + |
| 18A | 10/09/07 | Chapala X3R | Tift | 9 | 1 | | | S | + | | |
| 18B | 10/09/07 | Chapala X3R | Tift | 9 | 1 | | | S | + | | |
| 18C | 10/09/07 | Chapala X3R | Tift | - | 1 | | | - | + | + | + |
| 18D | 10/09/07 | Chapala X3R | Tift | 9 | 1 | | | S | + | | |
| 18E* | 10/09/07 | Chapala X3R | Tift | 10 | | | 1 | S | + | + | + |
| 18F | 10/09/07 | Chapala X3R | Tift | 10 | 1 | | | S | + | | |

**Table 2.1** *(continued from preceding page)*

| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Copper tolerance† | | | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tolerant | Intermediate | Sensitive | | | | |
| 19A | 10/09/07 | Naza | Tift | - | 1 | | | - | + | | |
| 19C | 10/09/07 | Naza | Tift | - | 1 | | | - | + | | |
| 19D | 10/09/07 | Naza | Tift | - | 1 | | | - | + | | |
| 20A | 10/09/07 | Var. 0287 | Tift | - | 1 | | | - | + | | |
| 20B | 10/09/07 | Var. 0288 | Tift | 10 | 1 | | | S | + | | |
| 20C | 10/09/07 | Var. 0289 | Tift | 10 | 1 | | | S | + | + | + |
| 20D | 10/09/07 | Var. 0290 | Tift | 10 | 1 | | | S | + | | |
| 20E | 10/09/07 | Var. 0291 | Tift | 10 | 1 | | | S | + | | |
| 20F | 10/09/07 | Var. 0292 | Tift | 10 | 1 | | | S | + | | |
| 21A | 10/09/07 | Cubanelle | Tift | 10 | 1 | | | S | + | + | + |
| 21D | 10/09/07 | Cubanelle | Tift | 10 | 1 | | | S | + | | |
| 21F | 10/09/07 | Cubanelle | Tift | - | 1 | | | - | + | | |
| 22A | 10/09/07 | Bell | Tift | - | 1 | | | - | + | + | + |
| 22B | 10/09/07 | Bell | Tift | - | | 1 | | - | + | | |
| 22D | 10/09/07 | Bell | Tift | - | 1 | | | - | + | | |
| 22E | 10/09/07 | Bell | Tift | - | 1 | | | - | + | | |
| 22F | 10/09/07 | Bell | Tift | 9 | 1 | | | S | + | + | + |
| 23A* | 10/09/07 | Aquiles | Tift | 4 | | | 1 | S | + | + | + |
| 23B | 10/09/07 | Aquiles | Tift | - | 1 | | | - | + | | |
| 23C | 10/09/07 | Aquiles | Tift | 9 | 1 | | | S | + | + | + |
| 23D | 10/09/07 | Aquiles | Tift | 10 | 1 | | | S | + | | |
| 23E | 10/09/07 | Aquiles | Tift | 10 | 1 | | | S | + | + | + |
| 24A | 10/09/07 | Jalapeno | Tift | 4 | 1 | | | S | + | | |
| 24B | 10/09/07 | Jalapeno | Tift | 9 | 1 | | | S | + | | |
| 24C | 10/09/07 | Jalapeno | Tift | 9 | 1 | | | S | + | | |
| 24D* | 10/09/07 | Jalapeno | Tift | - | 1 | | | - | + | + | + |
| 24E | 10/09/07 | Jalapeno | Tift | - | 1 | | | - | + | | |
| 24F | 10/09/07 | Jalapeno | Tift | - | 1 | | | - | + | + | + |
| 25A | 10/09/07 | Jalafuego | Tift | 10 | 1 | | | S | + | + | + |
| 25B | 10/09/07 | Jalafuego | Tift | 10 | 1 | | | S | + | | |

**Table 2.1** *(continued from preceding page)*

| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Copper tolerance† | | | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tolerant | Intermediate | Sensitive | | | | |
| 25C | 10/09/07 | Jalafuego | Tift | - | 1 | | | - | + | | |
| 25D | 10/09/07 | Jalafuego | Tift | 10 | | | 1 | S | + | | |
| 25F | 10/09/07 | Jalafuego | Tift | 9 | 1 | | | S | + | | |
| 27A | 10/09/07 | Plato | Colquitt | 9 | | 1 | | S | + | + | + |
| 27C | 10/09/07 | Plato | Colquitt | 9 | 1 | | | S | + | + | + |
| 27D | 10/09/07 | Plato | Colquitt | 10 | 1 | | | S | + | | |
| 28A | 10/09/07 | Plato | Colquitt | 10 | 1 | | | S | + | | |
| 28B | 10/09/07 | Plato | Colquitt | 10 | | | 1 | S | + | + | + |
| 28C | 10/09/07 | Plato | Colquitt | - | 1 | | | - | + | | |
| 28D | 10/09/07 | Plato | Colquitt | - | 1 | | | - | + | | |
| 28E | 10/09/07 | Plato | Colquitt | 9 | 1 | | | S | + | + | + |
| 29A | 05/15/08 | Aristotle | Sumter | 10 | 1 | | | S | + | + | + |
| 29B* | 05/15/08 | Aristotle | Sumter | 10 | | 1 | | S | + | + | + |
| 29C | 05/15/08 | Aristotle | Sumter | 10 | 1 | | | S | + | + | + |
| 30A | 05/21/08 | Aristotle | Sumter | 10 | 1 | | | S | + | + | + |
| 30B | 05/21/08 | Aristotle | Sumter | 10 | 1 | | | S | + | | |
| 30C | 05/21/08 | Aristotle | Sumter | 10 | 1 | | | S | + | + | + |
| 30E | 05/21/08 | Aristotle | Sumter | 6 | 1 | | | S | + | | |
| 32A | 05/21/08 | Aristotle | Sumter | 10 | | | 1 | S | + | + | + |
| 33A | 05/21/08 | Bell | unknown | 9 | | 1 | | S | + | + | + |
| 33B | 05/21/08 | Bell | unknown | 9 | | 1 | | S | + | + | + |
| 34B | 06/26/08 | Bell | unknown | 9 | 1 | | | S | + | + | + |
| 34C | 06/26/08 | Bell | unknown | 9 | 1 | | | S | + | + | + |
| 34D | 06/26/08 | Bell | unknown | 9 | 1 | | | S | + | | |
| 7443 | 2008 | Bell | unknown | 10 | 1 | | | S | + | | |
| 7444 | 2008 | Bell | unknown | 4 | | 1 | | S | + | + | + |
| 7445 | 2008 | Bell | unknown | 9 | 1 | | | S | + | | |
| 7446 | 2008 | Bell | unknown | 9 | 1 | | | S | + | + | + |
| Green A | 2008 | Bell | unknown | 9 | 1 | | | S | + | | |
| Green B | 2008 | Bell | unknown | 9 | 1 | | | S | + | + | + |

**Table 2.1** *(continued from preceding page)*

| Strain ID | Date Collected | Pepper Cultivar or type | County | Race | Copper tolerance† | | | Pathogenicity on ECW | ELISA | Gram Reaction | Oxidase Reaction |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Tolerant | Intermediate | Sensitive | | | | |
| Green C | 2008 | Bell | unknown | 9 | 1 | | | S | + | | |
| Green D | 2008 | Bell | unknown | 9 | 1 | | | S | + | + | + |
| Green P | 2008 | Bell | unknown | 9 | 1 | | | S | + | | |
| Green Q | 2008 | Bell | unknown | 9 | | 1 | | S | + | + | + |
| Green R | 2008 | Bell | unknown | 9 | 1 | | | S | + | + | + |
| Green S | 2008 | Bell | unknown | 9 | 1 | | | S | + | | |
| Green T | 2008 | Bell | unknown | 9 | | 1 | | S | + | | |
| Total | | | | 119 | 123 | 15 | 17 | | 155 | 62 | 62 |
| | | | total Cu tolerance | % | 79.4 | 9.7 | 11.0 | | | | |
| | | | | % | | 89.0‡ | 11.0 | | | | |

*Strains tested in Cu tolerance liquid assay and PFGE

†Results from standard plate assay on 200ppm $CuSO_4$ amended media

‡Total percentage of strains tolerant to Cu, including the tolerant and intermediate strains

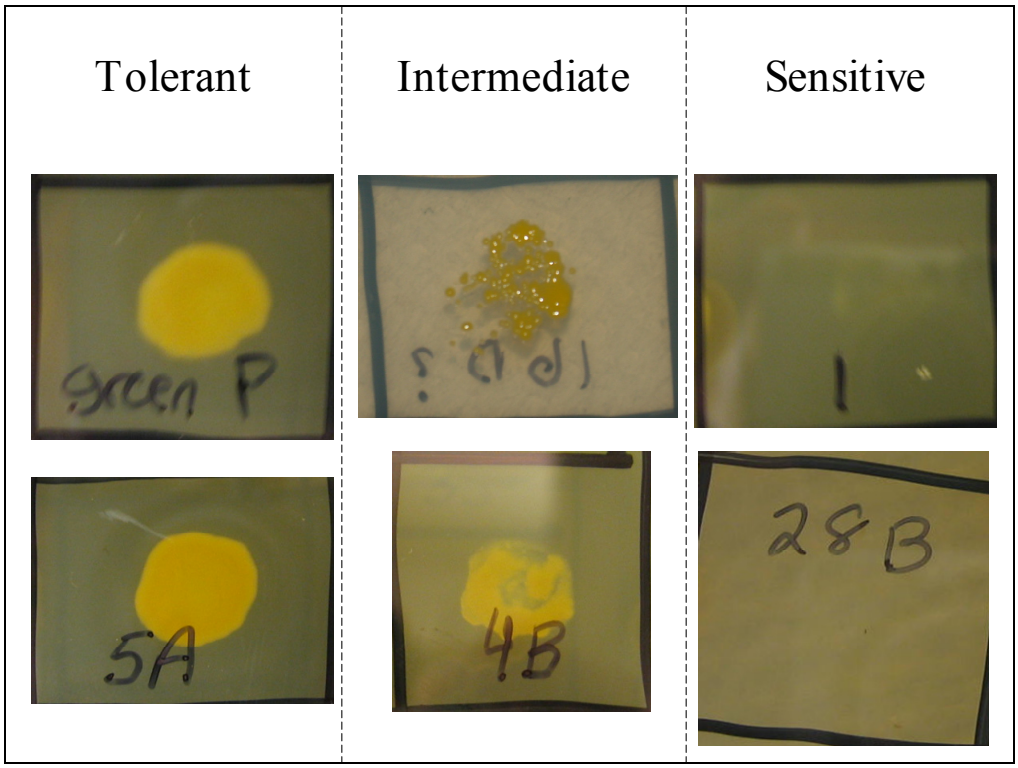


**Fig. 2.1 – Copper tolerance groupings of *Xanthomonas axonopodis* pv. *vesicatoria* strains using a copper amended agar plate assay.** Images demonstrate tolerance groups (tolerant, intermediate, and sensitive). The tolerant group was defined by full, confluent growth after 2 days. Intermediate strains were defined by having uneven, variable growth after 2 days. The sensitive group comprised strains that had no observable growth after 2 days.

# CHAPTER 3

# COMPARATIVE ANALYSIS OF COPPER TOLERANCE TESTING METHODS FOR *XANTHOMONAS AXONOPODIS* PV. *VESICATORIA*

Garton, Jr., J. E. and Langston, Jr., D. B. To be submitted to Phytopathology.

**Abstract** Copper-based fungicides are widely used to control bacterial leaf spot of peppers caused by *Xanthomonas axonopodis* pv. *vesicatoria*. Currently field strains are tested for their tolerance to copper by applying a suspension containing 1 x $10^6$ CFU/ml on a standard growth medium amended with $CuS0_4 \cdot 5H_2O$ and monitoring the growth for 48 hours. Research was conducted to test *X. a.* pv. *vesicatoria* strains on nutrient broth (NB) alone, NB plus 125, 250, and 500 ppm $CuSO_4 \cdot 5H_2O$ in a liquid assay. Strains from three predetermined tolerance levels (tolerant, intermediate, and sensitive) were chosen. Strains tested on NB alone showed similar growth rates in all three groupings. In the presence of copper, strains took longer to reach exponential growth phase and copper reduced growth in most strains. Individual strains displayed varying levels of copper tolerance in liquid media. In NB with 500 ppm $CuSO_4$, all strains were negatively affected by the presence of copper. Results obtained from the liquid and spot assays were dissimilar. The liquid assay provides more information for each strain and field studies need to be conducted in order to determine which test is more accurate.

**Key Words** *Xanthomonas axonopodis* pv. *vesicatoria*, *Xanthomonas campestris* pv. *vesicatoria*, *Capsicum annuum*, copper-tolerance, bactericide resistance

Bacterial diseases can be devastating on many crops each growing season in Georgia. In hot, humid environments that receive abundant amounts of rainfall, bacterial leaf spot (BLS) of pepper (*Capsicum annuum*) causes considerable losses in bell peppers by leaf spots, defoliation, fruit spots, and reduction in yield (10). BLS is caused by the gram-negative bacterium *Xanthomonas axonopodis* pv. *vesicatoria* (23) (also referred to as *Xanthomonas campestris* pv. *vesicatoria*).

Currently growers utilize pesticides and resistance genes to manage BLS. Other strategies, such as sanitation, disease-free seed and transplants, crop rotation, and applications of antibiotics, are utilized to help reduce inoculum levels and damage to bell pepper fruit and foliage. Copper-based bactericides have been the most widely used pesticide to prevent BLS and other foliar bacterial and fungal pathogens (24). However, in the past five years, copper bactericides have demonstrated reduced efficacy against BLS (16). In many areas where peppers and tomatoes are grown, the presence of copper-tolerant strains of *X. a.* pv. *vesicatoria* (9, 12, 13, 17) have been reported.

Copper is an essential element required for many cellular functions, in both eukaryotic and prokaryotic organisms. It is utilized in numerous enzymes involved in respiration, which includes oxygenases and electron transport proteins (7). Therefore, most cells need trace amounts of copper. However, when present at higher concentrations copper can also be toxic to bacterial cells. Copper ions will readily generate free radicals that can damage DNA and lipid membranes (24). Since copper plays a dual role, bacteria need to regulate the cellular concentration of copper ions. Many different copper regulation mechanisms have been studied (3, 5, 20, 22) but there seems to be no unifying link between these mechanisms.

Knowing the distribution and frequency of copper-tolerant strains that affect pepper would be useful for making disease management decisions. To determine whether a particular strain is tolerant or sensitive to copper, several testing methods using different types of agar media have been used to determine copper tolerance in bacteria. Several studies have used nutrient agar, or nutrient-rich media, amended with 200 μg/ml (μg/ml = ppm) of $CuSO_4 \cdot 5\ H_20$ to screen strains for tolerance (15, 16, 17, 21). This plating method is considered a standard assay for copper tolerance in *X. a.* pv. *vesicatoria*.

In studies conducted at the University of Georgia (Tifton campus) in 2004 thru 2006, 57 of 63 strains (90.5%) collected from BLS field epidemics were tolerant to copper when streaked onto nutrient agar amended with 5 g of glucose and 50 ppm $CuSO_4$ (Langston and Sanders, unpublished data). These results indicated that *X. a.* pv. *vesicatoria* strains with copper tolerance were isolated from peppers grown in Georgia. The mechanism of copper tolerance in *X. a.* pv. *vesicatoria* is still unknown and is thought to be controlled by genes on plasmids and/or the chromosome (24).

The objectives of this study were to survey the frequency of copper tolerant strains occurring in pepper production fields in Georgia from 2007 thru 2008 and to test a new liquid assay technique for determining copper tolerance/sensitivity in selected strains of *X. a.* pv. *vesicatoria*. Associating DNA fingerprints with a strain's ability to tolerate copper was also examined.

## Materials and Methods

**Isolation of strains**. From June of 2007 thru September of 2008, 155 pepper samples with BLS symptoms were collected from fields and greenhouses across southern Georgia. All samples were collected from commercial plantings of hot, specialty, and bell pepper cultivars.

Each sample consisted of a single, symptomatic leaf detached from a region of a plant displaying BLS symptoms. Samples were collected from fields with BLS outbreaks by sampling plants at the four corners of the field and one in the center of the field. If the field displaying BLS symptoms was approximately 1.6 Ha or larger, collections in the center of the area was increased to two or three samples. Leaf samples were placed in clear plastic bags, labeled, and transported to the laboratory. Bacterial strains were isolated from diseased leaves by removing a section of tissue from the outer region of a leaf spot with a sterile scalpel and macerating the tissue in 100 µL of sterile tap water. The scalpel, forceps, and inoculating needle used in the isolation were surface-sterilized by dipping in 90% alcohol and flamed with an alcohol burner between individual samples. The tissue sample was incubated in the water for approximately 1 min before a quad-loop (Globe Scientific, INC., U.S.A.) was used to remove approximately 10 ul of the suspension. The suspension was streaked onto a nutrient agar (Difco Laboratories, Detroit, MI) and yeast dextrose carbonate (YDC) media (19) and incubated for 24 to 48 hours at 28°C. After incubation, single yellow, circular, butyrous, shiny colonies were sub-cultured to obtain pure cultures. Plates were incubated at 28°C for 2 to 5 days. Colonies from pure cultures were suspended in 15% glycerol and stored at -80°C.

**Identification of strains.** The morphological characteristics of bacterial strains on NA and YDC were recorded. An ELISA based test kit (Product code 1099, Adgen Phytodiagnostics: Neogen Europe Ltd., Europe) was used to verify that the strains were identified as *X. a.* pv. *vesicatoria* according to the manufacturers instructions. All strains were assayed for pathogenicity on the susceptible pepper cultivar Early California Wonder. A sub-sample (n=62 strains) was arbitrarily selected and tested for Gram reaction and oxidase activity. Gram reactions were determined using the 3% potassium hydroxide string test (19). Strains that

became viscous were considered gram-negative. Oxidase reactivity was tested on a chemical based test kit (BBL BD Dryslide Oxidase) according to the manufacture's instructions. Strains producing a dark purple color on the test slide within 10 seconds were determined to be oxidase-positive.

**Determination of copper tolerance with plate assay.** Strains of *X. a.* pv. *vesicatoria* were removed from storage at -80°C, streaked onto NA and incubated at 28°C for 2 days. Cell suspensions containing 1 x $10^8$ CFU/ml ($A_{600nm}$ = 0.1) in sterile tap water were prepared for each sample and 25 µl (~ 1 x $10^5$ CFU/drop) were spotted onto NA and NA amended with 200 ppm $CuSO_4 \cdot 5H_20$ (5 g of peptone, 3 g of beef extract, 15 g of agar, and 0.20 g of $CuSO_4 \cdot 5H_20$ in 1 L of distilled water) and 500 ppm $CuSO_4 \cdot 5H_20$. Plates were incubated for 2 days at 28°C. Strains plated onto NA were used as controls. Strains were visually inspected for growth and grouped into tolerant, intermediate, and sensitive categories according to their colony morphology on copper (Fig. 2.1). Plates were incubated at 28°C for a week after the initial screening to monitor late growth.

**Screening strains for copper tolerance with liquid assay.** Strains were removed from storage suspensions containing 1 x $10^8$ CFU/ml and were prepared as previously described. Nine strains (18E, LTF2, 23A, 17B, 29B, 5G, 12A, 15G, and 24D) were selected for further testing from each copper tolerance level group, tolerant, intermediate, and sensitive. Each strain was diluted 10-fold to a suspension containing 1 x $10^7$ CFU/ml. Two microliters of each suspension were mixed with 198 µl of liquid media to give a final bacterial concentration of 1 x $10^5$ CFU/ml. The samples were dispensed into individual wells of a 100-well reader plate (Thermo Labsystems Honeycomb 2, USA). Treatments consisted of liquid nutrient broth (NB) as a control, and NB amended with 125, 250, and 500 ppm $CuSO_4 \cdot 5H_20$, respectively. Each

treatment was replicated 10 times. The reader plate was inserted into a BioScreen C machine (Growth Curves, USA) and continuously shaken at the medium setting for 24 hours at 28°C. Optical density (OD) measurements were recorded every 30 min. A pre-heat cycle (28°C) was run for 5 min prior to the first reading in each well. After preheating, each individual well was set to zero OD. OD values were recorded for each well and displayed as a curve with OD (600 nm) on the y-axis and time on the x-axis on BioScreen software (BioScreen EZ Experiment Software). An area under the population optical density growth curve (AUODC) was generated from the curve for each strain for each replicate. Mean AUODC values were generated for each strain individually and for each tolerance grouping. Each tolerance group mean was derived from three strains, each with 10 replications. Groups and individual strains were analyzed statistically by ANOVA (Tukey's test) using Minitab® software (version 15, Minitab, Inc.). Means given the same letter do not significantly differ at P=0.05 using ANOVA with n=30 (tolerance groups) and n=10 (individual strains).

**Pulsed field gel electrophoresis (PFGE).** Restriction enzyme digestion and pulsed field gel electrophoresis was used to genetically compare the nine strains evaluated for copper tolerance. Cultures were removed from storage and plated on NA for two days at 28°C. Cultures were inoculated into 4 ml of nutrient broth and grown overnight at 28°C with continuous shaking. Broth cultures were adjusted to an OD of 0.2 (600nm) and an aliquot of 1.5 ml was centrifuged for 2 min at 10,000 G. The supernatant was discarded and cells were resuspended in 1 ml sterile distilled water and centrifuged again. The supernatant was discarded and the remaining pellet was resuspended in 500 µl of sterile 1x Tris acetate EDTA (TAE) buffer (242 g of Tris base, 57.1 ml of acetic acid, glacial, and 37.2 g of EDTA disodium salt in 1 L of deionized water for a 50x solution). Five hundred microliters of room temperature low melting

point agar (LMP) solution (198 µl of 1M Tris at pH 8.0, 200 µl of 1M $MgCl_2$, 8 µl of 250 mM EDTA at pH 8.0, and 0.4 g of Sea Plaque GTG Agarose (FMC BioProducts) in 19.6 ml of distilled water) were added to the pellet and mixed thoroughly. One milliliter of solution was pipetted into block molds (Bio-Rad, Hercules, CA) incubated at 4°C for 20 minutes to solidify. After drying, blocks were placed into sterile poly propylene tubes with 2 ml of lysing solution (1000 µl of 25% sarcosyl, 625 µl of 20% sodium lauroyl sulfate, 50 µl of 250 mM EDTA at pH 9.5, and 15 mg of proteinase K in 23.325 ml of sterile, distilled water). Tubes were incubated in a water bath at 50-55°C overnight. The lysing solution was decanted within 24 hrs and the blocks were transferred to new tubes containing 2 ml of sterile 250 mM EDTA at pH 8.0 for storage at 4°C.

The blocks were removed from the storage tubes and plugs were cut to fit into the gel. Plugs were transferred into 1.5 ml micro-centrifuge tubes containing 1 ml of sterile 1x TAE and incubated for 2.5 hours at room temperature. TAE was changed six times to dilute the EDTA. Plugs were incubated in 200 µl of 1x restriction buffer for 15-30 min at room temperature then transferred into 200 µl of restriction digestion mix (200 µl per plug of 1x restriction buffer and 3 µl of *SpeI*) and incubated at 37°C overnight. After digestion, plugs were placed in 500 µl of fresh lysing solution (without the proteinase K) in sterile micro-centrifuge tubes and incubated at 50-55°C in a water bath for 2 hrs. The plugs were then removed, placed into new tubes with 500 µl of fresh lysing solution and incubated at room temperature for 2 hrs. During this time, a gel (100 ml of 0.5x TBE (54 g of Tris Base, 27.5 g of Boric acid, and 40 ml of 250 mM EDTA at pH 8.0 in 1 L of water) and 1 g of Pulsed field certified agarose) was prepared and the plugs were inserted into the wells. Plugs were covered with LMP solution for electrophoresis. The Bio-Rad CHEF-DRIII (Bio-Rad Laboratories, Richmond, CA) machine was used to separate the

restriction fragments with the following program (initial time: 5 seconds, final time: 45 seconds, run time: 22 hours, included angle: 120°, volts: 6 V/cm). After electrophoresis the gel was stained with ethidium bromide for 20 min followed by a rinse with deionized water for 40 min. The gel was observed under ultraviolet (UV) light on a transilluminator and photographed.

## Results

**Strain identification.** All strains were found to be *X. a.* pv. *vesicatoria*. All 52 sub-sample strains tested were gram-negative and oxidase negative. Colonies on NA were yellow, circular, butyrous, and shiny while the colonies on YDC were round, convex, bright yellow, and mucoid.

**Copper tolerance using the (standard) plate assay.** Results indicated that out of 155 *X. a.* pv. *vesicatoria* strains, 89% were tolerant and 11% were sensitive to 200 ppm $CuSO_4 \cdot 5H_20$ in amended media (Table 2.1). Within the strains that were tolerant, 79.4% were estimated to be tolerant and 9.7% were determined to have intermediate tolerance (Table 2.1). No strains grew on 500 ppm $CuSO_4 \cdot 5H_20$ after 2 days. The highly tolerant strains exhibited full, confluent growth after 2 days on the amended media while the intermediate strains displayed uneven, variable colony growth within the spot after 2 days (Fig. 2.1). Sensitive strains exhibited no growth after one week.

**Copper tolerance using the BioScreen C.** In the control treatment (0 ppm $CuSO_4 \cdot 5H_20$), the sensitive strains showed a significantly higher AUODC than the intermediate strains, but neither differed from the tolerant strains (Fig. 3.1). At a concentration of 125 ppm $CuSO_4 \cdot 5H_20$, the tolerant group demonstrated significantly greater growth ($P$=0.05) compared to both the intermediate and sensitive tolerance groups (Fig. 3.2). At a concentration of 250 ppm $CuSO_4 \cdot 5H_20$, the mean AUODC of the sensitive group was significantly lower ($P$=0.05) from both the

intermediate and tolerant groups (Fig. 3.3). At the highest copper concentration (500 ppm $CuSO_4 \cdot 5H_20$), growth with all groups demonstrated a negative mean AUODC and were not significantly different ($P$=0.05) (Fig. 3.4). Strains were also analyzed individually to determine the accuracy of grouping strains into copper tolerance groups (Fig. 3.5). In the control treatment (0 ppm $CuSO_4 \cdot 5H_20$), there were no differences between the strains that define each copper-tolerant group (Fig. 3.6). Strain LTF2 was found to have the highest OD value after 24 hrs (Fig. 3.5). Sensitive strain LTF2 demonstrated a significantly higher AUODC than intermediate strains 5G which had the lowest AUODC (Fig. 3.6). At 125 ppm $CuSO_4 \cdot 5H_20$, strains 15G and LTF2 had the highest mean OD value after 24 hrs (Fig. 3.7). These two strains demonstrated mean AUODC values that were significantly greater than all other strains regardless of tolerance group (Fig. 3.8). The other strains tested showed a delay in growth but were still increasing at 24 hrs (Fig. 3.7). Intermediate strain 29B demonstrated a similar AUODC to the sensitive strains 18E and 23A (Fig. 3.8). The other sensitive strain, LTF2, was similar to another tolerant strain. Strain LTF2, which had a good growth curve at 125 ppm $CuSO_4 \cdot 5H_20$, showed poor growth at 250 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.9). At 250 ppm $CuSO_4 \cdot 5H_20$, seven of the nine strains had significantly different mean AUODC values from each other (Fig. 3.10). Strain 24D, a tolerant strain, demonstrated a negative mean AUODC (Fig. 3.10) and poor growth (Fig. 3.9). Four of the nine strains had a higher mean OD value after 24 hrs in 250 ppm $CuSO_4 \cdot 5H_20$ than 125 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.7 and Fig. 3.9). Five strains had reduced mean OD values after 24 hrs at 250 ppm $CuSO_4 \cdot 5H_20$ compared to the control treatment (0 ppm $CuSO_4 \cdot 5H_20$). At 500 ppm $CuSO_4 \cdot 5H_20$, all strains demonstrated a negative mean AUODC and OD growth curve and there were no significant differences among strains (Fig. 3.11 and Fig. 3.12).

**Pulsed field gel electrophoresis (PFGE).** After comparing the genetic fingerprints of the nine strains, three groups with distinct profiles were observed. Strains 12A, 29B, and 5G were classified as group 1. All demonstrated a similar banding pattern with three distinctive bands within the distinguishing region of the lane (Fig. 3.13). Strains 23A, 24D, 17B, 18E, and 15G were classified as group 2 and demonstrated only two bands in the distinguishing region of the lane (Fig. 3.13). Strain LTF 2 was classified as group 3 and demonstrated a unique pattern of four faint bands within the distinguishing region of the lane (Fig. 3.13). Group 1, consisted of copper tolerant and intermediate strains according to the plate assay. Group 2 consisted of all three copper tolerance groups. The single strain in group 3 was classified as sensitive to copper according to the plate assay.

## Discussion

In this study, the majority of *X. a.* pv. *vesicatoria* strains collected from peppers from a survey of southern Georgia were tolerant to copper (Table 2.1). Copper tolerance has been reported in other locations (16, 17, 18) and studies have been conducted that found tolerant strains in the field, but at a much lower frequency than reported in this study (13). Currently in Georgia, the frequency of copper tolerant strains observed is 89% (n=155) (Table 2.1). A survey conducted in Georgia from 2004 thru 2006 (n=63) found a similar result in that 90.5% of the strains were copper-tolerant on amended media (Langston and Sanders, unpublished). Strains collected in this study also exhibited a range in copper tolerance on copper amended media, as 79.4% were classified as tolerant and 9.7% were classified as having intermediate tolerance. Due to this range of tolerance, strains that survived in copper-rich conditions were considered tolerant rather than resistant. Menkissoglu and Lindow (1991) defined copper-resistant bacteria as strains that can tolerate 10 to 80 x higher copper concentrations than do the sensitive strains of

the same species and copper-tolerant bacteria as strains that are poorly controlled by the standard applications of copper bactericides. Other definitions for resistance include the ability of a strain to exclude or overcome the effect of a damaging factor (1). Phytopathogenic copper-tolerant bacteria are also considered to exhibit a quantitative, rather than qualitative, resistance to copper compounds (14). Our data support those findings and reflect that copper tolerance in *X. a.* pv. *vesicatoria* is more quantitative than qualitative. Strains used in this study demonstrated varying levels of tolerance to different concentrations of copper. Different strains can tolerate and survive copper concentrations that other strains of the same species can not. However, all were affected at high copper concentrations. The data shows that *X. a.* pv. *vesicatoria* strains are tolerant to certain copper concentrations but killed at higher, more lethal copper concentrations. No strains were found to be completely resistant to copper bactericides at the 500 ppm level.

At the highest concentration of copper, 500 ppm $CuSO_4 \cdot 5H_20$, the OD (600 nm) values recorded by the BioScreen C machine gradually decreased over time. Consequently, after 24 hrs the sample measured an absorbance value lower than the initial absorbance value. *X. a.* pv. *vesicatoria* cells may be decreasing in number or size with longer exposure to the high copper concentration. It can be assumed that in an environment with a high copper concentration that the cells were lysing and settling to the bottom of the well. Since all strains showed a reduction in OD value at 500 ppm $CuSO_4 \cdot 5H_20$, it was concluded that this copper concentration was lethal to all strains.

When comparing the two copper tolerance testing methods, variable results were observed. At a similar copper concentration, the tolerant and intermediate strains had similar growth results when compared on the plate assay and the liquid BioScreen assay (Fig. 2.1 and Fig. 3.3). However, in a liquid medium, sensitive strains grew in copper concentrations of 250

ppm and below in contrast to no growth observed on copper amended agar media. No strains grew at 500 ppm $CuSO_4 \cdot 5H_2O$ in either assay. To verify the results of the liquid assay, strains were analyzed individually. Variability in copper tolerance was observed among strains in the same copper tolerance group. At 250 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.8), a similar copper concentration to the plate assay, seven of the nine strains showed significantly different mean AUODC values. Five strains showed a reduction in mean OD value after 24 hrs in 250 ppm $CuSO_4 \cdot 5H_20$ compared to 0 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.5 and Fig. 3.9). Three of these five strains were classified as sensitive on the standard plate assay. Some of the strains classified as intermediate or tolerant to copper with the plate assay were similar to sensitive strains in the liquid assay. When individual optical density growth curves were analyzed (Fig. 3.9), two strains (LTF2 and 24D) did not increase in OD after 24 hrs. All of the other strains were increasing in OD. Some of the strains had a delay in OD increase in the presence of copper. Due to this delay in the presence of copper, more incubation time may be required for growth for some of the strains. Because of the high degree of variability among strains at different copper concentrations, the assays in this study may not be the most effective in determining the level of copper tolerance in a field collected strain without experimentation in field trials. This assay could be important in determining if strains can grow in the presence of copper and the time required for a strain to reach a rapid growth phase. Results from these studies may be more useful if compared to a bioassay on *X. a.* pv. *vesicatoria* strains or field trials with similar copper concentrations. Even strains that were shown to have no growth on the plate assay demonstrated minimal growth in liquid copper conditions. In most cases, the strains classified as sensitive showed a reduction in growth after 24 hrs compared to the control treatment. Other studies have shown that variability in copper sensitivity can occur in strains from one population to another

(12). These data indicate that copper tolerance levels in strains differ when tested individually and should not be grouped. Whether it has to do with a copper tolerance plasmid acquisition, copper tolerance mechanisms, or the induction of those copper tolerance mechanisms, individual strains appear to have different reactions to different copper concentrations in liquid media.

Some strains even grew better in an environment with a low copper concentration in liquid media. The data shows that in some strains there may be a fitness cost (or benefit) for copper tolerance. Two strains (LTF2 and 15G) had higher mean AUODC values in the presence of 125 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.7) than when exposed to 0 ppm $CuSO_4 \cdot 5H_20$ (Fig. 3.5). Seven of the nine strains demonstrated a reduction in AUODC value from 0 ppm to 125 ppm $CuSO_4 \cdot 5H_20$. Four strains (15G, 12A, 29B, and 17B) demonstrated higher mean OD values after 24 hrs in 250 ppm $CuSO_4 \cdot 5H_20$ compared to 0 ppm $CuSO_4 \cdot 5H_20$. Thus strains with a high tolerance to copper may be less fit in the absence of copper (5). Strains may have evolved to be so efficient at surviving in high-copper environments that the tolerance mechanism may have more functions than just resisting toxic copper ions (5). It is possible that these strains may have more efficient copper transports systems, copper storage systems, and more efficient translocation pumps (4, 5). In low-copper conditions, tolerant strains may require more copper ions than sensitive strains. This low-copper condition may have a negative effect on the fitness of tolerant strains. Lee *et al.* (1990) state that without the genes responsible for controlling copper ion transport and utilization of copper ions interacting directly or indirectly with those regulating copper tolerance, bacteria could become copper depleted at low copper concentrations.

The same strains tested in the liquid assay were also analyzed by restriction enzyme digestion and PFGE. This was performed to determine if the genetic banding patterns could be

used to distinguish the copper tolerance groups among strains (Fig. 3.13). One similar region of the restriction digestion profile of all nine strains demonstrated a unique banding pattern that could distinguish strains. No direct comparisons could be linked to copper tolerance group. The only connection that could be determined between the PFGE banding patterns and the strains was in group 2. All of the strains in this group were from the same grower but from different collection sites and pepper cultivars or types.

The data collected in this study indicate that copper tolerance is present in a high percentage of the *X. a.* pv. *vesicatoria* strains infecting peppers in Georgia. Growers need to be careful and precise when applying copper-based bactericides and use them in a manner that does not promote copper tolerant strains but maximizes the effectiveness of the chemical. These tactics include applying higher labeled rates, tank mixing, utilizing bactericides with different modes of action, and using bactericides as a preventative control. Current recommendations for control of BLS include tank mixing copper bactericides with ethylenebisdithiocarbamate compounds, such as mancozeb, (8) to prevent copper tolerance. These compounds can be useful for reducing disease in pepper fields, but do not appear to provide adequate control in many fields. Future testing with mancozeb compounds will give more information on mancozeb effectiveness and tolerance and the importance of using these chemicals with copper bactericides against copper tolerant *X. a.* pv. *vesicatoria* strains.

## Literature cited


1 Agrios, G. N. 2005. *Plant Pathology*. 5th Edition. Elsevier Academic Press, Burlington, USA.

2 Basim, H., Stall, R. E., Minsavage, G. V., and Jones, J. B. 1999. Chromosomal gene transfer by conjugation in the plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria*. Phytopathology 89:1044-1049.

3 Brown, N. L., Rouch, D. A., and Lee, B. T. 1992. Copper resistance determinants in bacteria. Plasmid 27:41-51.

4 Cervantes, C., and Gutierrez-Corona, F. 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev. 14:121-138.

5 Cooksey, D. A. 1993. Copper uptake and resistance in bacteria. Mol. Microbiol. 7(1):1-5.

6 Cooksey, D. A., Azad, H. R., Cha, J. S., and Lim, C. K. 1990. Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. 56:431-435.

7 Garcia-Horsman, J. A., Barquera, B., Rumbley, R., Ma, J., and Gennis, R. B. 1994. The superfamily of heme-copper respiratory oxidases. J. Bacteriol. 176:5587-5600.

8 Holmes, G. J. and Kemble, J. M. (senior editors). 2009. Southeastern U.S. Vegetable Crop Handbook. Vance Publishing Corp.

9 Jones, J. B., Woltz. S. S., Jones, J. P., and Portier, K. L. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* in tomato leaves treated with copper bactericides. Phytopathology 81:714-719.

10 Kousik, C. S., Sanders, D. C., and Ritchie, D. F. 1996. Mixed genotypes combined with copper sprays to manage bacterial spot of bell peppers. Phytopathology 86:502-508.

11 Lee, B. T., Brown, N. L., Rogers, S., Bergemann, A., Camakaris, J., and Rouch, D. A. 1990. Bacterial response to copper in the environment: Copper resistance in *Escherichia coli* as a model system. In "Metal Speciation in the Environment", NATO ASI Series, Vol. G23, pp. 625-632.

12 Marco, G. M. and Stall, R. E. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv. *vesicatoria* that differ in sensitivity to copper. Plant Disease 67:779-781.

13 Martin, H. L. and Hamilton, V. A. 2004. Copper tolerance in Australian populations of *Xanthomonas campestris* pv. *vesicatoria* contributes to poor field control of bacterial spot of pepper. Plant Disease 88:921-924.

14 Menkissoglu, O. and Lindow, S. E. 1991. Chemical forms of copper on leaves in relation to the bactericidal activity of cupric hydroxide deposits on plants. Phytopathology 81:1263-1270.

15 Mirik, M., Aysan, Y., and Cinar, O. 2007. Copper-resistant strains of *Xanthomonas axonopodis* pv. *vesicatoria* in the eastern Mediterranean region of Turkey. J. Plant Pathol. 89(1):153- 154.

16 Pernezny, K., Nagata, R., Havranek, N., and Sanchez, J. 2008. Comparison of two culture media for determination of the copper resistance of *Xanthomonas* strains and their usefulness for the prediction of control with copper bactericides. Crop Protection 27:256-262.

17 Ritchie, D. F. and Dittapongpitch, V. 1991. Copper- and streptomycin-resistant strains and host differentiated races of *Xanthomonas campestris* pv. *vesicatoria* in North Carolina. Plant Disease 75:733-736.

18 Sahin, F. and Miller, S. A. 1996. Characterization of Ohio strains of *Xanthomonas campestris* pv. *vesicatoria*, causal agent of bacterial spot of pepper. Plant Disease 80:773-778.

19 Schaad, N. W., Jones, J. B., and Chun, W. (Eds.) Laboratory guide for identification of plant pathogenic bacteria. Third Edition. APS Press.

20 Silver, S. and Walderhaug, M. 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56:195-228.

21 Stall, R. E., Loschke, D. C., and Jones, J. B. 1986. Linkage of copper resistance and avirulence loci on a self-transmissible plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology 76:240-243.

22 Trevors, J. T. 1987. Copper resistance in bacteria. Microbiol. Sci. 4:29-31.

23 Vauterin, L., Rademaker, J., and Swings, J. 2000. Synopsis on the taxonomy of the genus *Xanthomonas*. Phytopathology 90:677-682.

24 Voloudakis, A. E., Reignier, T. M., and Cooksey, D. A. 2005. Regulation of resistance to copper in *Xanthomonas axonopodis* pv. *vesicatoria*. Appl. Environ. Microbiol. 71(2):782-789.

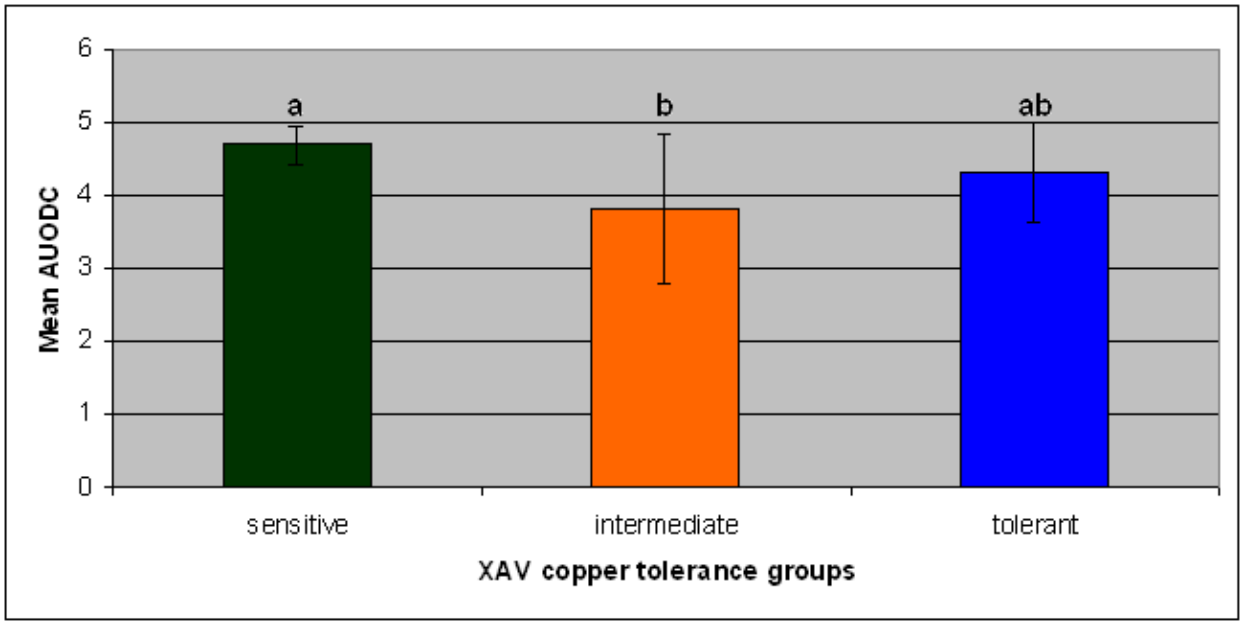


**Fig. 3.1 – Analysis of *Xanthomonas axonopodis* pv. *vesicatoria* copper tolerance groups with the BioScreen C liquid growth assay at 0 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for each tolerance group, tolerant, intermediate, and sensitive, and for the control treatment of liquid nutrient broth with 0 ppm $CuSO_4 \cdot 5H_20$. Means followed by the same letter are not significantly different $P$=0.05 using ANOVA (Tukey's test) with $n$=30. Means for each tolerance group were derived from three strains and 10 replications.

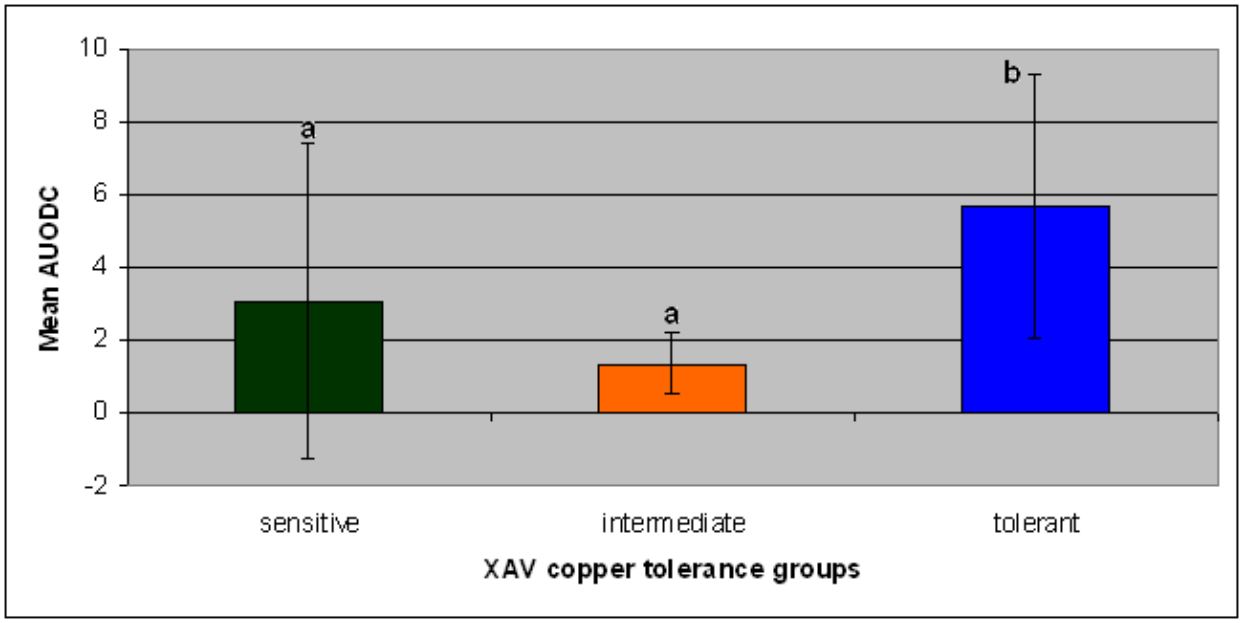


**Fig. 3.2 – Analysis of *Xanthomonas axonopodis* pv. *vesicatoria* copper tolerance groups with the BioScreen C liquid growth assay at 125 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for each tolerance group, tolerant, intermediate, and sensitive, and for treatment 1 of liquid nutrient broth with 125 ppm $CuSO_4 \cdot 5H_20$. Means followed by the same letter are not significantly different $P$=0.05 using ANOVA (Tukey's test) with $n$=30. Means for each tolerance group were derived from three strains and 10 replications.

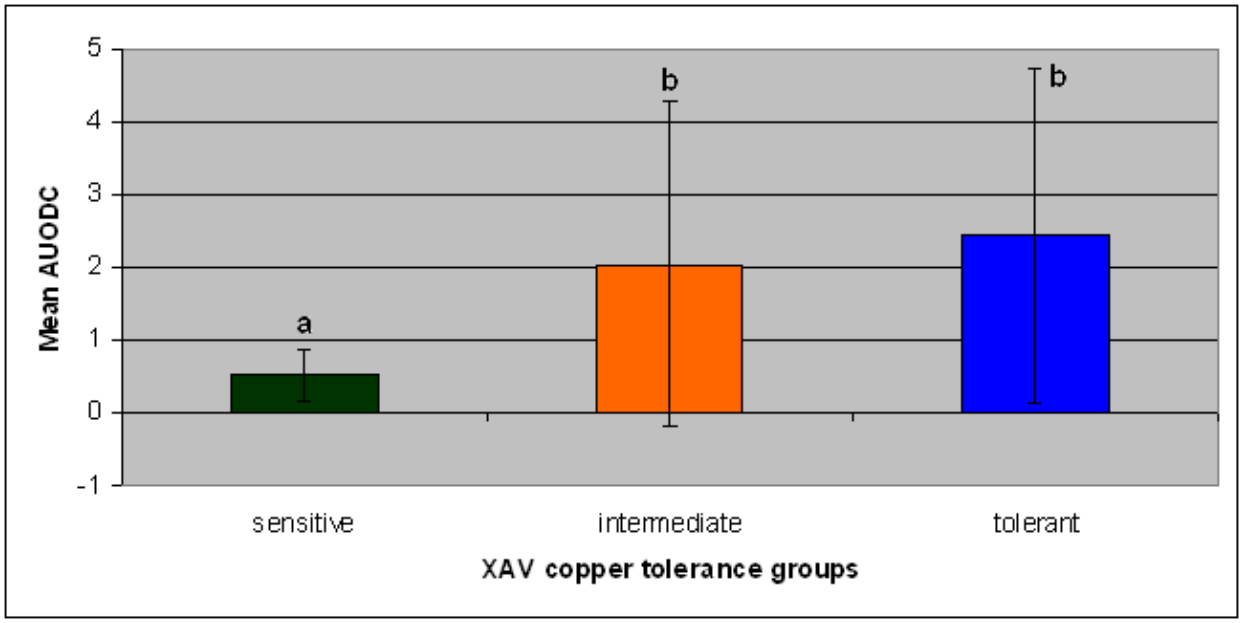


**Fig. 3.3 – Analysis of *Xanthomonas axonopodis* pv. *vesicatoria* copper tolerance groups with the BioScreen C liquid growth assay at 250 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for each tolerance group, tolerant, intermediate, and sensitive, and for treatment 2 of liquid nutrient broth with 250 ppm $CuSO_4 \cdot 5H_20$. Means followed by the same letter are not significantly different $P$=0.05 using ANOVA (Tukey's test) with $n$=30. Means for each tolerance group were derived from three strains and 10 replications.

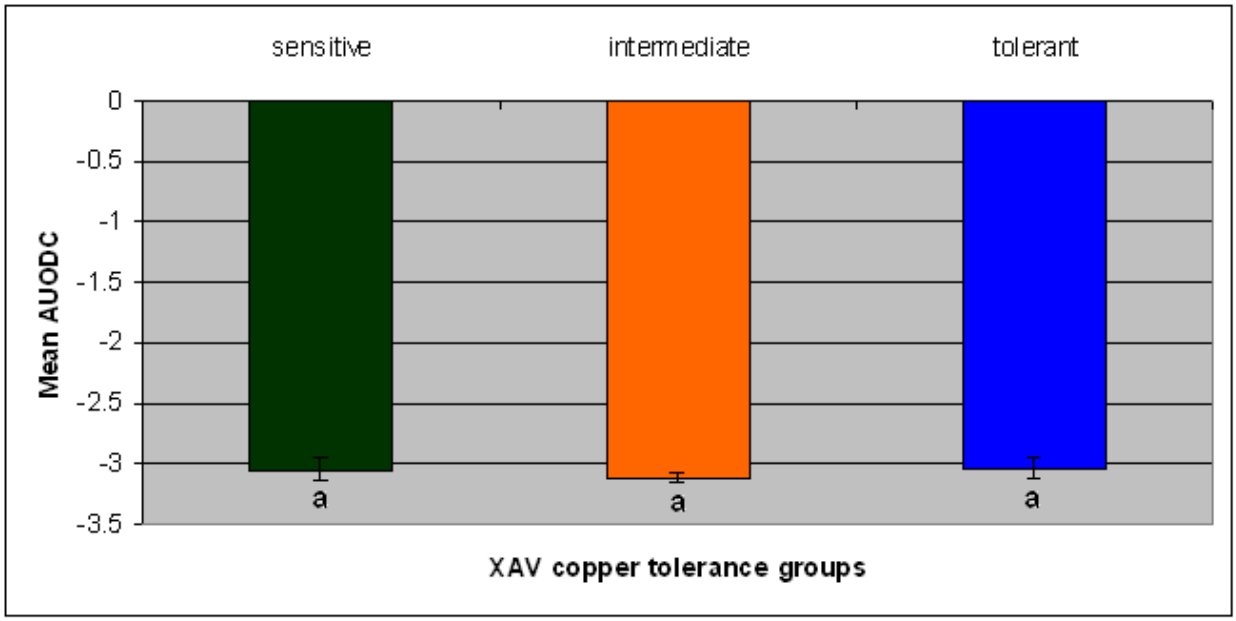


**Fig. 3.4 – Analysis of *Xanthomonas axonopodis* pv. *vesicatoria* copper tolerance groups with the BioScreen C liquid growth assay at 500 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for each tolerance group, tolerant, intermediate, and sensitive, and for treatment 3 of liquid nutrient broth with 500 ppm $CuSO_4 \cdot 5H_20$. Means followed by the same letter are not significantly different $P$=0.05 using ANOVA (Tukey's test) with $n$=30. Means for each tolerance group were derived from three strains and 10 replications.

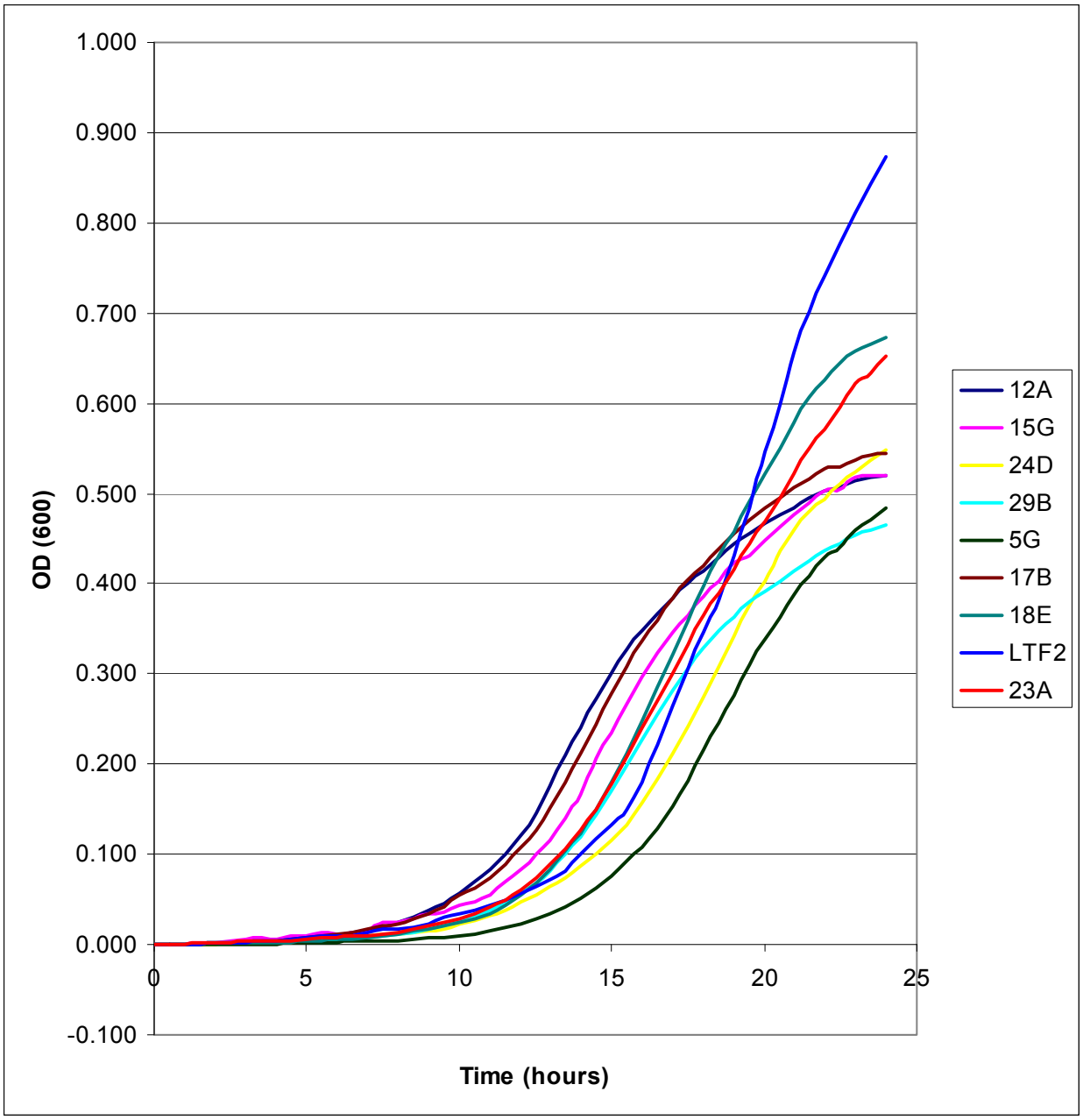


**Fig. 3.5 – *Xanthomonas axonopodis* pv. *vesicatoria* optical density growth curves in 0 ppm $CuSO_4 \cdot 5H_20$.** Graph represents the mean optical density growth curves (n=10) for each strain as recorded by the BioScreen C liquid assay for the control treatment (0 ppm $CuSO_4 \cdot 5H_20$) after 24 hours at 28°C.

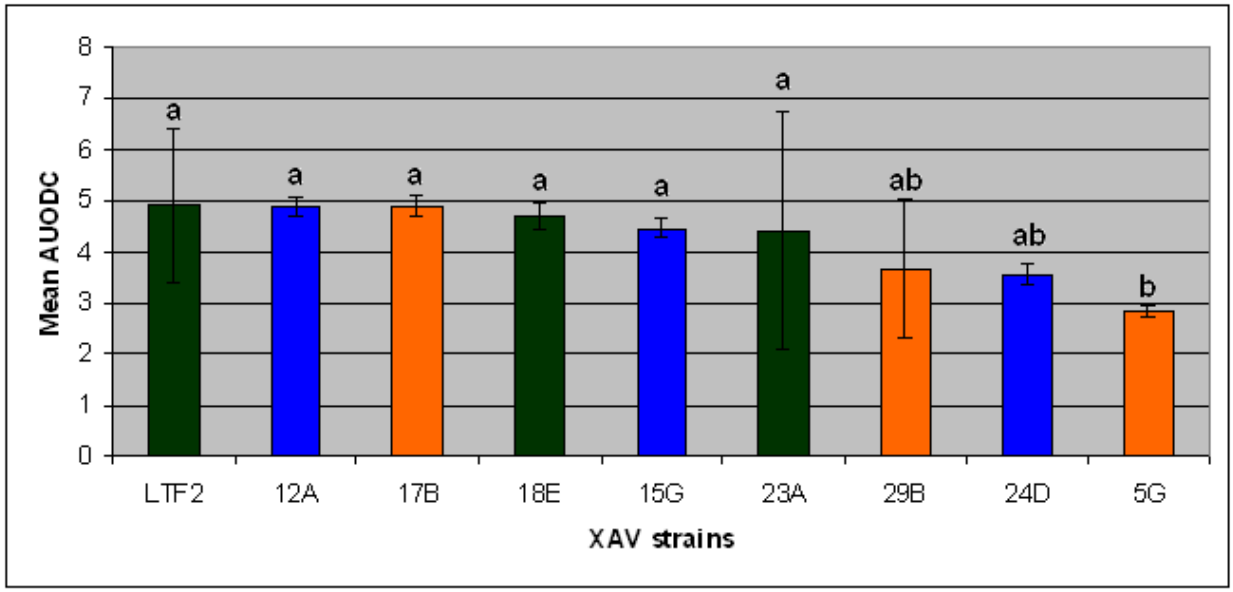


**Fig. 3.6 – Analysis of individual *Xanthomonas axonopodis* pv. *vesicatoria* strains for copper tolerance on the BioScreen C liquid growth assay in 0 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for the control treatment (0 ppm $CuSO_4 \cdot 5H_20$) for each individual strain tested by the liquid copper tolerance assay. The original tolerance groups, determined by the plate assay, are labeled by color. The green represents the sensitive group, the orange represents the intermediate group, and the blue represents the tolerant group. Means followed by the same letter are not significantly different $P$=.05 using ANOVA (Tukey's test) with $n$=10. Means for each strain were derived from 10 replications.

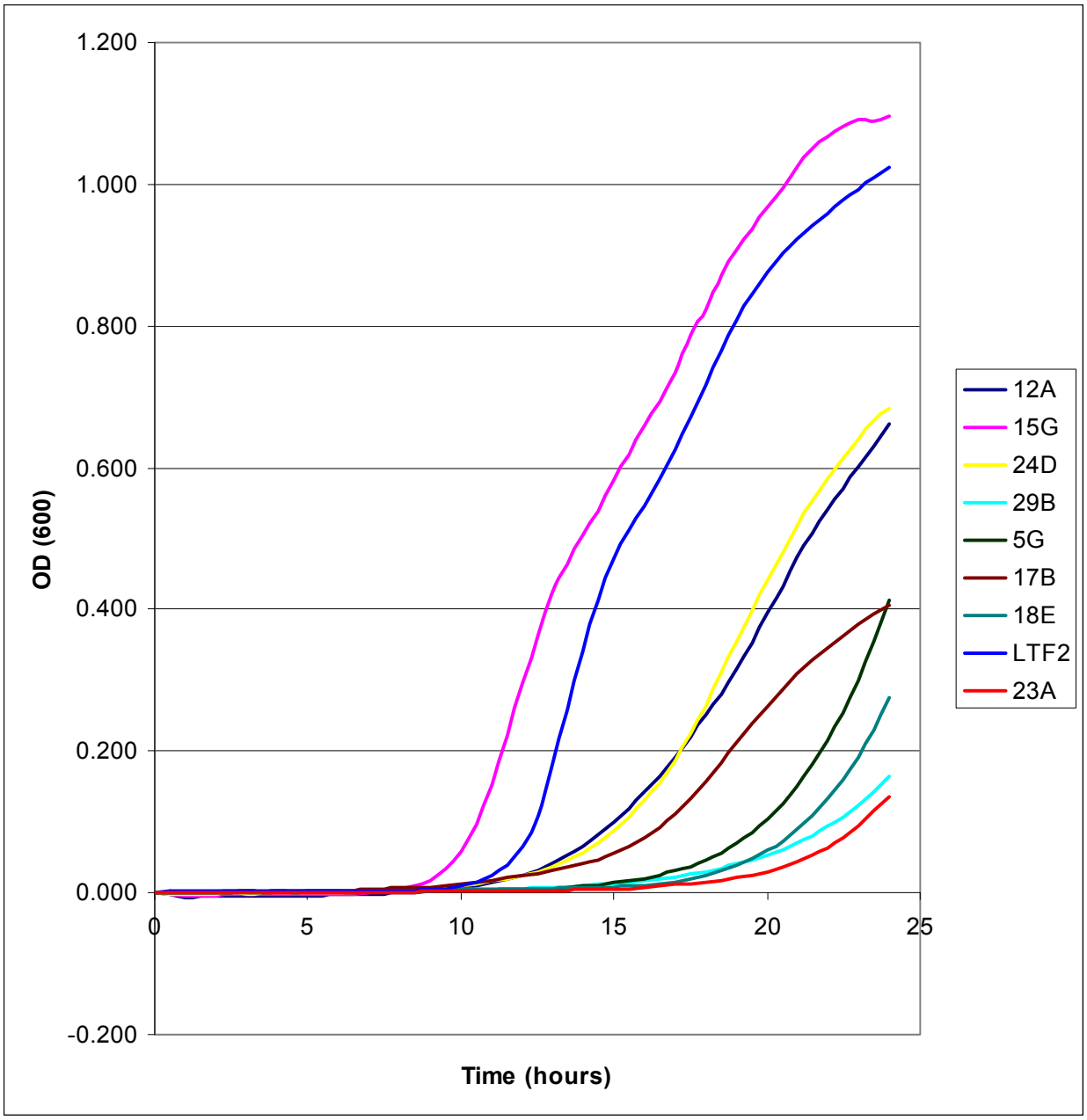


**Fig. 3.7 - *Xanthomonas axonopodis* pv. *vesicatoria* optical density growth curves in 125 ppm $CuSO_4 \cdot 5H_20$.** Graph represents the mean optical density growth curves (n=10) for each strain as recorded by the BioScreen C liquid assay for treatment 1 (125 ppm $CuSO_4 \cdot 5H_20$) after 24 hours at 28°C.

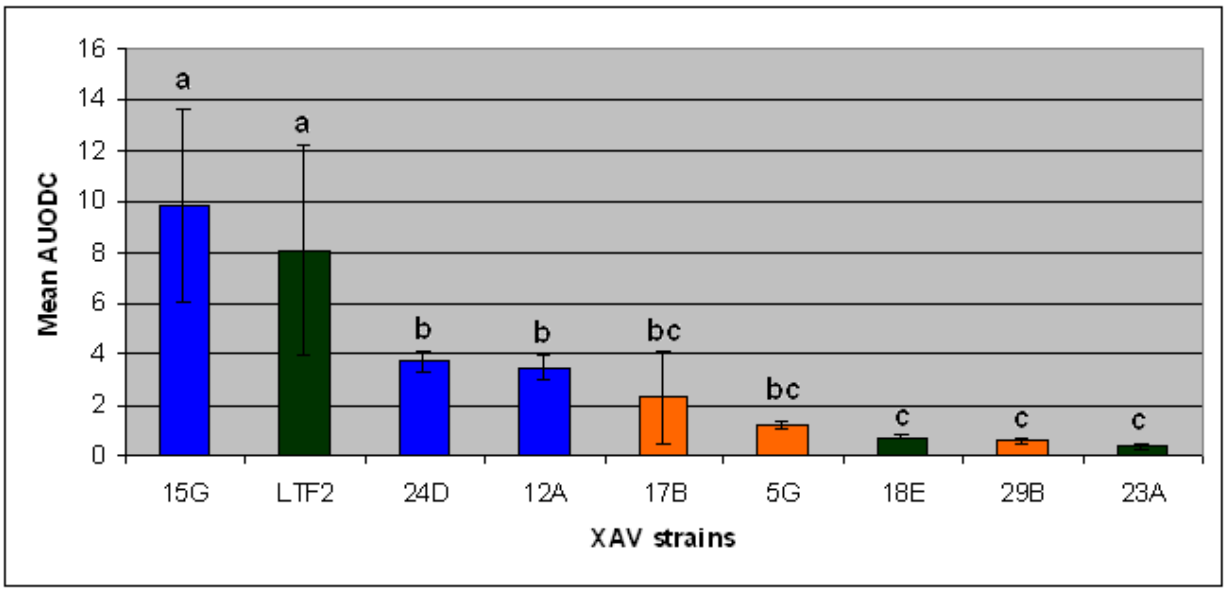


**Fig. 3.8 – Analysis of individual *Xanthomonas axonopodis* pv. *vesicatoria* strains for copper tolerance on the BioScreen C liquid growth assay in 125 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for treatment 1 (125 ppm $CuSO_4 \cdot 5H_20$) for each individual strain tested by the liquid copper tolerance assay. The original tolerance groups, determined by the plate assay, are labeled by color. The green represents the sensitive group, the orange represents the intermediate group, and the blue represents the tolerant group. Means followed by the same letter are not significantly different $P$=.05 using ANOVA (Tukey's test) with $n$=10. Means for each strain were derived from 10 replications.

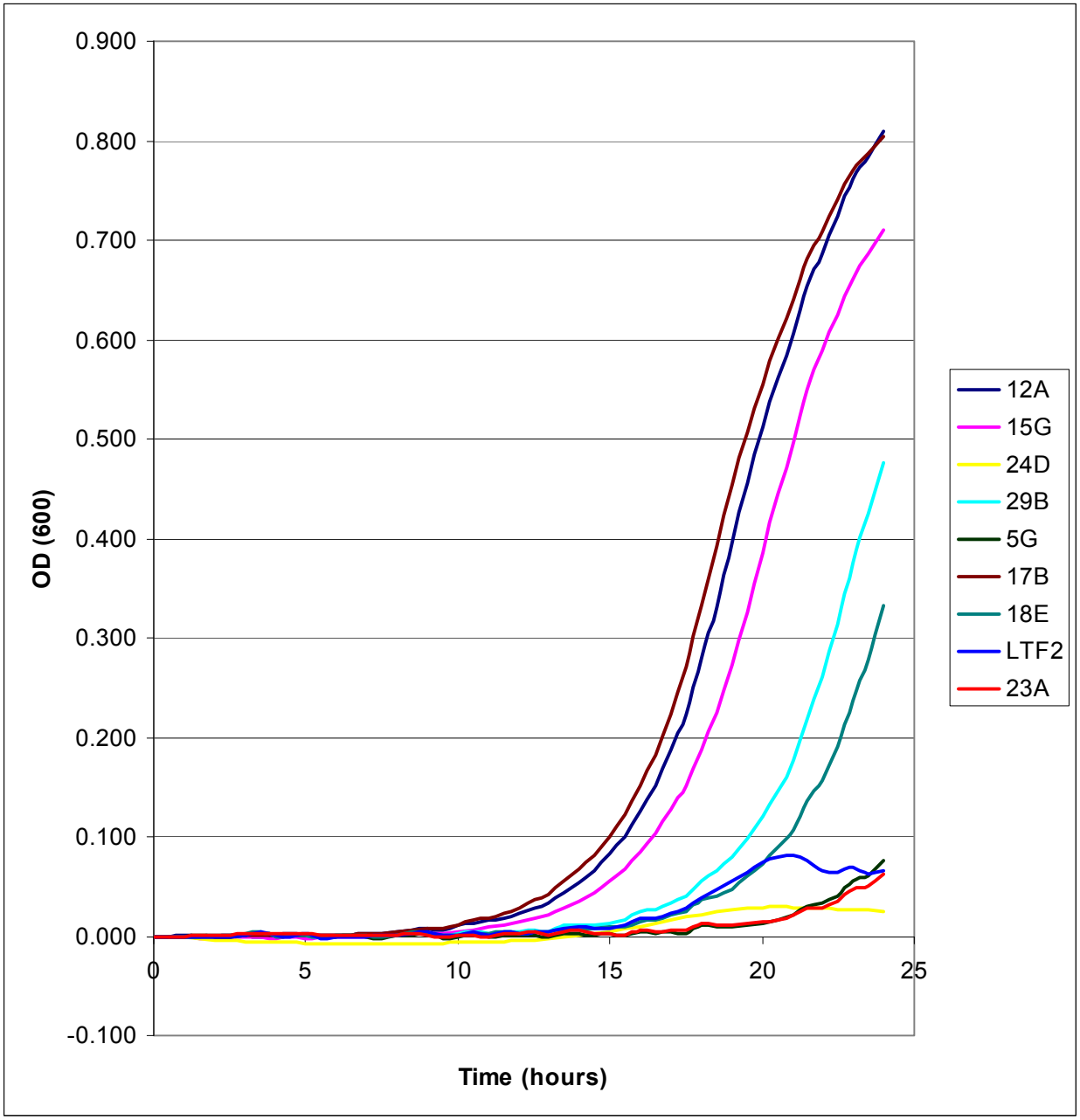


**Fig. 3.9 - *Xanthomonas axonopodis* pv. *vesicatoria* optical density growth curves in 250 ppm $CuSO_4 \cdot 5H_20$.** Graph represents the mean optical density growth curves (n=10) for each strain as recorded by the BioScreen C liquid assay for treatment 2 (250 ppm $CuSO_4 \cdot 5H_20$) after 24 hours at 28°C.

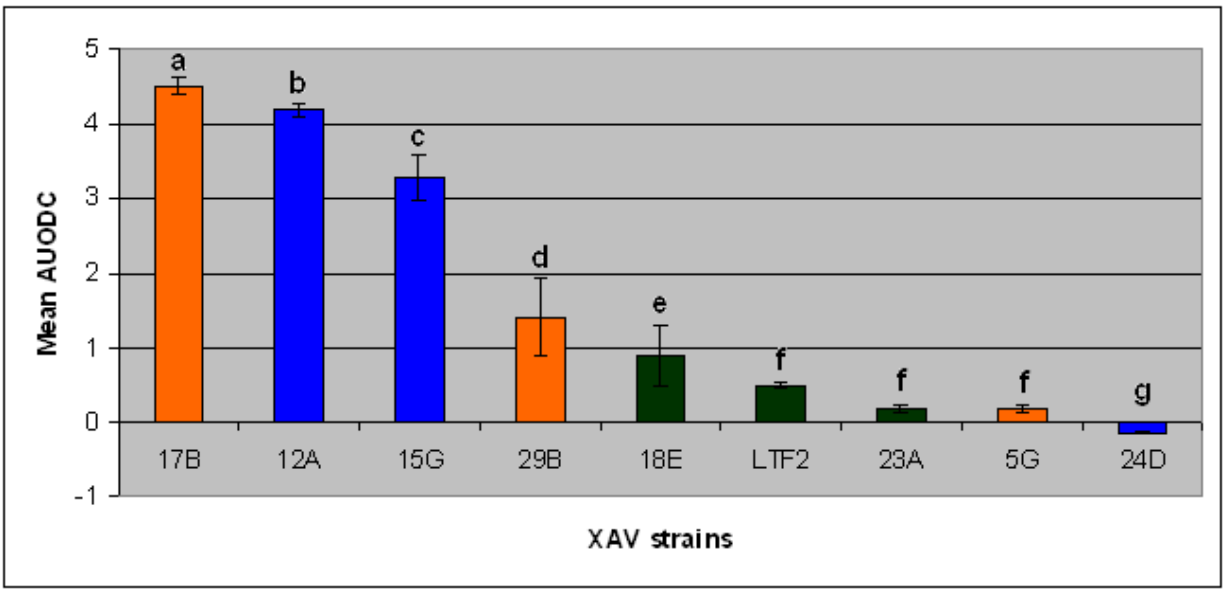


**Fig. 3.10 – Analysis of individual *Xanthomonas axonopodis* pv. *vesicatoria* strains for copper tolerance on the BioScreen C liquid growth assay in 250 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for treatment 2 (250 ppm $CuSO_4 \cdot 5H_20$) for each individual strain tested by the liquid copper tolerance assay. The original tolerance groups, determined by the plate assay, are labeled by color. The green represents the sensitive group, the orange represents the intermediate group, and the blue represents the tolerant group. Means followed by the same letter are not significantly different $P$=.05 using ANOVA (Tukey's test) with $n$=10. Means for each strain were derived from 10 replications.

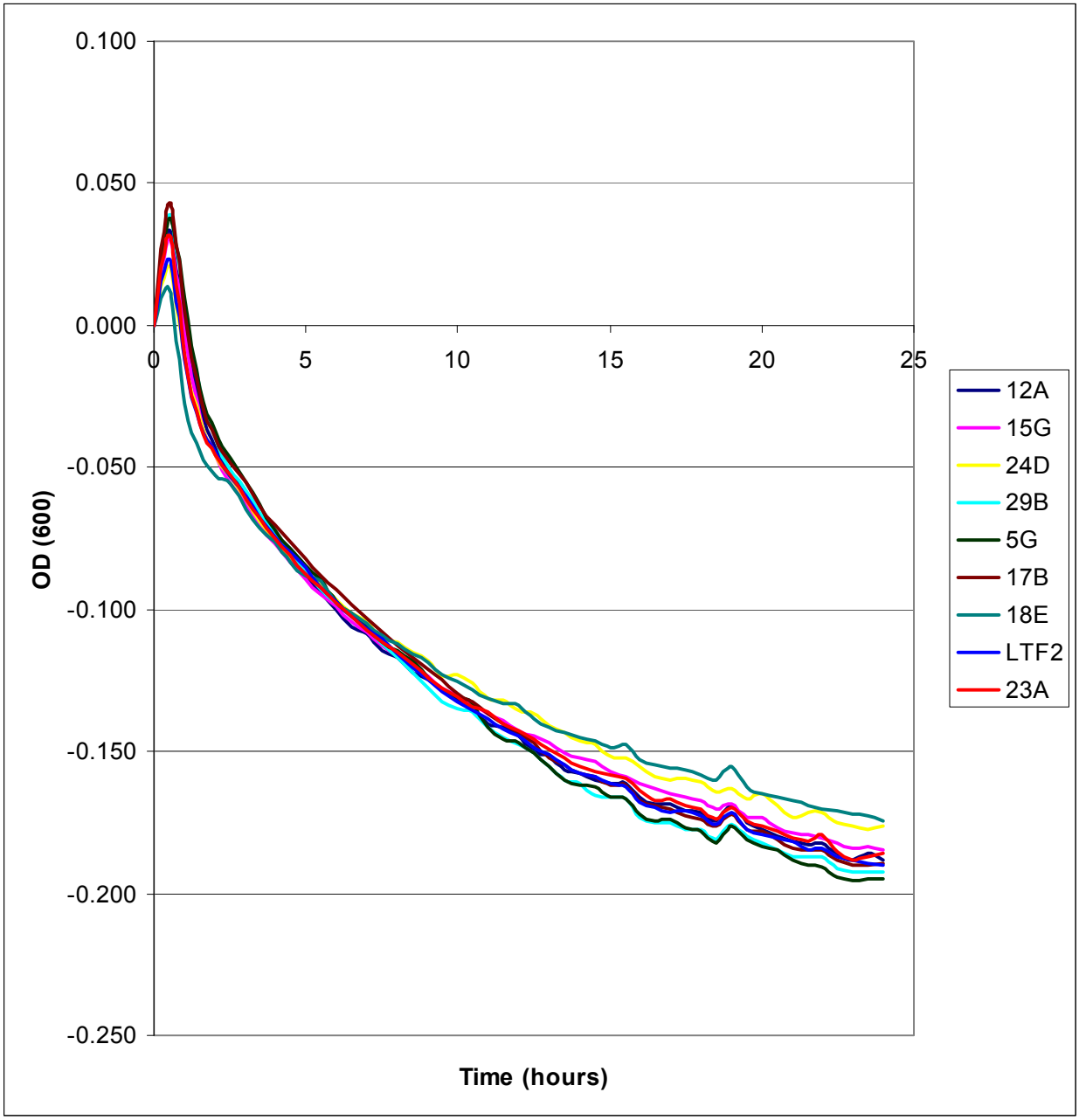


**Fig. 3.11 - *Xanthomonas axonopodis* pv. *vesicatoria* optical density growth curves in 500 ppm $CuSO_4 \cdot 5H_20$.** Graph represents the mean optical density growth curves (n=10) for each strain as recorded by the BioScreen C liquid assay for treatment 3 (500 ppm $CuSO_4 \cdot 5H_20$) after 24 hours at 28°C.

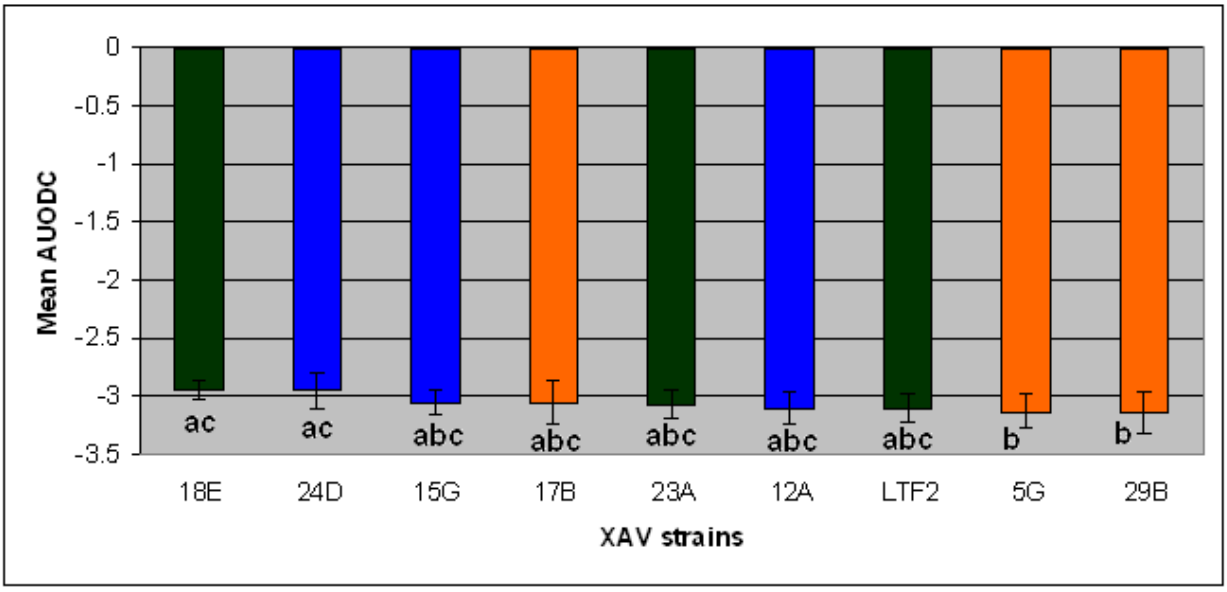


**Fig. 3.12 – Analysis of individual *Xanthomonas axonopodis* pv. *vesicatoria* strains for copper tolerance on the BioScreen C liquid growth assay in 500 ppm $CuSO_4 \cdot 5H_20$.** Chart represents the mean area under the optical density curve (AUODC) for treatment 3 (500 ppm $CuSO_4 \cdot 5H_20$) for each individual strain tested by the liquid copper tolerance assay. The original tolerance groups, determined by the plate assay, are labeled by color. The green represents the sensitive group, the orange represents the intermediate group, and the blue represents the tolerant group. Means followed by the same letter are not significantly different *P*=.05 using ANOVA (Tukey's test) with *n*=10. Means for each strain were derived from 10 replications.

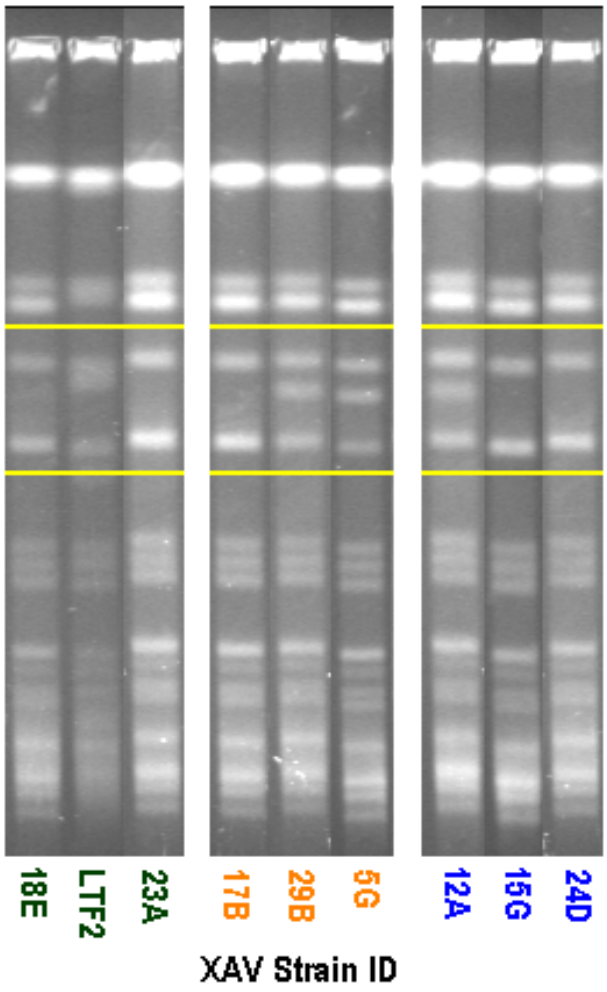


**Fig. 3.13 – Restriction profile of pulse field gel electrophoresis (PFGE) image of nine *Xanthomonas axonopodis* pv. *vesicatoria* strains digested with *SpeI*.** Strains of three copper tolerance groups, tolerant, intermediate, and sensitive, were tested and labeled in blue, orange, and green, respectively. The region of each strain that has distinctive bands which can be used to differentiate the restriction profile is outlined between the two yellow lines. Based on the patterns observed in this zone, three groups were defined. Group one consisted of 12A, 29B, and 5G. Group two was made up of strains 23A, 24D, 17B, 18E, and 15G. Finally, group three consisted of the single strain LTF 2.

# CHAPTER 4

# CONCLUSION

The studies presented provide a better understanding of the distribution and frequency of races and copper tolerance of *Xanthomonas axonopodis* pv. *vesicatoria* (also referred to as *Xanthomonas campestris* pv. *vesicatoria*), the causal agent of bacterial leaf spot (BLS) on peppers, in Georgia. BLS has been argued to be one of the most important diseases of peppers in the southeastern U.S (Pernezny and Collins, 1997; Leite et al., 1995). Previous research has focused on many aspects of the pathogen's life cycle, pathogenicity, genetics, and virulence, but no survey has been conducted to determine the distribution of the pathogen in Georgia with respect to race and copper tolerance. New techniques have been identified that have increased the effectiveness of control measures, yet the disease is still a problem. Data presented in this thesis help researchers and pepper growers to better understand the efficacy, or lack thereof, of current control measures. This data is also an indication of what direction we need to explore in the near future in order to implement more effective disease management tactics.

From these studies, seven of the 11 described races were found throughout Georgia from 2007 thru 2008. The majority of strains (50.4%) was pepper race 10. Race 10 *X. a.* pv. *vesicatoria* strains can cause disease on peppers that carry any of the four current resistance genes. It was also determined that most of the pepper race 10 *X. a.* pv. *vesicatoria* strains were isolated from Colquitt and Tift County in Georgia. Other races collected were pepper race 9, 8, 7, 6, 4, and 3.

Populations of strains collected from field outbreaks of BLS consisted of mainly copper tolerant strains. With copper-based bactericides being widely used across Georgia, losing effectiveness would be detrimental to disease management. Previous studies in other states, as well as in past years in Georgia, have shown the presence of copper-tolerant *X. a.* pv. *vesicatoria* strains. In this study, 89% of the strains tested on copper amended agar media were tolerant to copper. Copper was still found to be effective at high concentrations (500 ppm $CuSO_4 \cdot 5\ H_2O$). It is unknown if 500 ppm $CuSO_4 \cdot 5\ H_2O$ is phytotoxic to pepper plants and is likely to exceed labeled tolerances. Lower concentrations of copper can still be used to reduce the rate of disease development but do not provide complete BLS control. Strains were also found to have a high degree of variability in growth to varying or different copper concentrations. Data from these studies showed that results of copper tolerance assays can be different depending on the method used, and some strains may be more fit in the presence of copper. Testing under liquid copper conditions appears to be a more sensitive technique for field-collected *X. a.* pv. *vesicatoria* strains rather than plating on copper amended agar. Experiments using pulse field gel electrophoresis were also conducted to determine the genetic relatedness among strains with different levels of copper tolerance. Three groups of distinct banding patterns could be seen, but no correlations to copper tolerance, were obvious.

The information presented in this research provides the vegetable industry with crucial insight on the resistance management of a very important disease and the copper tolerance of the pathogen. The research also leaves us with questions for the future. The testing methods described previously may be helpful in determining copper tolerance, but it would be interesting to understand the tolerance of other chemicals used in control against BLS, such as mancozeb or copper and mancozeb mixtures. Other advances in management for BLS that would be

beneficial to the research community and growers would be the discovery or development of new bactericides that could be used either as preventatives before BLS outbreaks or as curatives which would help alleviate damage to pepper plants while infected.

The use of plant resistance genes against BLS has been another management strategy utilized for many years. Scientific advances in new pepper resistance genes to BLS that are more durable would also be beneficial. Developments in accurate seed testing is another procedure that could help determine if commercial seed is infested with *X. a.* pv. *vesicatoria* and help to provide valuable insight on limiting the spread of the bacterium at the seed level.

One very important aspect of this research that should be addressed in the future is the determination of how closely related the copper testing methods are to actual field conditions. Laboratory testing for copper tolerance is a very useful tool in determining if collected strains are tolerant to certain concentrations of copper. As shown in this study, different testing methods can provide different results. If some of the results of the two testing methods are artifacts of the procedure, then an accurate determination of the copper tolerance in strains collected in field epidemics is hard to achieve. In the plate assay, it was discovered that strains demonstrated a high percentage of copper tolerance and that sensitive strains could not grow in the presence of copper. In the liquid assay, it was found that the growth of the majority of strains was affected in the presence of copper and that the sensitive strains were able to grow. Only one sensitive strain was found to have a reduction in growth using the liquid assay. Some factors that may be contributing to these differences include lower amounts of free copper ions present due to the binding of the ions to other molecules in the solid media, the lack of contact between the bacterial cells and copper ions in solid media, extremely high or low concentrations of copper

surrounding the bacterial cells, or the development of a barrier of dead bacteria cells that buffer copper concentrations on the amended agar media.

Throughout this study, other experiments were attempted in order to understand more about the disease cycle and tolerance to other chemicals. Polymerase chain reaction (PCR) was used to detect avirulence genes that were present within a given strain. However, since avirulence genes in bacteria can be present but in the recessive form or have small base-pair mutations, the use of PCR was determined not to be useful. The purpose of locating the expressed avirulence genes was to determine if PCR could be used to determine the race of *X. a.* pv. *vesicatoria* strains.

Real-time PCR and conventional PCR were used to detect the copper resistance genes present in a given strain. Primer sets were designed specifically for *copA*, *copA-2*, *copB, copL*, *copM*, *copG*, *copG-2*, and *copF*, all of which are copper resistance genes described in *Xanthomonas* sp. or similar related *Pseudomonas* sp. None of the genes previously mentioned could be verified by PCR in the tested strains. It is possible that the primer sets created are not specific enough for each gene and more primer optimization is needed before they can be used. There is also a possibility that the copper resistant genes in the *X. a.* pv. *vesicatoria* strains collected may be different to those of the reference gene sequences. This experiment was performed to determine if the level of copper tolerance observed on a plate with copper amended agar media could be correlated to the number of copper resistance genes present.

Other experiments that were attempted were to study BLS disease onset and mancozeb tolerance testing. Disease onset of *X. a.* pv. *vesicatoria* is an important factor that should be considered throughout the study. Bacterial populations may be surviving on or within seeds (Bashan and Okon, 1986; Leite et al., 1995), on transplants in the greenhouse, or on early stages

of the host until conditions are conducive for disease development and spread. During the course of the study, some commercial greenhouses that produced pepper transplants were visited and visually inspected for BLS. No symptoms were present during the inspection and *X. a.* pv. *vesicatoria* was not isolated from the plants sampled. Yet after some time in the field, those same transplants developed a devastating outbreak of BLS (strain 12A-J, bell pepper type, Colquitt County). The time it takes bacteria to reach a concentration needed for infection may be long. Strain growth may be delayed for survival or environmental reasons (Boelema, 1985). Another theory, the theory of independent action, is that bacterial cells need to be at high enough number to establish infection because only a small percentage can cause disease (Boelema, 1985). In either case, the time required for symptom development is unknown. More research is needed to determine the disease onset and time until symptom development in *X. a.* pv. *vesicatoria*. Therefore, seeds from one commercial pepper cultivar with resistance to BLS, Aristotle, and one susceptible pepper cultivar to BLS, Early California Wonder, were vacuum infiltrated with a 1 x $10^6$ CFU/ml suspension of three different *X. a.* pv. *vesicatoria* races, pepper race 0, 6, and 10, and grown under greenhouse conditions for eight to 10 weeks. The greenhouse environment was kept as hot and humid as possible in order to favor disease development. After 10 weeks in the greenhouse, no disease was observed. Plants had flowers and small to medium size fruit present. Once a week, leaves were sampled for bacteria and none were found. The objective for this experiment was to determine the time period until BLS symptom development on greenhouse transplants. Understanding how bacteria survive on infected seeds, or within a greenhouse, and how they remain dormant will help researchers understand how to test for seed infection and how to prevent devastating field epidemics.

Bacterial growth on mancozeb-amended liquid media was also conducted to test strains for tolerance to this chemical. Growers use mancozeb to increase the effectiveness of copper-based bactericides (Shukla and Gupta, 2004). Experiments were designed using the liquid assay as previously described, with concentrations of 5 ppm mancozeb and 5 ppm mancozeb plus 250 ppm $CuSO_4 \cdot 5\ H_2O$. Testing was performed on the same nine strains tested for copper tolerance. The results were extremely variable and could not be replicated in any of the three experimental replications. In some strains, the optical density of the sample-wells remained constant at zero for the 24 hr run. These results indicate that the bacteria were not growing in the presence of mancozeb. Also some strains had an immediate drop in optical density, at time point 0, followed by a slight increase in optical density after 24 hrs. More testing is needed to determine what factors may be influencing these results. Factors such as pH, experimental run time, mancozeb concentrations, mancozeb molecular interactions, and mancozeb stability should be considered. Dilution plating was also performed after the experiment to count *X. a.* pv. *vesicatoria* population numbers. Strains that demonstrated an optical density close to zero did not grow. Only strains 17B and 24D had colony growth on the dilution plates. Both of these strains demonstrated very low optical densities after 24 hours and other strains that showed similar optical densities did not grow on the dilution plates. These strains were also tested on nutrient agar, 50 ppm mancozeb, 500 ppm $CuSO_4 \cdot 5\ H_2O$, and 50 ppm mancozeb plus 200 ppm $CuSO_4 \cdot 5\ H_2O$ amended agar media. All strains on nutrient agar had growth after 48 hours. All other treatments were lethal to all tested strains except one. At 50 ppm mancozeb, strain 15G had minimal growth after 48 hours.

These studies presented provide evidence that strains of *X a.* pv. *vesicatoria* are present in commercial pepper fields with the ability to infect plants with resistance genes and capable of

surviving in copper-rich environments. Thus, further studies and advances in management strategies are needed to provide better control for BLS in Georgia.

## Literature cited

Bashan, Y. and Okon, Y. 1986. Internal and external infection of fruit and seeds of peppers by Xanthomonas campestris pv. vesicatoria. Can. J. Bot. 64:2865-2871.

Boelema, B. H. 1985. Dose-response relationships between phytopathogenic bacteria and their hosts. Euro. J. Plant Path. 91(4):189-202.

Leite, R. P., Jones, J. B., Somodi, G. C., Minsavage, G. V., and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* associated with pepper and tomato seed by DNA amplification. Plant Disease 79(9):917-922.

Pernezny, K. and Collins, J. 1997. Epiphytic populations of *Xanthomonas campestris* pv. *vesicatoria* on pepper: Relationships to host-plant resistance and exposure to copper sprays. Plant Disease 81:791-794.

Shukla, A and Gupta, S. K. 2004. Management of bacterial spot (*Xanthomonas campestris* pv. *vesicatoria*) through chemicals. J. Myco. Plant Pathol. 34(1):88-90.