ABSTRACT

BHATTARAI, KRISHNA. Screening for Bacterial Spot (*Xanthomonas* spp.) Resistance in Tomato (*Solanum lycopersicum* L.) and Microbe Associated Molecular Patterns. (Under the direction of committee co-chairs Dr. Dilip R. Panthee and Dr. Frank J. Louws).

Bacterial spot (BS), caused by four species of *Xanthomonas: X. euvesicatoria, X. vesicatoria, X. perforans* and *X. gardneri* in tomato (*Solanum lycopersicum* L.) results in severe loss in yield and quality by defoliation and the appearance of lesions on fruits, respectively. The combined industry standard for BS control (foliar applications Actigard rotated with copper plus mancozeb) does not offer sufficient protection, especially when weather conditions favor disease spread. Development of tomato cultivars with BS resistance is thus an important option to minimize losses. The objectives of this study were to: i) screen tomato lines for identification of resistance against BS in North Carolina, ii) analyze microbe associated molecular patterns for BS resistance in tomato and iii) conduct diversity analysis of tomato lines based on vegetative and reproductive traits. A total of 71 tomato lines (63 lines for objectives i and ii) were grown in the Method Road Greenhouse, Raleigh, NC and in the field at the Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, NC in 2013. The greenhouse experiment was designed in a randomized complete design with three replications; the field experiment was designed in a complete randomized block design with two replications. BS severity was rated in the greenhouse experiment based on severity of infection on leaves using Horsfall-Barratt (HB) scale (1945). Foliar disease incidence, in the field experiment, was rated every week for five weeks using the HB scale, and fruit disease incidence was measured by counting total number of lesions in 10 severely infected fruits from individual plants of each genotype. Eight phenotypic traits, including vegetative and reproductive traits, were measured using the

tomato descriptor. Disease scores were compared based on least square means, and several lines, including 74L-1W(2008), NC2CELBR, 081-12-1X-gsms, NC22L-1(2008) and 52LB-1(98) showed resistance to BS in either field or greenhouse conditions or both. There was no correlation between BS incidence of foliar and fruit symptoms (r = 0.12; *P*= 0.356) suggesting susceptibility/resistance in these two tissue types is not genetically related. Severity of fruit spot lesions and incidence of infected fruit was highly correlated (r = 0.90; *P* <0.0001) indicating incidence is an efficient method to score fruit resistance traits. Luminol based assays were conducted using the *Xanthomonas* specific flagellin (*xcc22*) peptide designed from *Xanthomonas campestris pv. campestris* as well as flagellin 22 (*flg22*) and flagellin 28 (flgII-28) peptides designed from *Pseudomonas aeruginosa* to assess reactive oxygen species (ROS) production in each tomato genotype. *X. perforans,* race T4, a prevalent race in North Carolina, was cultured on yeast dextrose chalk (YDC) agar for 24-48 hours at 30°C, diluted in dH_2O and inoculated on plants using a backpack sprayer using a inoculum concentration of $2\text{-}5x10^8$ CFU/ml. Total and maximum ROS production using *xcc22* peptide showed more than 24% and 27% negative correlation with foliar area under disease progress curve scores which were significant (*P* = 0.049 and 0.032). Significant negative correlation, although low, suggests a use for luminol based ROS assays in screening tomato genotypes for BS resistance. For objective iii, five principal components accounted for more than 92% of the phenotypic variation in the tested genotypes. A dendrogram displayed six clusters, each showing unique fruit characteristics and was based on hierarchical average-cluster analysis methods. Genotypes in each of these distinct clusters can assist future breeding efforts in developing tomato varieties with specific fruit characteristics. Five genotypes resistant to BS had *S. pimpinellifolium* L3707 in their

pedigree, indicating future research should focus on screening this genotype and developing a mapping population to identify and map genes regulating BS resistance. This work highlights that BS is a major economic issue in NC and that development of screening methods linked to increased host resistance could offer an important contribution to future integrated BS management programs.

Screening for Bacterial Spot (*Xanthomonas* spp.) Resistance in Tomato (*Solanum lycopersicum* L.) and Microbe Associated Molecular Patterns

by
Krishna Bhattarai

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APPROVED BY:

_____	_____
Dr. Dilip R. Panthee	Dr. Frank J. Louws
Committee Co-chair	Committee Co-chair

_____
Dr. John D. Williamson

DEDICATION

To my father Sudan Kumar Bhattarai, mother Maya Bhattarai and brother Kritan Bhattarai

BIOGRAPHY

Krishna Bhattarai was born in Nepalgunj, Banke, Nepal. He grew up with a younger brother, Kritan, in Rajapur, Bardia, Nepal. After completion of School Leaving Certificate (SLC) from Gulariya, Bardia, Nepal he completed high school in Kathmandu, Nepal. He joined the Institute of Agriculture and Animal Science (IAAS), Tribhuvan University (TU), Nepal for a Bachelor of Science in Agriculture degree in 2006. He studied at IAAS in Paklihawa, Rupendehi, Nepal for the first two years and the last two years at IAAS in Rampur, Chitwan, Nepal thereby completing his undergraduate degree, with Plant Breeding as an elective, in 2010. After graduation he started working as a Project Officer for the Association for Social Transformation and Humanitarian Assistance (ASTHA) – Nepal, a non-government organization in 2011. He started his Master of Science degree with the Department of Horticultural Science, North Carolina State University in 2012. His research focused on developing genetic host resistance to bacterial spot of tomato at the Mountain Horticultural Crops Research and Extension Center, tomato breeding program in Mills River, NC.
Mr. Bhattarai is a member of the American Society of Horticultural Science and Pi Alpha Xi (a national honor society for horticulture, NCSU).

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INTRODUCTION

Bacterial spot (*Xanthomonas* spp.) is caused by four different species of *Xanthomonas: X. euvesicatoria, X. vesicatoria, X. perforans and X. gardneri* in tomato (*Solanum lycopersicum* L.) (Jones et al., 2000; Jones et al., 2005) (Table 1.1). This disease was first reported in tomato in South Africa around 1914 (Doidge, 1921). In the United States, it was reported for the first time in the north central States in 1918 in canning tomato varieties (Gardner and Kendrick, 1921). Pohronezny and Volin (1983) reported that yield loss can range from 17 to 66% depending on late and early infections, respectively. Direct effects of bacterial spot on tomato production are yield loss due to defoliation and quality loss due to appearance of lesions on fruits (Pohronezny and Volin, 1983). Indirect effects are sun scald, black shoulder and cracking of fruits due to exposure of fruits to direct sunlight and rain because of defoliation resulting in further yield and quality losses (Scott and Jones, 1986).

HOST PATHOGEN AND ENVIRONMENT

Tomato and pepper are two vegetable crops affected by bacterial spot. These crops are cultivated worldwide in warm weather conditions and high rainfall that favor the development of this disease. Host, pathogen and environmental relationships of tomato and bacterial spot disease is given in Figure 1.2. *Xanthomonas* spp. can survive for more than 10 years in seed (Bashan et al., 1982b), either externally or internally. This can act as source of primary inoculum. When the seeds are infested externally, cotyledons can be infected upon contact with the seed coat and thus develop lesions. Bacteria present on these lesions can be splashed onto top of the leaves or to other plants. This pathogen can survive in the soil,

possibly in the rhizosphere of non-host plants (Bashan et al., 1982a), but has a limited survival period of days to weeks, so they are most often found associated with debris from diseased or infected plants. Survival of this pathogen in solanaceous weed species is reported to be minimal (Jones et al., 1986) but volunteer plants are potentially important sources of inoculum (Ritchie, 2000). Volunteer tomato seedlings, for instance, were shown to be a primary origin of bacterial spot in Florida (Jones et al., 1984). Secondary spread of this pathogen occurs through surface and overhead irrigation, wind driven rain (Sherf and MacNab, 1986, Goode and Sasser, 1980) and, cultural practices like pruning, stringing and spraying pesticides with high-pressure sprayers. The pathogen enters through natural openings like stomata and hydathodes and through injuries created by wind driven sand, cultural practices and other factors (Jones et al., 1991).

Bacterial spot is a common disease in warm and humid weather conditions. An optimum temperature of 27°C (Gardner and Kendrick, 1923) and frequent rainfall and high humidity (Diab et al., 1982a) favors this disease. Diab et al., (1982b) reported that temperatures above 30°C, but not over 35°C favor the disease. This is why it is a common disease in tomato grown in the fields of sub-tropical and tropical regions and in glasshouses, seed borne infection is a major concern.

Symptoms of this disease can be seen on all foliar parts such as stem, leaf, calyx and fruit. During the initial stages of disease onset, small, yellow-green lesions occur on young leaves. As the disease progresses, dark, water soaked, greasy lesions on the leaves expand to 0.25-0.5 cm wide and can be surrounded by a yellow halo. As lesions coalesce on lower leaves, the whole leaf turn yellow to dark brown and ultimately senesces. Lesions on fruits generally

appear when disease pressure is high during flower anthesis and early fruit development. Tomato fruits don't have stomata but falling of fruit hairs present on the fruit surface creates injuries and opening on the fruit surface. These openings, injures abrasions and punctures act as an entrance for the bacteria (CABI and EPPO, unpublished data). Raised lesions up to 0.5 cm wide, generally surrounded by a pale green halo, can be seen on fruits. Appearance of lesions on fruits decreases the quality resulting in economic losses.

MANAGEMENT OF BACTERIAL SPOT

Despite technological advances and research on chemical control of bacterial diseases, there is no bactericide, that has been developed, which can efficiently control plant bacterial diseases. Bacterial spot is difficult to control during favorable weather conditions of optimum temperature and frequent rainfall (Pohronezny and Volin, 1983). Chemical, biological and breeding strategies have been of limited success in finding an effective control for this disease (Jones and Jones, 1985; Jones and Scott, 1986; Scott and Jones, 1986; Louws et al, 2001). In the 1950s use of streptomycin to control bacterial diseases was common, but bacteria eventually developed resistance (Stall and Thayer, 1962; Thayer and Stall, 1961). Copper containing bactericides were used intensively starting in the 1960s to control the disease and continue to be used. Copper mixed with ethylenebisdithiocarbamate (EBDC) fungicides such as maneb or mancozeb was found to be efficient in controlling bacterial spot in fields (Marco and Stall, 1983) but their effectiveness decreased due to development of resistance to copper by the bacteria (Jones and Jones, 1985; Kousik and Ritchie, 1996; Pohronezny et al., 1992). Bacterial spot strains in North Carolina, for instance, are reported to be resistant to both copper and streptomycin (Ritchie and Dittapongpitch, 1991). Poor

disease control by existing chemical pesticides directed efforts towards identification of alternative methods for bacterial spot disease management (Obradovic et al., 2005). Louws et al. (2001) reported the effective control of bacterial spot in eastern North America with Acibenzolar-*S*-methyl (Actigard 50WG), a plant activator that induces systemic acquired resistance (SAR) in plants to limit pathogenicity of bacteria. Use of host-range mutant bacteriophages have also been found to control race 1 and race 3 bacterial spot strains (Balogh et al., 2003; Flaherty et al., 2000).

Crop protection compounds are not able to sufficiently control *Xanthomonas,* and hence developing genetic resistance against bacterial spot has become a priority in breeding programs (Horvath et al., 2012). Current management practices for bacterial spot are use of pathogen-free seed and transplants, elimination of volunteer plants, regular application of a copper based pesticides mixed with EDBCs and rotated with actigard, use of bacteriophages and use of cultivars that have some resistance/tolerance to the pathogen.

BREEDING TOMATO FOR BACTERIAL SPOT RESISTANCE

Five races (T1 through T5) of *Xanthomonas* causing BS in tomato have been recognized based on the differential formation of a hypersensitive response (HR) on specific host genotypes (Jones et al., 2005; Jones et al., 2000; Jones et al., 2004). Hawaii 7998 (H7998) produced HR against *X. euvesicatoria* race T1 (Jones and Scott, 1986) and the nature of resistance was multigenic (Wang et al., 1994; Whalen et al., 1993; Yu et al., 1995) and additional genetic factors (Somodi et al., 1996; Wang, 1992). PI114490 exhibited non-hypersensitive resistance to *X. vesicatoria* race T2 with the resistance being additive and controlled by two genes (Scott et al., 2003). *X. perforans* race T3 has an antagonistic effect to

race T1 in *in-vitro* experiments (Jones et al., 1998). Race T3 has displaced race T1 and is reported to overcome the hypersensitivity of H7998 in Florida (Jones et al., 1998). Hawaii 7981 (H7981), PI 126932 and PI128216 are reported to show HR against race T3 (Scott et al., 1995) . The resistance of H7981 is controlled by an incompletely dominant gene *Xv3* (Scott et al., 1996). Currently *X. perforans* race T4 is the predominant race found in North Carolina (Louws et al., unpublished data). LA716 exhibits HR against race T4 due to the presence of the *avrXv4* gene in the race T4 pathogen (Astua-Monge et al., 2000). Cultivated tomato (*Solanum lycopersicum* L.) does not have effective resistance against bacterial spot. Tomato breeding programs have been trying to identify bacterial spot resistant genes in wild relatives to incorporate these into advanced tomato cultivars. However, it has been difficult to get resistance to all known available races of the pathogen into tomato varieties having desired horticultural traits. Resistance development for bacterial spot management is difficult due to the multigenic nature of resistance and limited availability of resistant sources. The pathogen has been found to be able to overcome resistance due to mutations that alter the race, emergence of new races already present in the field, or migration of new races from other regions of the world. Given the growing environmental and economic concern over excessive use of pesticides, developing resistance against bacterial spot becomes an even more important approach to minimize crop losses due to BS. Finally, to provide broad resistance, there is a critical need to develop tomato lines that harbors horizontal resistance to all races of the pathogen.

PLANT IMMUNE SYSTEM

Foliar bacterial plant pathogens enter inside plant tissue through stomata, hydathodes or injuries. Unlike animals, plants do not have mobile defender cells and a somatic adaptive immune system (Jones and Dangl, 2006). Instead, they depend upon performed defenses and on induced or systemic responses to infection sites (Dangl and Jones, 2001). Jones and Dangl (2006) described two distinct branches of the plant immune system. One uses transmembrane pattern recognition receptors (PRRs) present in the plasma membrane of the plant cell. These identify slowly evolving microbial-associated molecular patterns (MAMPs) or pathogen associated molecular patterns (PAMPs) such as flagellin in bacteria (Zipfel and Felix, 2005). The second uses nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) or polymorphic nucleotide binding leucine rich receptors (NB-LRRs) protein products, which act largely inside the cell, and are encoded by most R genes (Dangl and Jones, 2001). Jones and Dangl (2006) proposed a four phased 'zigzag' model to illustrate the mechanism in plant cells whereby PAMPs are recognized by PRRs and the resulting PAMP-triggered immunity (PTI) checks further spread of the pathogen in the plant. Once restricted, the pathogen develops effectors that restore virulence and allows continued disease development. This is called effector triggered susceptibility (ETS). Plants frequently harbor NB-LRR proteins specifically able to recognize the effector and are thus able to restrict pathogen spread, a response called effector triggered immunity (ETI). PTI thus amplifies the ETI responses leading to disease resistance and usually HR at the site of infection. Natural selection in the pathogen results in avoidance of ETI by shedding or diversifying the recognized effector gene or by acquiring additional effectors that are able to suppress ETI.

Similarly, natural selection in plants leads in new R specificities with which the plant is again able to establish ETI and the cycle continues as show in Figure 1.1. Bacterial flagellins are prevalent class of PAMPs that are recognized by the innate immune systems of plants (Gomez-Gomez and Boller, 2002). In *Arabidopsis thaliana*, a transmembrane receptor kinase with a leucine-rich repeat (LRR) extracellular domain, FLAGELLIN SENSING2 (FLS2), is required for flagellin perception and flagellin elicited defense activation (Gomez-Gomez and Boller, 2000). FLS2 binds flagellin (Chinchilla et al., 2006) and activates downstream defense responses.

Identification of PAMP recognition specificity across plant families might provide a means of developing resistance to a wide range of pathogens. Lacombe et al. (2010) reported an increase in resistance to a range of phytopathogenic bacteria, from different genera, in the solanaceous crops *Nicotiana benthamiana* and *Solanum lycopersicum* after transfer of EFR, a PRR from the cruciferous plant *Arabidopsis thaliana* which confers responsiveness to bacterial elongation factor thermal unstable (EF-Tu). This technique has several advantages over current practices of managing diseases and development of host resistance mechanisms. Utilizing the innate immune system response of a plant to control a pathogen would lead to decreased pesticides use and, ameliorate the associated financial, health and environmental costs (Lacombe et al., 2010). Classical breeding, which predominantly targets R-gene mediated resistance, can be "broken down" often rapidly by evolving pathogens like *Xanthomonas.* In contrast, it would be more difficult for a pathogen to modify PAMPs-mediated resistance, since the targeted molecule recognized by PRRs is frequently associated with the fitness of the pathogen. Therefore, resistance through PRRs is hypothesized to be

more durable than obtained through classical breeding methods that tend to rely on use of R genes. Rapid transfer of new PRRs to elite varieties and into crops through transgenic expression can expedite such resistance breeding (Lacombe et al., 2010). Successful pathogens are able to avoid recognition by PRRs by evolving new or modified effectors, but PTI activation is still able to reduce pathogen populations and thus decrease the probability of emergence of new virulent pathogens and the breakdown of resistance (Brun et al., 2010). Lacombe et al. (2010) proposed that combinations of several PRRs as well as combining PRRs along with R proteins could enable broad spectrum resistance against multiple genera of plant pathogens with the promising potential for durability under field conditions, by recognizing widely distributed effectors.

Perception of PAMPs from pathogens by the host cell leads to rapid activation of defense mechanisms such as reinforcement of cell walls by callose deposition, production of reactive oxygen species (ROS), and induction of numerous defense-related genes (Zipfel et al., 2006). Plants can perceive PAMPs derived from the component various microbial structures such as flagellin (FLG), lipopolysaccharides (LPS), bacterial cold-shock protein (CSP), and EF-Tu from bacteria, cell wall polysaccharides such as chitin and the fungal sterol ergosterol from true fungi (Nurnberger et al., 2004; Zipfel and Felix, 2005), cell wall β-glucan, the pep13 epitope conserved in cell wall transglutaminases, and secreted lipotransfer proteins termed elicitins (Nurnberger et al., 2004). Some of these PAMPs, like FLG, are perceived by a wide range of hosts whereas some, like CSP and EF-Tu seemed to be identified by the orders of *Solanales* and *Brassicales* respectively (Zipfel et al., 2006). PRRs like EFR (Zipfel et al.,

2006) and FLS2 are able to perceive effector molecules like EF-Tu and flagellin (Zipfel et al., 2006).

ROS are generated by excitation or incomplete reduction of molecular oxygen during cellular metabolism in aerobic organisms (Halliwell, 2006). They are not always harmful to the cell and spatial and temporal fluctuations of ROS levels can act as signals required for growth, development, tolerance to abiotic stresses, proper response to pathogens and initiation of cell death (Apel and Hirt, 2004; Foreman et al., 2003; Gechev and Hille, 2005). These highly reactive intermediates are produced when molecular dioxygen (O_2) accepts either energy or electrons released from different reactions inside the cell. Superoxide ($O_2^{\cdot-}$) is formed when an O_2 molecules are reduced. $O_2^{\cdot-}$ has a short half-life of 2 to 4 μs. Further reduction of $O_2^{\cdot-}$ leads to the formation of hydrogen peroxide (H_2O_2) which is relatively stable and has a half-life of 1 ms. ROS are produced at various locations inside the cell. Chloroplasts are a major site of ROS production in plants (Asada, 2006) where $O_2^{\cdot-}$ for instance can be formed by electron leakage from Fe-S centers of photosystem I or reduced ferrodoxin to O_2 (Mehler reaction) which is then metabolized to H_2O_2 by superoxide dismutase (SOD). ROS are produced in peroxisomes and glyoxysomes during photorespiration and fatty acid oxidation, respectively (del Rio et al., 2006). $O_2^{\cdot-}$ and H_2O_2 are produced from NADH dehydrogenase, ubiquinone radical and complex III during mitochondrial respiration (Moller, 2001). Mitochondrial ROS production is comparatively lower than that of chloroplasts due to lack of light energy-absorbing chlorophyll pigments but they are important regulators of stress adaptation and programmed cell death (Robson and Vanlerberghe, 2002). Apoplastic enzymes like the plasmalemma-embedded NAD(P)H oxidases and cell wall-associated

peroxidases are thought to be the main producers of $O_2^{\cdot-}$ and H_2O_2 (Sagi and Fluhr, 2006) in the extra cellular matrix respectively. ROS generated by apoplastic enzymes take part in the oxidative burst as a part of the HR to pathogens but are also thought to regulate cell growth, development and cell death (Foreman et al., 2003; Gapper and Dolan, 2006; Gechev and Hille, 2005; Sagi and Fluhr, 2006; Torres et al., 2002). Unfavorable environmental conditions like cold, heat, drought, high-light and heavy metal stress also result in increased ROS production. ROS controlled cell death occurs during aleurone cell death, leaf senescence, a number of abiotic stresses and, HR and many allelopathic plant-plant interactions. During HR-mediated cell death, a biphasic burst of NADPH-dependent ROS production occurs which leads to the onset of cell death in the proximity of the infection and triggers signals that migrate to neighboring tissues to initiate distant micro-hypersensitive responses and to induce systemic acquired resistance (SAR) (Alvarez et al., 1998). ROS also interacts with auxin, abscisic acid (ABA) and jasmonic acid to regulate growth, stomatal closure and wounding responses (Kwak et al., 2006). Increased synthesis of ethylene and salicylic acid is observed under pathogen attack and abiotic stress which results in production of ROS (Wang et al., 2002).

Detailed protocols for measuring various PTI-associated phenotypes, induction of reporter genes, callose deposition, activation of mitogen-activated protein kinases (MAPKs), and a luciferase-based reporter system have been reported (Nguyen et al., 2010). Despite being conserved across the plant kingdom, PTI-specific responses and underlying molecular mechanisms may differ between species. For instance, tomato recognizes a 15 amino acid flagellin peptide from *Escherichia coli* but *Arabidopsis* does not (Felix et al., 1999; Meindl et

al., 2000). Bacterial flagellins are perceived by both *Solanaceae* and *Brassicaceae* families but the response to different peptides or protein epitopes is different (Nguyen et al., 2010). For instance, the *xcc22* region of *Xanthomonas* flagellin is known for the detectable elicitation of *Arabidopsis* defense responses (Sun et al., 2006). Polymorphisms in *xcc22* has been reported (Sun et al., 2006) and sequences from the protein that elicited ROS production in *Arabidopsis* was used in Oxidative burst assay to measure ROS production in tomato lines in North Carolina. The oxidative burst, a rapid and well characterized response to PAMPs, is the basis of a high-throughput assay and can be used in characterizing PAMP responsiveness in diverse accessions and mapping populations (Lloyd et al., 2014). Quantitative assessment of ROS production between *Arabidopsis* leaf tissue and live *Pseudomonas syringae* pv *tomato* has been previously reported (Smith and Heese, 2014). PAMP-triggered responses are mostly congruent (Wan et al., 2008; Zipfel et al., 2006) and have been reported not to necessarily induce microbe-specific immunity (Ferrari et al., 2007; Zipfel et al., 2004). Lloyd et al. (2014) suggested the possibility of PAMPs being used to predict the extent of PTI responsiveness to quantitative resistance to distinct pathogens. Here we assess resistance in diverse tomato lines and compare the BS resistance with ROS production, one of the PTI responses, using three different flagellin peptides: flg22, flgII-28 and *xcc22* as a means of ROS induction in tomato.

RESEARCH OBJECTIVES

The major objectives of this research were:

1. Screening analysis of tomato lines in greenhouse and field conditions for identification of BS disease resistance

2. Screening analysis of tomato lines using oxidative burst or pathogen associated molecular pattern assay and comparison to greenhouse and field disease severity
3. Diversity analysis of tomato lines using morphological traits

REFERENCES

Alvarez, M., R. Pennell, P. Meijer, A. Ishikawa, R. Dixon, and C. Lamb. 1998. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 92(6):773-784.

Apel, K. and H. Hirt. 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55:373-399.

Asada, K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. 141(2):391-396.

Astua-Monge, G., G.V. Minsavage, R.E. Stall, C.E. Vallejos, M.J. Davis, and J.B. Jones. 2000. Xv4-vrxv4: a new gene-for-gene interaction identified between *Xanthomonas campestris* pv *vesicatoria* race T3 and the wild tomato relative *Lycopersicon pennellii*. Mol. Plant-Microbe Interact. 13(12):1346-1355. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=20003026134&site=ehost-live&scope=site>.

Balogh, B., J. Jones, M. Momol, S. Olson, A. Obradovic, P. King, and L. Jackson. 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. Plant Dis. 87(8):949-954.

Bashan, Y., S. Diab, and Y. Okon. 1982a. Survival of *Xanthomonas campestris* pv *vesicatoria* in pepper seeds and roots, in symptomless and dry leaves in non-host plants and

in the soil. Plant Soil 68(2):161-170. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=19831393025&site=ehost-live&scope=site>.

Bashan, Y., Y. Okon, and Y. Henis. 1982b. Long-Term Survival of *Pseudomonas* s*yringae* pv *tomato* and *Xanthomonas campestris* pv *vesicatoria* in Tomato and Pepper Seeds. Phytopathology 72(9):1143-1144.

Brun, H., A. Chevre, B.D.L. Fitt, S. Powers, A. Besnard, M. Ermel, V. Huteau, B. Marquer, F. Eber, M. Renard, and D. Andrivon. 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytol. 185(1):285-299.

CABI and EPPO. Data sheets on Quarantine pests *Xanthomonas vesicatoria*. EPPO quarantine pest. https://www.eppo.int/QUARANTINE/bacteria/Xanthomonas_vesicatoria/XANTVE_ds.pdf

Chinchilla, D., Z. Bauer, M. Regenass, T. Boller, and G. Felix. 2006. The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18(2):465-476.

Dangl, J.L. and J.D.G. Jones. 2001. Plant pathogens and integrated defence responses to infection. Nature (London) 411(6839):826-833.

<http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=20013085090&site=ehost-live&scope=site>.

del Rio, L., L. Sandalio, F. Corpas, J. Palma, and J. Barroso. 2006. Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiol. 141(2):330-335.

Diab, S., Y. Bashan, and Y. Okon. 1982b. Studies of infection with Xanthomonas campestris pv vesicatoria, causal agent of bacterial scab of pepper in Israel. Phytoparasitica 10(3):183-191. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=19831389553&site=ehost-live&scope=site>.

Doidge, E.M. 1921. A tomato canker. Ann. Appl. Biol. 7(4):407-430.

Felix, G., J. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 18(3):265-276.

Ferrari, S., R. Galletti, C. Denoux, G. De Lorenzo, F.M. Ausubel, and J. Dewdney. 2007. Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiol. 144(1):367-379.

Flaherty, J., J. Jones, B. Harbaugh, G. Somodi, and L. Jackson. 2000. Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. HortScience 35(5):882-884.

Foreman, J., V. Demidchik, J. Bothwell, P. Mylona, H. Miedema, M. Torres, P. Linstead, S. Costa, C. Brownlee, J. Jones, J. Davies, and L. Dolan. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422(6930):442-446.

Gapper, C. and L. Dolan. 2006. Control of plant development by reactive oxygen species. Plant Physiol. 141(2):341-345.

Gardner, M. and J. Kendrick. 1921. Bacterial spot of tomato. J. Agric. Res. 21:0123-0156.

Gardner, M.W. and J.B. Kendrick. 1923. Bacterial spot of Tomato and Pepper. Phytopathology 13(7):307-315 pp.

Gechev, T. and J. Hille. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. J. Cell Biol. 168(1):17-20.

Gomez-Gomez, L. and T. Boller. 2000. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell 5(6):1003-1011.

Gomez-Gomez, L. and T. Boller. 2002. Flagellin perception: a paradigm for innate immunity. Trends Plant Sci. 7(6):251-256.

Halliwell, B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 141(2):312-322.

Horvath, D.M., R.E. Stall, J.B. Jones, M.H. Pauly, G.E. Vallad, D. Dahlbeck, B.J. Staskawicz, and J.W. Scott. 2012. Transgenic Resistance Confers Effective Field Level Control of Bacterial Spot Disease in Tomato. Plos One 7(8):e42036.

Jones, J. and J. Scott. 1986. Hypersensitive Response in Tomato to *Xanthomonas campestris* pv. *vesicatoria*. Plant Dis. 70(4):337-339.

Jones, J., G. Lacy, H. Bouzar, R. Stall, and N. Schaad. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst. Appl. Microbiol. 27(6):755-762.

Jones, J., H. Bouzar, G. Somodi, R. Stall, K. Pernezny, G. El-Morsy, and J. Scott. 1998. Evidence for the preemptive nature of tomato race 3 of *Xanthomonas campestris* pv. *vesicatoria* in Florida. Phytopathology 88(1):33-38.

Jones, J., R. Stall, J. P. JONES, and K. Phoronezny. 1984. Survival of *Xanthomonas-Campestris* pv. *Vesicatoria* in Florida. Phytopathology 74(7):858-858.

Jones, J., S. Woltz, J. Jones, and K. Portier. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets treated with copper bactericides. Phytopathology 81(7):714-719.

Jones, J.B. and J.P. Jones. 1985. The effect of bactericides, tank mixing time and spray schedule on bacterial leaf spot of tomato. Proceedings of the Florida State Horticultural Society 98:244-247.

Jones, J.B., G.H. Lacy, H. Bouzar, G.V. Minsavage, R.E. Stall, and N.W. Schaad. 2005. Bacterial spot - worldwide distribution, importance and review. Acta Horticulturae (695):27-33. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=20063067259&site=ehost-live&scope=site; http://www.actahort.org>.

Jones, J.B., H. Bouzar, R.E. Stall, E.C. Almira, P.D. Roberts, B.W. Bowen, J. Sudberry, P.M. Strickler, and J. Chun. 2000. Systematic analysis of xanthomonads (*Xanthomonas* spp.) associated with pepper and tomato lesions. Int. J. Syst. Evol. Microbiol. 50 Pt 3:1211-1219.

Jones, J.B., K.L. Pohronezny, R.E. Stall, and J.P. Jones. 1986. Survival of *Xanthomonas campestris pv. vesicatoria* in Florida on tomato crop residue, weeds, seeds, and volunteer tomato plants. Phytopathology 76(4):430-434. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=19861318075&site=ehost-live&scope=site>.

Jones, J.D.G. and J.L. Dangl. 2006. The plant immune system. Nature (London) 444(7117):323-329. <http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=20063212665&site=ehost-live&scope=site>.

Kousik, C. and D. Ritchie. 1996. Race shift in *Xanthomonas campestris* pv *vesicatoria* within a season in field-grown pepper. Phytopathology 86(9):952-958.

Kwak, J., V. Nguyen, and J. Schroeder. 2006. The role of reactive oxygen species in hormonal responses. Plant Physiol. 141(2):323-329.

Lacombe, S., A. Rougon-Cardoso, E. Sherwood, N. Peeters, D. Dahlbeck, H.P. van Esse, M. Smoker, G. Rallapalli, B.P.H.J. Thomma, B. Staskawicz, J.D.G. Jones, and C. Zipfel. 2010. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nat. Biotechnol. 28(4):365-U94.

Lloyd, S.R., H. Schoonbeek, M. Trick, C. Zipfel, and C.J. Ridout. 2014. Methods to Study PAMP-Triggered Immunity in *Brassica* Species. Mol. Plant-Microbe Interact. 27(3):286-295.

Louws, F., M. Wilson, H. Campbell, D. Cuppels, J. Jones, P. Shoemaker, F. Sahin, and S. Miller. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. Plant Dis. 85(5):481-488.

Marco, G. and R. Stall. 1983. Control of bacterial spot of pepper initiated by strains of *Xanthomonas campestris* pv *vesicatoria* that differ in sensitivity to copper. Plant Dis. 67(7):779-781.

Moller, I. 2001. Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu. Rev. Plant Physiol. Plant Mol. Biol. 52:561-591.

Nguyen, H.P., S. Chakravarthy, A.C. Velasquez, H.L. McLane, L. Zeng, H. Nakayashiki, D. Park, A. Collmer, and G.B. Martin. 2010. Methods to Study PAMP-Triggered Immunity Using Tomato and *Nicotiana benthamiana*. Mol. Plant-Microbe Interact. 23(8):991-999.

Nurnberger, T., F. Brunner, B. Kemmerling, and L. Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198:249-266.

Obradovic, A., J. Jones, M. Momol, S. Olson, L. Jackson, B. Balogh, K. Guven, and F. Iriarte. 2005. Integration of biological control agents and systemic acquired resistance inducers against bacterial spot on tomato. Plant Dis. 89(7):712-716.

Pohronezny, K. and R.B. Volin. 1983. The effect of bacterial spot on yield and quality of freshmarket tomatoes. HortScience 18:69-70.

Pohronezny, K., R. Stall, B. Canteros, M. Kegley, L. Datnoff, and R. Subramanya. 1992. Sudden Shift in the Prevalent Race of *Xanthomonas campestris* pv *vesicatoria* in Pepper Fields in Southern Florida. Plant Dis. 76(2):118-120.

Ritchie, D. and V. Dittapongpitch. 1991. Copper-Resistant and Streptomycin-Resistant Strains and Host Differentiated Races of *Xanthomonas campestris* pv *vesicatoria* in North-Carolina. Plant Dis. 75(7):733-736.

Ritchie, D.F. 2000. Bacterial spot of pepper and tomato. *The Plant Health Instructor*. DOI: 10.1094/PHI-I-2000-1027-01 Updated 2007.

Robson, C. and G. Vanlerberghe. 2002. Transgenic plant cells lacking mitochondrial alternative oxidase have increased susceptibility to mitochondria-dependent and -independent pathways of programmed cell death. Plant Physiol. 129(4):1908-1920.

Sagi, M. and R. Fluhr. 2006. Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol. 141(2):336-340.

Scott, J. and J. Jones. 1986. Sources of Resistance to Bacterial Spot in Tomato. HortScience 21(2):304-306.

Scott, J., D. Francis, S. Miller, G. Somodi, and J. Jones. 2003. Tomato bacterial spot resistance derived from PI 114490; Inheritance of resistance to race T2 and relationship across three pathogen races. J. Am. Soc. Hort. Sci. 128(5):698-703.

Scott, J., J. Jones, G. Somodi, and R. Stall. 1995. Screening Tomato Accessions for Resistance to *Xanthomonas campestris* pv *vesicatoria*, Race T3. HortScience 30(3):579-581.

Scott, J.W., R.E. Stall, J.B. Jones, and G.C. Somodi. 1996. A single gene controls the hypersensitive response of Hawaii 7981 to race 3 (T3) of the bacterial spot pathogen. 46:23.

Sherf, A. F. and A. A. MacNab, 1986. Vegetable Diseases and Their Control. Wiley, New York.

Smith, J.M. and A. Heese. 2014. Rapid bioassay to measure early reactive oxygen species production in *Arabidopsis* leave tissue in response to living *Pseudomonas syringae*. Plant Methods 10:6.

Somodi, G., J. Jones, J. Scott, J. Wang, and R. Stall. 1996. Relationship between the hypersensitive reaction and field resistance to tomato race 1 of *Xanthomonas campestris* pv *vesicatoria*. Plant Dis. 80(10):1151-1154.

Stall, R.E. and P.L. Thayer. 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Dis. Rptr. 46:389-392.

Sun, W., F. Dunning, C. Pfund, R. Weingarten, and A. Bent. 2006. Within-species flagellin polymorphism in *Xanthomonas campestris* pv *vesicatoria* and its impact on elicitation of *Arabidopsis* FLAGELLIN SENSING2-dependent defenses. Plant Cell 18(3):764-779.

Thayer, P. and R. Stall. 1961. A survey of *Xanthomonas vesicatoria* resistance to streptomycin.

Torres, M.A., J.D.G. Jones, and J.L. Dangl. 2002. *Arabidopsis* gp91phox homologues AtrbohD and AtrobohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. U. S. A. 99(1):517-522. <http://search.ebscohost.com/login.aspx?direct=true&db=agr&AN=IND23320499+&site=ehost-live&scope=site>.

Wan, J., X. Zhang, D. Neece, K.M. Ramonell, S. Clough, S. Kim, M.G. Stacey, and G. Stacey. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell 20(2):471-481.

Wang, J. 1992. Resistance to *Xanthomonas campestris* pv *vesicatoria* in tomato. PhD Diss. Univ. Florida, Gainsville.

Wang, J., J. Jones, J. Scott, and R. Stall. 1994. Several Genes in *Lycopersicon esculentum* Control Hypersensitivity to *Xanthomonas campestris* pv *vesicatoria*. Phytopathology 84(7):702-706.

Wang, K., H. Li, and J. Ecker. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14:S131-S151.

Whalen, M., J. Wang, F. Carland, M. Heiskell, D. Dahlbeck, G. Minsavage, J. Jones, J. Scott, R. Stall, and B. Staskawicz. 1993. Avirulence Gene *AvrRxv* from *Xanthomonas campestris* pv *vesicatoria* Specifies Resistance on Tomato Line Hawaii-7998. Mol. Plant-Microbe Interact. 6(5):616-627.

Yu, Z., J. Wang, R. Stall, and C. Vallejos. 1995. Genomic Localization of Tomato Genes that Control a Hypersensitive Reaction to *Xanthomonas campestris* pv *vesicatoria* (Doidge) Dye. Genetics 141(2):675-682.

Zipfel, C. and G. Felix. 2005. Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. 8(4):353-360.

<http://search.ebscohost.com/login.aspx?direct=true&db=lah&AN=20053127009&site=ehost-live&scope=site>.

Zipfel, C., G. Kunze, D. Chinchilla, A. Caniard, J. Jones, T. Boller, and G. Felix. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125(4):749-760.

Zipfel, C., S. Robatzek, L. Navarro, E. Oakeley, J. Jones, G. Felix, and T. Boller. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428(6984):764-767.

Table 1.1. Species of *Xanthomonas* causing bacterial spot in tomato, classification of their races and resistant lines for those races

Race	Species	Molecular Phylogeny	Comments	Resistant Germplasm
Race T1	*X. euvesicatoria*	A group strains		Hawaii 7998, PI 114490
Race T2	*X. vesicatoria*	B group strains	possibly also includes *X. perforans*, C group strains; *X. euvesicatoria*, A group strains; and *X. gardneri*, D group strains	PI 114490
Race T3	*X. perforans*	C group strains	possibly also includes *X. gardneri*, D group strains	Hawaii 7981, PI 128216, PI 126932
Race T4	*X. perforans*	C group strains		LA 716, PI 114490
Race T5	*X. perforans*	C group strains		
	X. gardneri	D group strains		

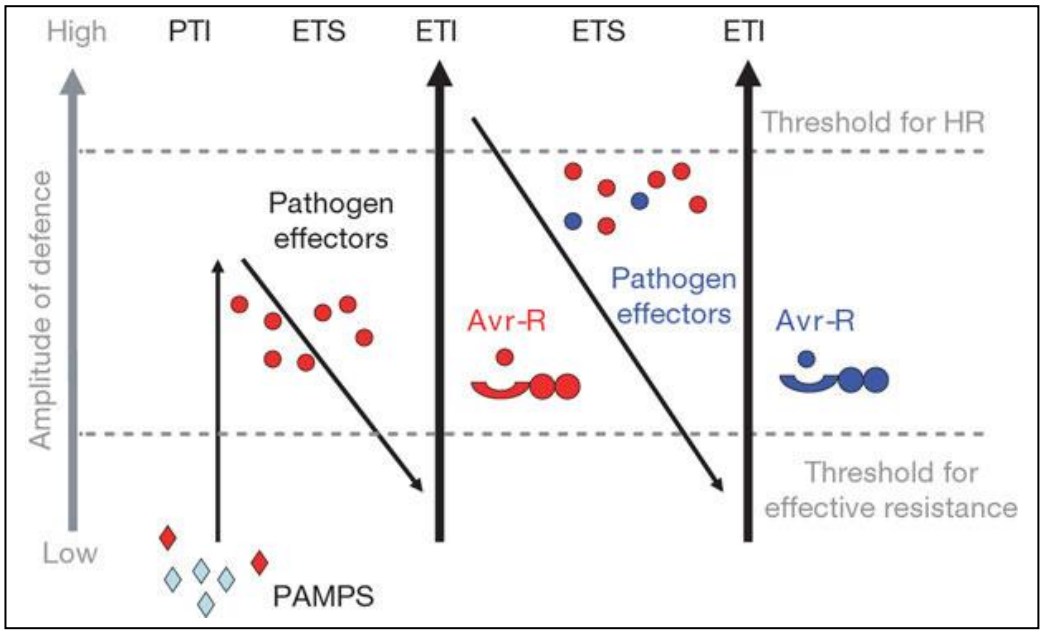


Figure 1.1: A zigzag model illustrates the quantitative output of the plant immune system. In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)—these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI.

(Reproduced from: Jones and Dangl, 2006)

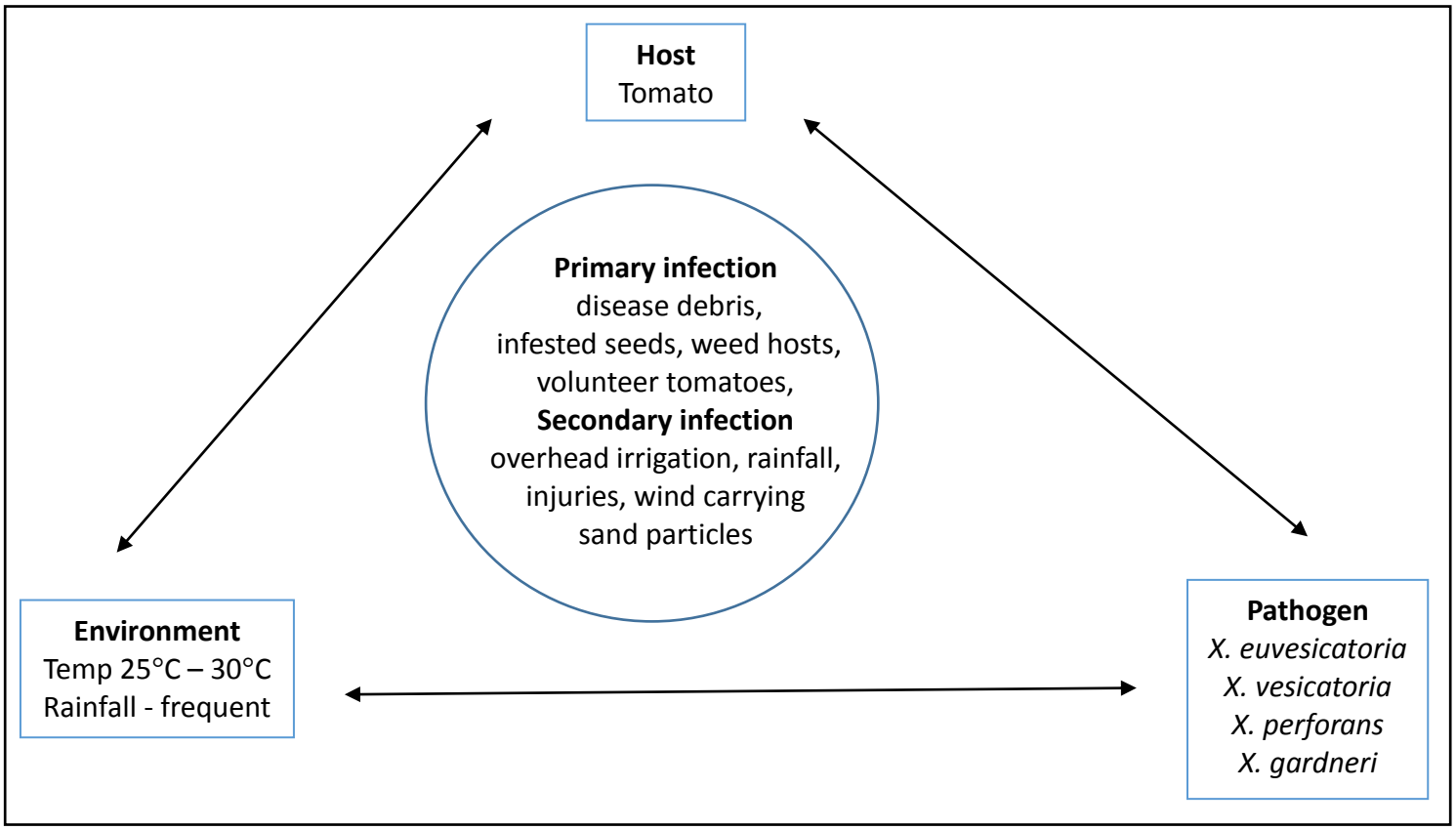


Figure 1.1. Host, pathogen and environmental conditions for bacterial spot development in tomato

CHAPTER 2 SCREENING TOMATO (*SOLANUM LYCOPERSICUM* L.) LINES FOR BACTERIAL SPOT (*XATHOMONAS* SPP.) RESISTANCE IN NORTH CAROLINA

ABSTRACT

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop in the world. Bacterial spot (BS), caused by four species of *Xanthomonas: X. euvesicatoria, X. vesicatoria, X. perforans and X. gardneri*, of tomato results in severe loss in yield and quality by defoliation and appearance of lesions on fruits, respectively. The present commonly used chemical control practices of spraying copper and mancozeb mixes is not effective under conditions of high disease pressure and when the weather conditions are favorable for disease spread. Thus, developing resistance against bacterial spot is a critical priority for the industry in order to minimize crop losses. Sixty three advanced, heirloom and wild tomato lines were screened under greenhouse and field conditions for BS resistance. *Xanthomonas perforans* race T4, was found to be a prevalent strain in North Carolina. Isolate 9, characterized as *X. perforans* race T4 was cultured in yeast dextrose chalk (YDC) agar medium for 24-48 hours at 30°C. Bacterial cultures were diluted with distilled water to achieve a standardized inoculum an optical density of 0.3 at 600 nm (2-5x10^8 CFU/ml) using spectrophotometer and serial dilution. Plants were inoculated by spraying and disease incidence was measured by using the Horsfall and Barratt (1945) scale. Tomato lines 74L-1W(2008), NC2CELBR, 081-12-1X-gsms, NC22L-1(2008) and 52LB-1(98) showed resistance to BS in field and/or greenhouse. These lines were derived from *S. pimpinellifolium* L3707. Screening of L3707 followed by developing a mapping populations and mapping resistance genes might be useful for developing resistance against BS in future breeding programs.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable crop in the world. Bacterial spot (BS), caused by four species of *Xanthomonas: X. euvesicatoria, X. vesicatoria, X. perforans and X. gardneri* (Jones et al., 2000; Jones et al., 2005), in tomato results in severe loss in yield and quality by defoliation and appearance of lesions on fruit (Cox, 1966; Pohronezny and Volin, 1983). BS is a very common disease in warm and humid weather conditions. An optimum temperature of 27°C (Gardner and Kendrick, 1923) and frequent rainfall favor this disease. In Western North Carolina, dew at night, warm summer temperatures and frequent wind-driven rains provide an optimum phyllosphere environment for the growth and spread of the pathogen.

Different methods of controlling BS have been available over the years. Streptomycin was effective for years but development of bacterial resistance to streptomycin has been documented (Lai et al., 1977; Stall and Thayer, 1962). Never-the-less, it remains a core component of an integrated pest management (IPM) program in the NC in transplant production facilities where most strains are sensitive (Louws, personal communication). Present common chemical control practices of spraying copper and mancozeb mixes is not effective if disease pressure is high and when weather conditions are favorable for pathogen spread (Jones et al., 1991b; Jones et al., 1991; Jones and Jones, 1985). In addition, copper resistance in pathogen populations is prevalent. Acibenzolar-S-methyl (Actigard; Syngenta, Basel, Switzerland), originally developed as a fungicide, is helpful to control BS inducing systemic acquired resistance (SAR) (Louws et al., 2001). Bacteriophages are also reported to control BS (Balogh et al., 2003; Flaherty et al., 2000).

Five races (T1 to T5) have been recognized based on induction of hypersensitive responses (HR) on differential lines of host genotypes (Jones et al., 2005; Jones et al., 2000; Jones et al., 2004). Hawaii 7998 (H7998) produced an HR against *X. euvesicatoria* race T1 (Jones and Scott, 1986) and the nature of resistance was reported to be multigenic (Wang et al., 1994; Whalen et al., 1993; Yu et al., 1995) and on additional genetic factors because of the low correlation between HR and field resistance (Somodi et al., 1996; Wang, 1992). PI114490 exhibited a non-hypersensitive resistance to *X. vesicatoria* race T2, with resistance being reported as additive and controlled by two genes (Scott et al., 2003). *X. perforans* race T3 has an antagonistic effects against race T1 in *in-vitro* experiments (Jones et al., 1998). Race T3 has displaced race T1 in most production fields and overcame the hypersensitivity of H7998 (Jones et al., 1998). Hawaii 7981 (H7981), PI 126932 and PI128216 are reported to show HR against race T3 (Scott et al., 1995). The resistance of H7981 was known to be controlled by an incompletely dominant gene *Xv3* (Scott et al., 1996). Currently *X. perforans* race T4 is the predominant race found in the fields of North Carolina (Louws et al., unpublished data). LA716 exhibited HR against race T4 (Astua-Monge et al., 2000).

Cultivated tomato (*Solanum lycopersicum*) does not have effective durable resistance against BS. Tomato breeding programs have been trying to identify BS resistant genes in wild relatives and incorporate them into advanced cultivated tomato selections. However, it has been difficult to develop resistance to all known races of BS in tomato lines that also have desired horticultural traits. Resistance development for BS is difficult due to the apparent multigenic nature of resistance and the limited availability of resistance. The pathogen is able to overcome host resistance due to mutations that alter the race, emergence of new races

already present in the field, or migration of new races from other regions of the world. The objective of this study was to screen a diversity of tomato lines and to identify potential sources of resistance against the predominant strain of BS in North Carolina. This chapter reports the results of both greenhouse and field evaluations of tomato lines for race T4 resistance.

MATERIALS AND METHODS

Plant materials and plant growth

Sixty three tomato genotypes including lines from tomato breeding programs of North Carolina State University and University of Florida, heirlooms and wild lines (Table 2.1) were sown in 4P soil mixture (Fafard®, Florida, USA) in 24-cell flat trays for a greenhouse experiment in March, 2013 at the Method Road Greenhouse, North Carolina State University, Raleigh, NC. Six plants per genotype were planted in three replications in a completely randomized design. Plants in the greenhouse study were fertilized using a 20:20:20 ratio of nitrogen, phosphorus and potassium respectively. Standard greenhouse spraying for insects and fungal diseases (powdery mildew) were followed whereas copper was not applied. For the field experiment, sixty three genotypes were sown in flat bed metal trays in a standard seeding mix (2:2:1 (v/v/v) peat moss:pine bark:vermiculite with macro- and micro-nutrients (Van Wingerden International Inc., Mills River, NC) on May, 2013. After 10 days, seedlings were transplanted to 72-cell flats (56 cm X 28cm). After four weeks these plants were transplanted to the field at the Mountain Horticultural Crops Research and Extension Center, Mills River, NC (Figure 2.1). Six plants per plot were planted with plant to plant spacing of 45 cm and 150 cm distance between rows in two replications in a

randomized complete block design. The recommended management practices for fertilization, insect management and management of foliar fungal diseases were done according to standard recommendations (Ivors and Louws, 2013). Copper was not sprayed on the crop.

Inoculum preparation and inoculation

Plants in both the greenhouse and field studies were artificially inoculated with Isolate 9 (Figure 2.2), isolated from infected tissue in a field in North Carolina and characterized as *X. perforans* race T4 (by Dr. Jefferey B. Jones lab, University of Florida, Gainesville, Florida). The description of characterized isolates is given in Table 2.2. The strain was obtained in pure culture and stored at -80°C. Bacteria stored at -80°C was grown in Yeast Dextrose Chalk (YDC) agar medium (Lelliott and and Stead, 1987) for 24-48 hours at 28°C. Distilled water was poured over the culture plates and bacterial cells were suspended. Bacterial suspension was standardized at 0.3 optical density by using a LKB Biochrom Ultrospec II spectrophotometer (American Laboratory Training, USA) absorbance at 600 nm which is approximately $2\text{-}5\times10^{8}$ CFU/ml, as previously used by Hutton et al. (2010) and immediately used for inoculations.

For greenhouse inoculations, humidity in the immediate vicinity of the plants was maintained by using V5100NS humidifiers (Vicks Ultrasonic Humidifiers, Hudson, NY, USA) from 24 hours prior to the inoculation to 48 hours after inoculation and covering plants with white plastic. Spray inoculation to runoff from the foliage was performed on the plants 30 days after sowing using a hand sprayer. A second inoculation was done 15 days after the first inoculation and a third inoculation was done after another 15 days following the same

procedure. The first and second scorings were done after 15 and 21 days of the first inoculation and a scoring was done 30 days after the third inoculation.

In the field, spray inoculation was done uniformly on all plants using a backpack sprayer 30 days after transplanting. Plants were scored beginning 10 days post inoculation and every week for five weeks.

Disease evaluation

Greenhouse plants were scored for foliage symptoms using the most severely infected leaves of the plant through a modified use of the Horsfall-Barratt scale (1945) where 0% = 1, 1-3% = 2, 3-6% = 3, 6-12% = 4, 12-25% = 5, 25-50% = 6, 50-75% = 7, 75-87% = 8, 87-94% = 9, 94-97% = 10, 97-100% = 11 and 100% dead tissue = 12 (Figure 2.3).

Plants were scored in the field using the same scale as used in the greenhouse, except complete foliage was rated rather than the severity of infected leaves. Disease severity on the fruit was rated one time after the last foliar disease rating. Fruit disease incidence was rated by counting the number of BS lesions on ten severely infected fruits of a most infected plant among the six plants of a genotype.

Statistical analyses

Area under the disease progress curve (AUDPC) was calculated (Campbell and Madden, 1990) and used in further analysis. Analyses of variance were performed on AUDPC disease scores from the greenhouse and the field data using the GLM procedure from SAS Software version 9.3 (SAS Institute, Inc., Cary, NC). Least Squares of Means were calculated and lines were separated using LSD at $P = 0.05$ levels. Disease severity on fruits was calculated by counting total number of lesions on ten severely infected fruits of severely infected plant of

each plot. Disease incidence on fruits was calculated by counting the number of lesions on severely infected fruit of each plot. Correlation analysis was done using the Pearson product-moment correlation coefficient (Pearson, 1985).

RESULTS

Due to favorable temperatures and frequent rainfall during the field season, there was very high BS disease pressure in the field. Disease symptoms were seen on all foliar parts of a plant (Figure 2.4). Analysis of variance (ANOVA) of the greenhouse study revealed significant ($P<0.0001$) differences between the tomato lines with respect to BS disease scores (Table 2.3). The coefficient of determination (R-squared) and coefficient of variance (CV) were 0.59 and 19.12 respectively (Table 2.3). ANOVA of the field study demonstrated that there were significant differences among tomato lines for foliar ($P<0.0001$) and fruit disease scores ($P<0.0001$) (Table 2.3). The coefficient of determination for foliar and fruit disease scores were 0.85 and 0.76, respectively and the CV values were 6.18 and 70.38, respectively (Table 2.3). Separation of the means based on Tukey comparison are shown in Table 2.4. Least squares (LS) means of the AUDPC values based on BS disease scores of tomato lines in the greenhouse and field studies ranged from 15.33 to 40.52 with a standard error (SE) of 3.03 (Table 2.4) and 18.71 to 31.82 with SE of 1.14 (Table 2.4). LS means of BS disease score on fruits expressed as total number of lesions per ten fruits per plant in the field study ranged from 0.5 to 71 with a SE of 7.82. The AUDPC values of highly resistant and susceptible lines in the field experiment are shown in Figure 2.5.

Tomato lines G357-2(2011), PI114490-1-1, Favorite, FLA7600, 52LB-1(98), NC50-7, 30LB-1W were found to have high level of resistance in both greenhouse and field

conditions. These lines had lower AUDPC scores than 25 under both environmental conditions. In contrast, some lines exhibited resistance in greenhouse conditions but had equal to or above 25 AUDPC score under field conditions or vice-versa for instance line 47NC2 had the AUDPC score of 21.90 in the greenhouse condition whereas in field it had 31.82 and line NC22L-1 had an AUDPC score of 40.52 in the greenhouse however it had 23.08 in the field condition. Tomato lines 74L-1W(2008), 081-12-1X-gsms and NC22L-1(2008) did not show any resistance in the greenhouse experiment but appeared to be resistant in the field. AUDPC scores of highly resistance and susceptible lines in the field and greenhouse experiments are shown in figure 2.5 and 2.6 respectively.

Tomato lines that had above AUDPC score values of 27 or greater exhibited severe symptoms on foliage. Cherokee Purple, Stupice, 48BC-1(96), IRAT-L3, 38BC-2R(96), CRA66, 39BC-1(96), 38BC-1(96), Oxheart, NC714, PI134417, NC84173, NCEBR-8, NC123S and Aker's West Virginia were highly susceptible in both studies. Pearson correlation between the greenhouse disease score and field foliar BS disease score was relatively low (r=0.27) but significant (*P*=0.0293) (Table 2.5).

Tomato lines PI114490-1-1, Favorite and NC1CS were found to be the most resistant in the greenhouse study, whereas G357-2(2011) and 74L-1W(2008) with an AUDPC score of 18.71 and 19.9, respectively, were the most resistant lines in the field study. PI114490-1-1, which was most resistant line in the greenhouse study ranked fifth in resistance in the field study.

Average number of BS lesions on ten fruits per severely infected plant per plot ranged from 0.5 to 71. Genotypes 081-12-1x-gsms, 52LB-3(98) and Oxheart had on an average of 0.5

lesions and wild genotypes PI114490-1-1 and PI134417 had on average 1 lesion. Genotypes 15BC-4, 97E-2W(95), 38BC-2R(96) and 16BC-1(94) had the highest number of BS lesions: 43, 47.5, 53 and 71 respectively (Figure 2.7).

BS disease scores on fruits was missing for tomato lines NC1CS, NC1CELBR and NC714 but they were present in greenhouse and field study for foliar disease scores. 081-12-1X-gsms and PI114490-1-1 ranked second and fifth respectively in having the least number of BS disease score on fruits. PI114490-1-1 and 081-12-1X-gsms ranked fifth and seventh in having the least foliar disease score in field respectively and PI114490-1-1 was the most resistant line in the greenhouse study.

AUDPC values based on BS symptoms on foliage in the field did not correlate with the BS score on fruits (-10.88%) (Figure 2.8).

DISCUSSION

Significant BS disease symptoms were seen on fruits. Images of fruits of tomato genotypes resistant and susceptible to BS are shown in Figure 2.9. Generally, the appearance of fruit symptoms occurs when there is high disease pressure accompanied by moisture to maintain high mobility of the bacteria. When fruit hairs fall from the fruit, there is an injury or opening left for the bacteria to enter through the fruit surface and cause upraised black lesions (Louws, personal communication).

It is difficult to get secondary infection by bacterial spot disease in the greenhouse because a greenhouse is a closed chamber that restricts air movement, rain, and dew at night. Air entering the greenhouse is filtered. This removes dust and sand particles that are present in unfiltered air that are capable of causing injuries to plant tissues through which bacteria can

enter inside the plant. Lack of secondary infection makes it difficult to get full plant symptoms and thus score the whole plant using the Horsfall-Barratt scale. Hence for greenhouse-grown plants, only the most severely infected leaf of each line was scored using the Horsfall-Barratt scale.

Low correlation between greenhouse and field likely to be due largely to the difference in disease pressure and scoring method used in the two studies. Quantitative scoring of bacterial spot in greenhouse using Horsfall-Barratt scale is challenging. HR scoring is thus recommended for screening tomato lines in greenhouse studies. Somodi et al. (1994) scored HR and/or small and few lesions in greenhouse condition to rate the disease severity. Significant ($p<0.001$) correlation coefficients of 0.39 and 0.41 were also reported between HR in greenhouse and field BS disease score by Wang (1992). Although correlation between greenhouse and field disease scores were low, 0.27, this result is similar to results reported by Somodi et al. (1994) which showed a correlation between cotyledon-dip seedlings and field plants disease scores to be in between 0.28 to 0.34.

The total lack of correlation between foliar and fruit disease scores in the field experiment was unexpected. Different genes regulating the resistance of bacterial spot in fruits of tomato may be one of the reasons for this observation. Another possibility could be a difference in mechanisms of infection or mode of disease development on these plant parts. Further studies on the genetic basis or mode of infection may provide more information on this subject. High correlation between fruit disease incidence and severity indicates that any one of these disease ratings can be used to rate the disease symptoms on fruits.

Some genotypes, 74L-1W(2008), NC2CELBR, 081-12-1X-gsms, NC22L-1(2008) and 52LB-1(98), that showed some resistance for bacterial spot, have *S. pimpinellifolium* L3707 in their pedigrees (Figure 2.10 and 2.11). Using L3707 to develop a mapping population and map for the resistant genes would lead in identifying the genes for bacterial spot disease resistance. L3707 is also believed to have resistance to race T3 (Gardner, personal communication). *S. pimpinellifolium* L3707 could be screened to other races of *Xanthomonas* to identify if it has horizontal resistance.

The bacterial spot pathogen is phenotypically, biochemically and genetically diversified. New races are frequently identified before resistance to already existing races can be identified and incorporated into desired cultivars. Breeding programs are focused on developing resistance to specific races. Instead, future research should focus on developing horizontal resistance on tomato cultivars with a broad resistance. This can be a long term goal but will also prevent significant losses being caused by the disease at present.

REFERENCES

Astua-Monge, G., G.V. Minsavage, R.E. Stall, C.E. Vallejos, M.J. Davis, and J.B. Jones. 2000. Xv4-vrxv4: a new gene-for-gene interaction identified between *Xanthomonas campestris* pv. *vesicatoria* race T3 and the wild tomato relative *Lycopersicon pennellii*. Mol. Plant-Microbe Interact. 13(12):1346-1355.

Balogh, B., J. Jones, M. Momol, S. Olson, A. Obradovic, P. King, and L. Jackson. 2003. Improved efficacy of newly formulated bacteriophages for management of bacterial spot on tomato. Plant Dis. 87(8):949-954.

Campbell, C. L. and L. V. Madden. 1990. Introduction to Plant disease epidemiology. John Wiley and Sons, New York, USA.

Cox, R.S. 1966. The role of bacterial spot on tomato production in south Florida. Plant Dis. Rptr. 50:699-700.

Flaherty, J., J. Jones, B. Harbaugh, G. Somodi, and L. Jackson. 2000. Control of bacterial spot on tomato in the greenhouse and field with h-mutant bacteriophages. HortScience 35(5):882-884.

Gardner, M.W. and J.B. Kendrick. 1923. Bacterial spot of Tomato and Pepper. Phytopathology 13(7):307-315 pp.

Horsfall, J. G., and R. W. Barratt. 1945. An improved grading system for measuring plant diseases. Phytopathology 35: 655.

Hutton, S.F., J.W. Scott, and J.B. Jones. 2010. Inheritance of resistance to bacterial spot race T4 from three tomato breeding lines with differing resistance backgrounds. J. Am. Soc. Hort. Sci. 135(2):150-158.

Ivors K. L. and Louws F. J. 2013. 2013 North Carolina Agricultural Chemicals Manual. College of Agriculture and Life Sciences, North Carolina State University.

Jones, J.B., S.S. Woltz, R.O. Kelly, and G. Harris. 1991b. The role of ionic copper, total copper, and select bactericides on control of bacterial spot of tomato. Proc. Florida. State Hort. Soc. 104:257-258, 259.

Jones, J., S. Woltz, J. Jones, and K. Portier. 1991. Population dynamics of *Xanthomonas campestris* pv. *vesicatoria* on tomato leaflets treated with copper bactericides. Phytopathology 81(7):714-719.

Jones, J.B. and J.P. Jones. 1985. The effect of bactericides, tank mixing time and spray schedule on bacterial leaf spot of tomato. Proceedings of the Florida State Horticultural Society 98:244-247.

Jones, J.B., G.H. Lacy, H. Bouzar, G.V. Minsavage, R.E. Stall, and N.W. Schaad. 2005. Bacterial spot - worldwide distribution, importance and review. Acta Horticulturae (695):27-33.

Jones, J., H. Bouzar, G. Somodi, R. Stall, K. Pernezny, G. El-Morsy, and J. Scott. 1998. Evidence for the preemptive nature of tomato race 3 of *Xanthomonas campestris* pv. *vesicatoria* in Florida. Phytopathology 88(1):33-38.

Jones, J., H. Bouzar, R. Stall, E. Almira, P. Roberts, B. Bowen, J. Sudberry, P. Strickler, and J. Chun. 2000. Systematic analysis of xanthomonads (*Xanthomonas spp.*) associated with pepper and tomato lesions. Int. J. Syst. Evol. Microbiol. 50:1211-1219.

Jones, J., R. Stall, J. P. Jones, and K. Phoronezny. 1984. Survival of *Xanthomonas campestris* pv. *vesicatoria* in Florida. Phytopathology 74(7):858-858.

Jones, J., R. Stall, J. Scott, G. Somodi, H. Bouzar, and N. Hodge. 1995. A third tomato race of *Xanthomonas campestris* pv. *vesicatoria*. Plant Dis. 79(4):395-398.

Jones, J., G. Lacy, H. Bouzar, R. Stall, and N. Schaad. 2004. Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. Syst. Appl. Microbiol. 27(6):755-762.

Jones, J. and J. Scott. 1986. Hypersensitive Response in Tomato to *Xanthomonas campestris* pv *vesicatoria*. Plant Dis. 70(4):337-339.

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

Lai, M., N. Panopoulos, and S. Shaffer. 1977. Transmission of R-Plasmids among *Xanthomonas spp* and Other Plant Pathogenic Bacteria. Phytopathology 67(8):1044-1050.

Lelliott, R.A. and D.E. and Stead. 1987. Methods for the diagnosis of bacterial diseases of plants. In: Methods in Plant Pathology. Vol. 2 ed. T.F. Preece Series, British Society of Plant Pathology, Blackwell Scientific Publications, Oxford.

Louws, F., M. Wilson, H. Campbell, D. Cuppels, J. Jones, P. Shoemaker, F. Sahin, and S. Miller. 2001. Field control of bacterial spot and bacterial speck of tomato using a plant activator. Plant Dis. 85(5):481-488.

Pearson, K. 1895. Notes on regression and inheritance in the case of two parents. Proceedings of the Royal Society of London. 58 : 240–242.

Pohronezny, K. and R.B. Volin. 1983. The effect of bacterial spot on yield and quality of freshmarket tomatoes. HortScience 18:69-70.

Scott, J.W., R.E. Stall, J.B. Jones, and G.C. Somodi. 1996. A single gene controls the hypersensitive response of Hawaii 7981 to race 3 (T3) of the bacterial spot pathogen. 46:23.

Scott, J., D. Francis, S. Miller, G. Somodi, and J. Jones. 2003. Tomato bacterial spot resistance derived from PI 114490; Inheritance of resistance to race T2 and relationship across three pathogen races. J. Am. Soc. Hort. Sci. 128(5):698-703.

Scott, J., J. Jones, G. Somodi, and R. Stall. 1995. Screening Tomato Accessions for Resistance to *Xanthomonas campestris* pv *vesicatoria*, Race T3. HortScience 30(3):579-581.

Somodi, G., J. Jones, J. Scott, and J. Jones. 1994. Screening Tomato Seedlings for Resistance to Bacterial Spot. HortScience 29(6):680-682.

Somodi, G., J. Jones, J. Scott, J. Wang, and R. Stall. 1996. Relationship between the hypersensitive reaction and field resistance to tomato race 1 of *Xanthomonas campestris* pv *vesicatoria*. Plant Dis. 80(10):1151-1154.

Stall, R.E. and P.L. Thayer. 1962. Streptomycin resistance of the bacterial spot pathogen and control with streptomycin. Plant Dis. Rptr. 46:389-392.

Wang, J. 1992. Resistance to *Xanthomonas campestris* pv. *vesicatoria* in tomato. PhD Diss. Univ. Florida, Gainsville.

Wang, J., J. Jones, J. Scott, and R. Stall. 1994. Several Genes in *Lycopersicon esculentum* Control Hypersensitivity to *Xanthomonas campestris* pv *vesicatoria*. Phytopathology 84(7):702-706.

Whalen, M., J. Wang, F. Carland, M. Heiskell, D. Dahlbeck, G. Minsavage, J. Jones, J. Scott, R. Stall, and B. Staskawicz. 1993. Avirulence Gene Avrrxv from *Xanthomonas campestris* pv *vesicatoria* Specifies Resistance on Tomato Line Hawaii-7998. Mol. Plant-Microbe Interact. 6(5):616-627.

Yu, Z., J. Wang, R. Stall, and C. Vallejos. 1995. Genomic Localization of Tomato Genes that Control a Hypersensitive Reaction to *Xanthomonas campestris* pv *vesicatoria* (Doidge) Dye. Genetics 141(2):675-682.

Table 2.1. List of tomato lines evaluated for bacterial spot disease resistance in greenhouse (Method Rd., Raleigh, NC) and field (Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, NC, studies in 2013

Genotypes	Scientific name	Comment	Source
NC714	*Solanum lycopersicum*	Breeding line	NCSU
081-12-1X-gsms	*S. lycopersicum*	Breeding line	NCSU
15BC-4(94)	*S. lycopersicum*	Breeding line	NCSU
16BC-1(94)	*S. lycopersicum*	Breeding line	NCSU
16BC-2(94)	*S. lycopersicum*	Breeding line	NCSU
17BC-1(94)	*S. lycopersicum*	Breeding line	NCSU
30LB-1W(95)	*S. lycopersicum*	Breeding line	NCSU
31LB-1W(95)	*S. lycopersicum*	Breeding line	NCSU
38BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
38BC-2R(96)	*S. lycopersicum*	Breeding line	NCSU
39BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
45LB-1	*S. lycopersicum*	Breeding line	NCSU
46BC-2R(96)	*S. lycopersicum*	Breeding line	NCSU
47NC2	*S. lycopersicum*	Breeding line	NCSU
48BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-1R(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-3R(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-4R(96)	*S. lycopersicum*	Breeding line	NCSU
52LB-1	*S. lycopersicum*	Breeding line	NCSU
52LB-2	*S. lycopersicum*	Breeding line	NCSU
52LB-3	*S. lycopersicum*	Breeding line	NCSU
52LB-4	*S. lycopersicum*	Breeding line	NCSU
71BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
72E-1(96)	*S. lycopersicum*	Breeding line	NCSU
74L-1W(2008)	*S. lycopersicum*	Breeding line	NCSU
87E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
89E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-2W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-3W(95)	*S. lycopersicum*	Breeding line	NCSU
AkersWestVirginia	*S. lycopersicum*	Heirloom	
BlackfromTula	*S. lycopersicum*	Heirloom	
Brandywine	*S. lycopersicum*	Heirloom	
CherokeePurple	*S. lycopersicum*	Heirloom	

Table 2.1. Continued

CRA66	*S. lycopersicum*	Breeding line	France
Favorite	*S. lycopersicum*	Breeding line	
FD502-3-BK	*S. lycopersicum*	Breeding line	NCSU
Fla7600	*S. lycopersicum*	Breeding line	UFL
Fla8000	*S. lycopersicum*	Breeding line	UFL
Fla8233	*S. lycopersicum*	Breeding line	UFL
G357-1(2011)	*S. lycopersicum*	Breeding line	
G357-2(2011)	*S. lycopersicum*	Breeding line	
HI7981	*S. lycopersicum*	Breeding line	UFL
HI7997	*S. lycopersicum*	Breeding line	UFL
HI7998	*S. lycopersicum*	Breeding line	UFL
IRAT-L3	*S. lycopersicum*	Breeding line	France
Moneymaker	*S. lycopersicum*	Heirloom	
NC109	*S. lycopersicum*	Breeding line	NCSU
NC123S	*S. lycopersicum*	Breeding line	NCSU
NC161L-1W(2007)	*S. lycopersicum*	Breeding line	NCSU
NC1CELBR	*S. lycopersicum*	Breeding line	NCSU
NC1CS	*S. lycopersicum*	Breeding line	NCSU
NC22L-1(2008)	*S. lycopersicum*	Breeding line	NCSU
NC2CELBR	*S. lycopersicum*	Breeding line	NCSU
NC50-7	*S. lycopersicum*	Breeding line	NCSU
NC84173	*S. lycopersicum*	Breeding line	NCSU
NCEBR-6	*S. lycopersicum*	Breeding line	NCSU
NCEBR-8	*S. lycopersicum*	Breeding line	NCSU
OrangeStrawberry	*S. lycopersicum*	Heirloom	
Oxheart	*S. lycopersicum*	Heirloom	
PI114490-1-1	*S. lycopersicum var. cerasiforme*	Wild	USDA
PI134417	*S. habrochaites*	Wild	USDA
Rutgers	*S. lycopersicum*	Breeding line	Rutgers
Stupice	*S. lycopersicum*	Heirloom	
YellowPear	*S. lycopersicum*	Heirloom	
YellowStuffer	*S. lycopersicum*	Heirloom	

Table 2.2. Race characterization of representative isolates from different counties of North Carolina. It was characterized by Dr. Jeffery B. Jones Lab, Department of Plant Pathology, University of Florida, FL

Isolate	County	Species	Race
Isolate 1	Henderson	*Xanthomonas perforans*	T4
Isolate 2	Henderson	*X. perforans*	T4
Isolate 4	Henderson	*X. perforans*	T4
Isolate 8	Henderson	*X. perforans*	T4
Isolate 9	Guilford	*X. perforans*	T4
Isolate 10	Sampson	*X. perforans*	T4
Isolate 11	Columbus	*X. euvesicatoria*	T1

Table 2.3. Analysis of variance, coefficient of variance (CV) and coefficient of determination (R-squared) of greenhouse bacterial spot disease score, field bacterial spot foliar and fruit disease scores

Trait	Mean square	F value	R-square	CV
Greenhouse disease score	79.12	2.88***	0.589	19.1
Field foliar disease score	14.56	5.64***	0.847	6.2
Field fruit disease score	401.16	3.28***	0.763	70.4

*** Significant at level of 0.0001 probability

Table 2.4. Tukey-Kramer pairwise comparison of tomato genotypes derived from various genetic background for bacterial spot based on foliar bacterial spot disease severity under greenhouse and field conditions, and bacterial spot disease severity on fruits from field experiment of tomato genotypes in 2013. The table presents the least square means of area under disease progress curve values.

Genotype	Bacteria spot disease severity[w]		
	Greenhouse foliar[p]	Field foliar[p]	Field fruits[q]
081-12-1X-gsms	27.4a-d	22.7 e-k	0.5c
15BC-4(94)	28.5 a-d	24.1 c-k	43.0 a-c
16BC-1(94)	32.9 a-d	25.2 a-k	71.0 a
16BC-2(94)	32.9 a-d	26.6 a-j	37.0 a-c
17BC-1(94)	32.9 a-d	27.0 a-i	33.0 a-c
30LB-1W(95)	20.8 cd	24.5 b-k	21.0 bc
31LB-1W(95)	27.4 a-d	26.6 a-j	17.5 bc
38BC-1(96)	30.7a-d	28.4 a-g	19.5 bc
38BC-2R(96)	28.5 a-d	28.2 a-g	53.0 ab
39BC-1(96)	35.0 a-c	28.3 a-g	10.5 bc
45LB-1	32.9 a-d	26.1 a-j	8.5 bc
46BC-2R(96)	27.4 a-d	24.6 b-k	38.0 a-c
47NC2	21.9 b-d	31.8a	5.0 c
48BC-1(96)	35.0 a-c	28.0 a-g	29.5 a-c
48BC-1R(96)	27.4 a-d	24.9 a-k	10.5 bc
48BC-3R(96)	25.2 a-d	25.1 a-k	28.5 a-c
48BC-4R(96)	26.3 a-d	24.3 b-k	26.0 a-c
52LB-1	23.0 a-d	24.1 c-k	5.0 c
52LB-2	24.1 a-d	26.0 a-j	2.0 c
52LB-3	27.4 a-d	26.5 a-j	0.5 c
52LB-4	30.7 a-d	26.8 a-j	7.0 bc
71BC-1(96)	36.1 a-c	26.8 a-j	36.0 a-c
72E-1(96)	29.6 a-d	26.3 a-j	26.0 a-c
74L-1W(2008)	29.6 a-d	19.9 jk	9.5 bc
87E-1W(95)	28.5 a-d	23.4 e-k	22.0 bc

Table 2.4. Continued

89E-1W(95)	28.5 a-d	23.4 e-k	21.5 bc
97E-1W(95)	25.2 a-d	24.8 b-k	18.5 bc
97E-2W(95)	26.3 a-d	20.8 h-k	47.5 a-c
97E-3W(95)	31.8 a-d	26.5 a-j	23.0 bc
AkersWestVirginia	29.6 a-d	31.1ab	20.0 bc
BlackfromTula	25.2 a-d	27.2 a-i	20.0 bc
Brandywine	26.3 a-d	28.0 a-g	7.5 bc
CherokeePurple	30.7 a-d	27.9 a-g	3.0 c
CRA66	29.6 a-d	28.3 a-g	17.5 bc
Favorite	15.3 d	23.5 d-k	7.0 bc
FD502-3-BK	31.8 a-d	26.0 a-j	7.5 bc
Fla7600	24.1 a-d	23.5 d-k	15.5 bc
Fla8000	32.9 a-d	26.2 a-j	17.0 bc
Fla8233	19.7 cd	24.9 a-k	16.0 bc
G357-1(2011)	25.2 a-d	20.4 i-k	12.5 bc
G357-2(2011)	23.0 a-d	18.7 k	18.0 bc
HI7981	23.0 a-d	25.8 a-j	7.0 bc
HI7997	30.7 a-d	26.5 a-j	7.0 bc
HI7998	23.0 a-d	25.9 a-j	4.0 c
IRAT-L3	28.5 a-d	28.2 a-g	2.5 c
Moneymaker	26.3 a-d	26.0 a-j	14.0 bc
NC109	23.0 a-d	25.4 a-k	1.5 c
NC123S	28.0 a-d	30.9a-c	3.5 c
NC161L-1W(2007)	23.0 a-d	27.6 a-h	4.5 c
NC22L-1(2008)	40.5 a	23.1 e-k	2.5 c
NC2CELBR	29.6 a-d	22.2 f-k	9.0 bc
NC50-7	18.6 cd	24.4 b-k	5.5 bc
NC84173	39.4 ab	30.4 a-d	6.0 bc
NCEBR-6	25.2 a-d	29.5 a-e	8.0 bc
NCEBR-8	28.5 a-d	30.8 a-c	10.5 bc

Table 2.4. Continued

OrangeStrawberry	26.3 a-d	28.4 a-g	23.5 a-c
Oxheart	35.0 a-c	28.7 a-g	0.5 c
PI114490-1-1	15.3 d	21.7 g-k	1.0 c
PI134417	27.4 a-d	29.0 a-f	1.0 c
Rutgers	23.0 a-d	26.6 a-j	25.0 a-c
Stupice	30.7 a-d	27.9 a-g	13.0 bc
YellowPear	20.8 cd	25.9 a-j	4.5 c
YellowStuffer	27.4 a-d	24.0 c-k	14.5 bc

[p]Least squares means of area under disease progress curve (AUDPC) scores based on Horsfall-Barratt (1945) scale. Least Square means with the same letter are not significantly different from each other. AUDPC was calculated based on five different scores in the field, three scores from the greenhouse and one score on fruits from the field
[q]Disease severity measured by counting total number of bacterial spot lesions on ten severely infected fruits from individual plant of each genotype
[w]Significant at <0.05 level of probability respectively
Greenhouse, field and fruit disease severities had the standard error of 3.03, 1.14 and 7.82 respectively

Table 2.5. Pearson correlation coefficient of Greenhouse and field disease studies in 2013

	Greenhouse_AUDPC	Field_AUDPC
Field AUDPC	0.26*	
Fruit disease score	0.21^{NS}	-0.11^{NS}

*, ^{NS} Significant at level of 0.01 probability and non-significant, respectively

Figure 2.1. Field experimental trial at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759 in 2013

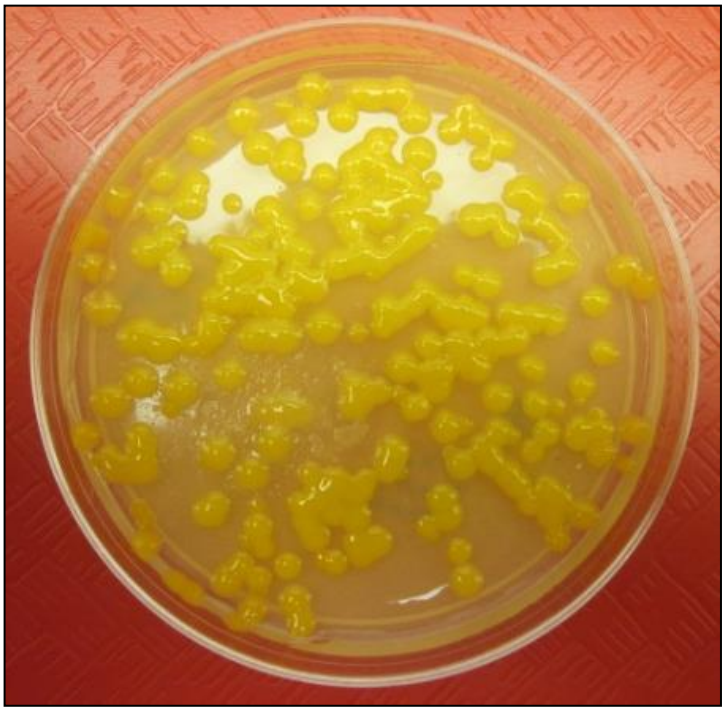


Figure 2.2. *Xanthomonas perforans*, characterized as race T4, culture in Yeast Dextrose Chalk (YDC) Agar medium

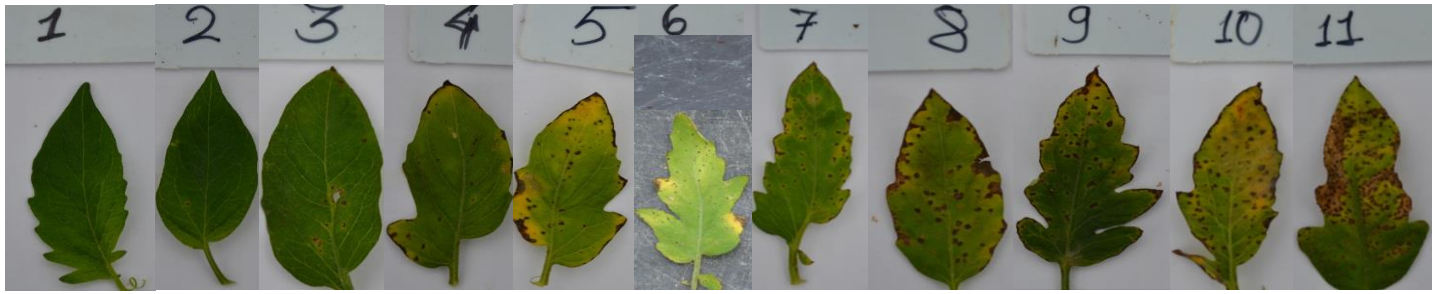


Figure 2.3. Modified Horsfall and Barrat scale used in scoring disease incidence in greenhouse experiment at Method Greenhouse, Raleigh, North Carolina, 26759

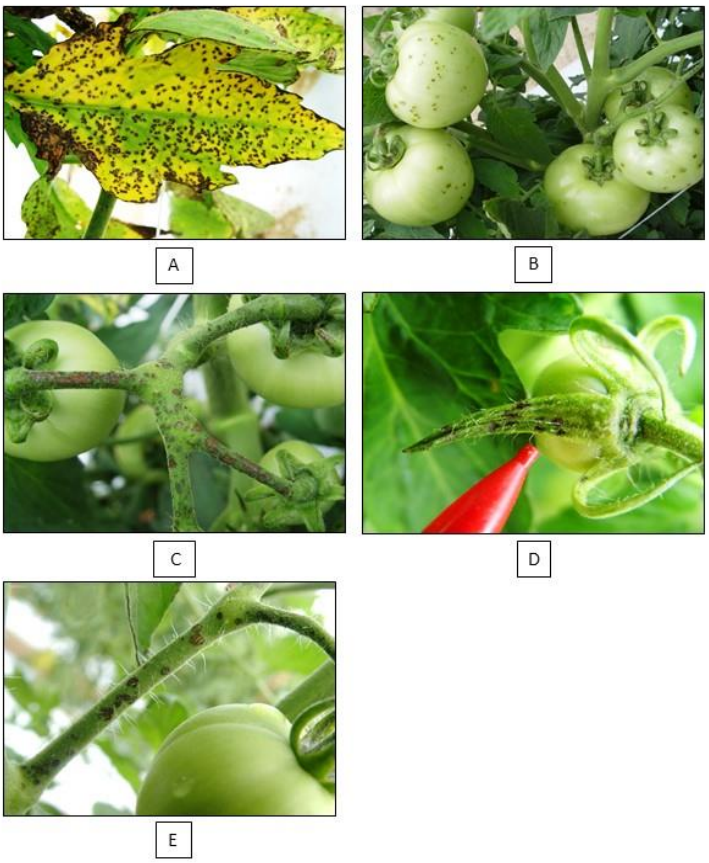


Figure 2.4. Bacterial spot disease symptoms seen on tomato foliar parts for instance A. leaf, B. fruits, C. Peduncles, D. calyx and E. stem in the field experiment at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759

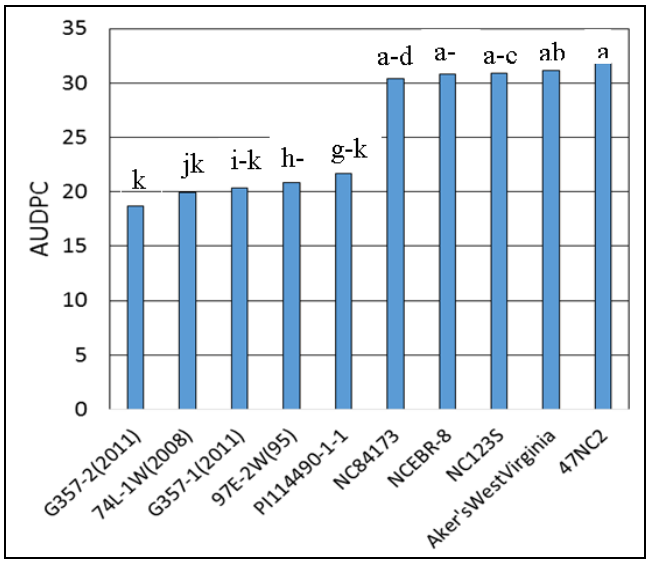


Figure 2.5. Foliar Area under disease progress curve of Bacterial Spot disease severity in the field experiment at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759 in 2013. The most susceptible and most resistant lines are shown.

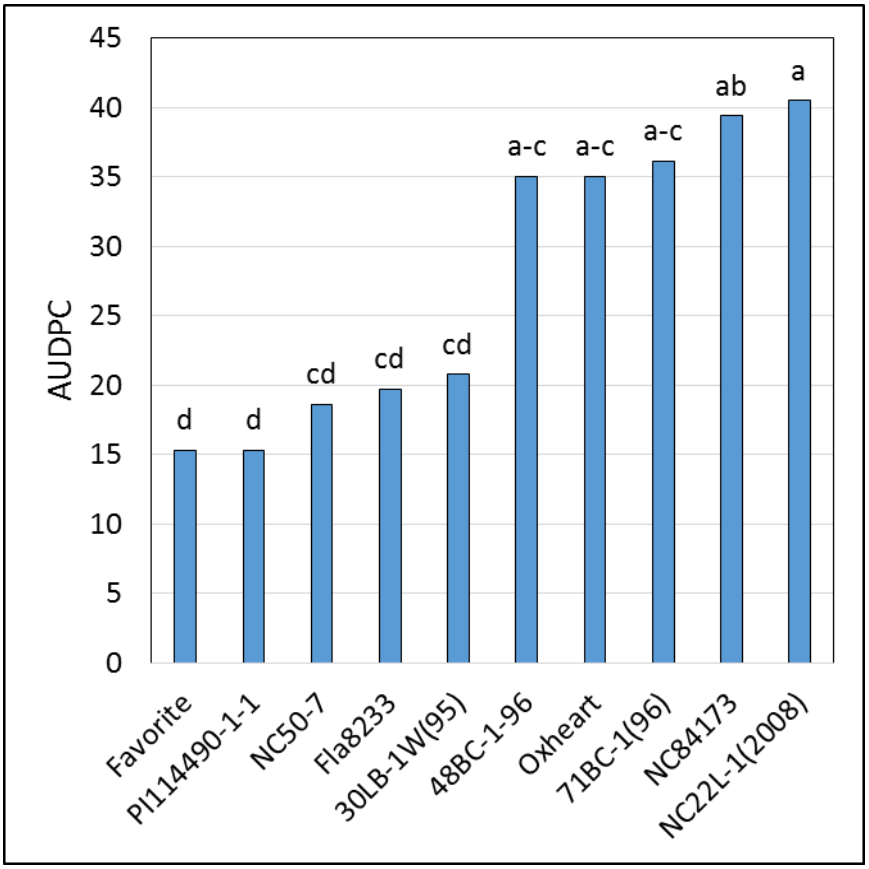


Figure 2.6. Foliar area under disease progress curve of bacterial spot disease severity in the greenhouse experiment at the Method Road Greenhouse, Raleigh, North Carolina, 27695 in 2013. The most susceptible and most resistant lines are shown.

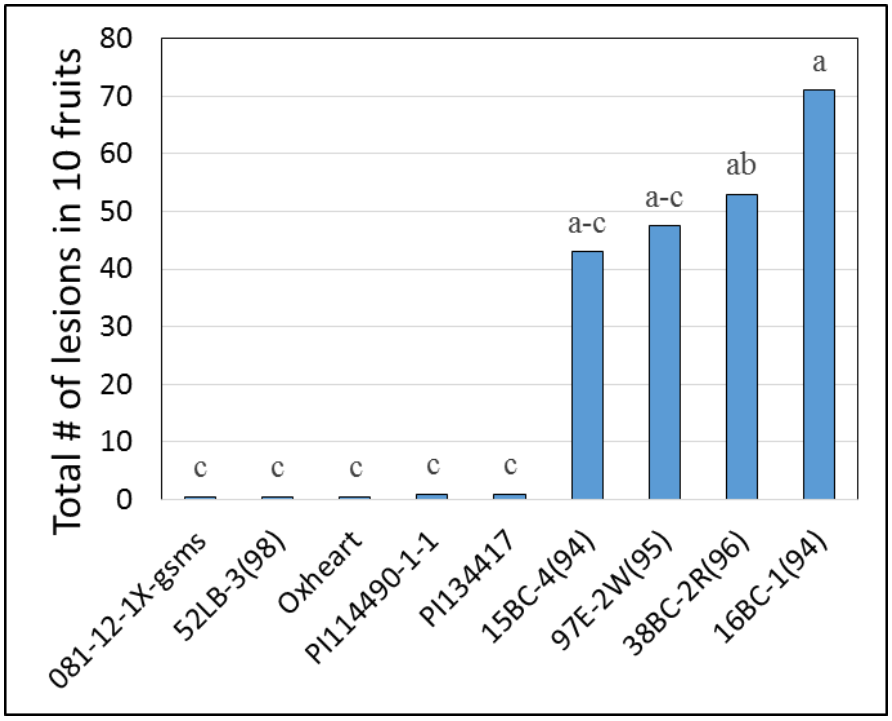


Figure 2.7. Bacterial spot severity on tomato fruits calculated by counting total number of lesions in ten severely infected fruits of a severely individual plant in a plot in the field experiment at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759 in 2013. The most susceptible and most resistant lines are shown.

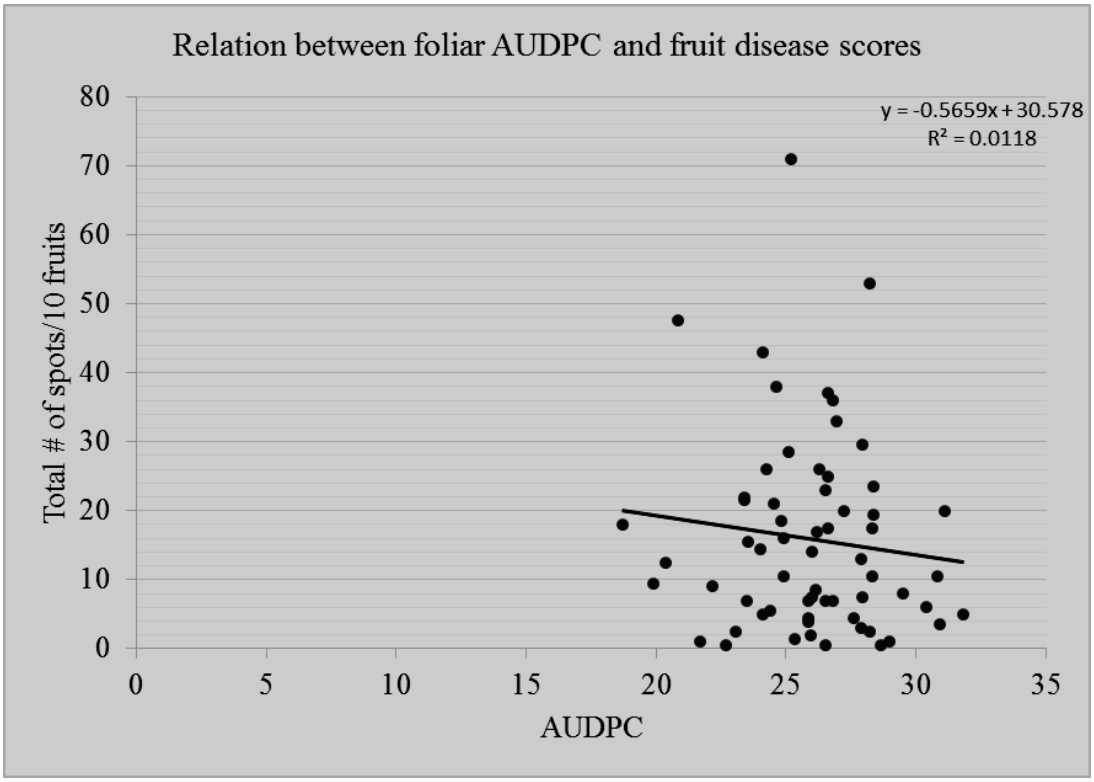


Figure 2.8. Simple Linear Regression between bacterial spot foliar area under disease progress curve and fruit disease severity on tomato fruits in field experiment at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759 in 2013

Susceptible line

38BC-2R

Resistant line

081-12(x)-1gsms-3

Figure 2.9. Fruits of resistant and susceptible tomato lines in the field experiment at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, 28759 in 2013

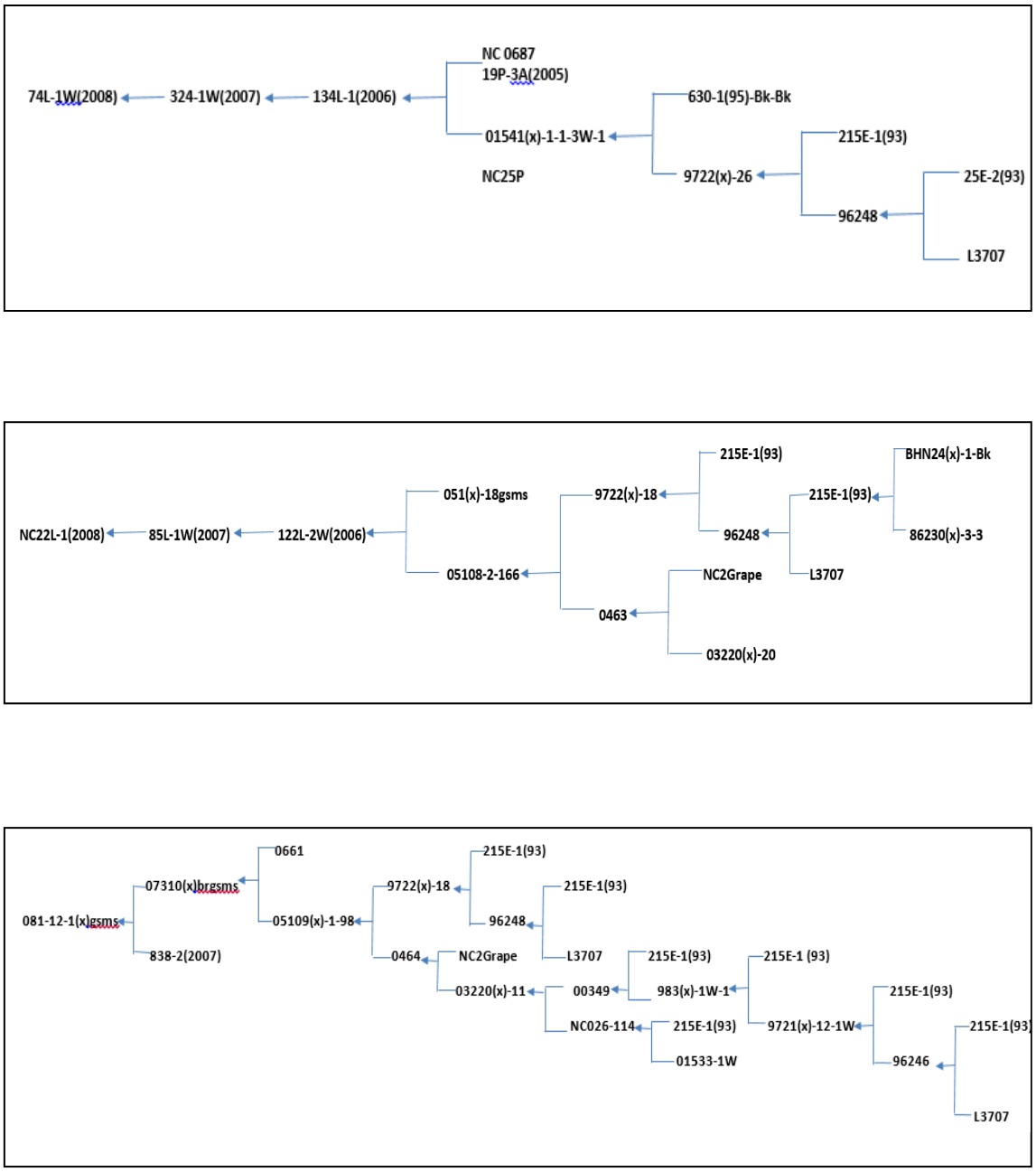
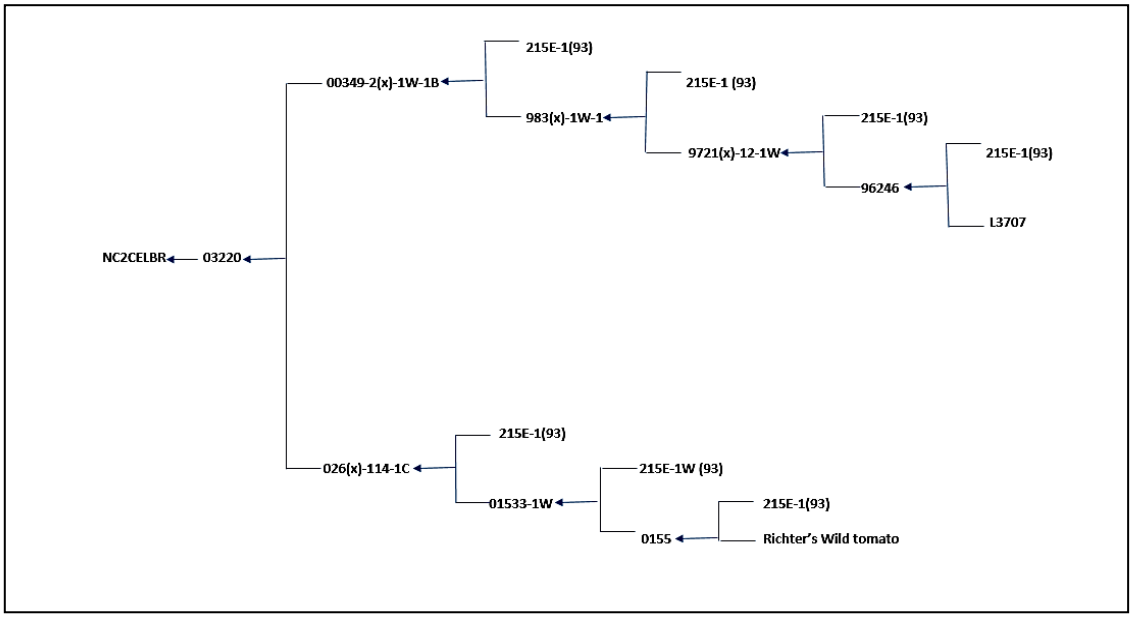


Figure 2.10. Pedigree information of genotypes 74L-1W(2008), NC22L-1W(2008), 081-12-1(x)gsms, NC2CELBR and 52LB-1(98) going back to *Solanum piminellifolium* L3707 line



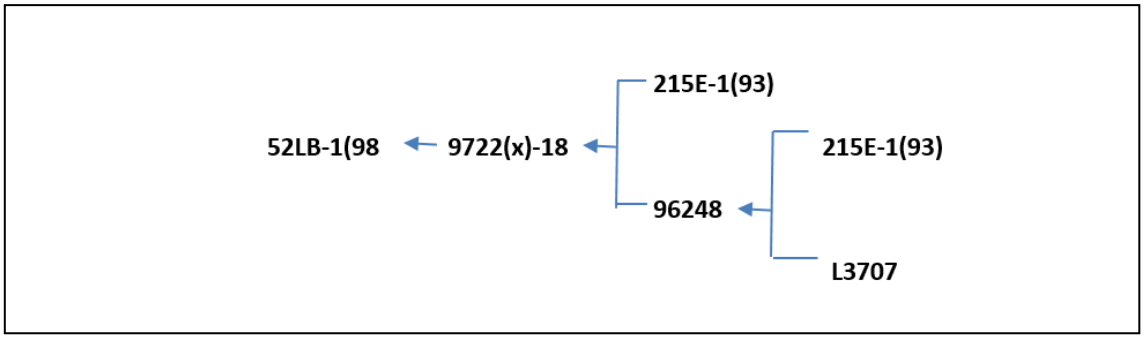


Figure 2.11: Pedigree information of genotypes NC2CELBR and 52LB-1(98) going back to *Solanum piminellifolium* L3707 line

CHAPTER 3 ANALYSIS OF MICROBE ASSOCIATED MOLECULAR PATTERN FOR BACTERIAL SPOT (*XANTHOMONAS* SPP.) RESISTANCE IN TOMATO (*SOLANUM LYCOPERSICUM* L.)

ABSTRACT

Plants depend on innate immune system for restricting pathogen that enters into the system through stomata, hydathodes or injuries. Pathogens or microbes possess conserved amino-acid sequence, known as pathogen associated molecular patterns (PAMPs) or microbe associated molecular pattern (MAMPs), in those structures, like chitin in fungi and flagellin in bacteria that are important for survival of those organisms. PAMPs/MAMPs are identified by pattern recognition receptors (PRRs) present in the plasma membrane of the plant cell. PAMPs, once identified in the cell, the defense mechanism initiates in the plant to restrict the spread of the pathogen. Production of reactive oxygen species (ROS) is one of the responses to be initiated rapidly and are considered to be first line of defense. The objective of the study was to conduct comparative analysis between production of ROS and bacterial spot (*Xanthomonas* spp.) (BS) resistance in tomato (*Solanum lycopersicum* L.). Sixty three genotypes were planted in greenhouse and field conditions. A luminol based assay was done using *Xanthomonas* specific flagellin 22 (*xcc22*) peptide designed from *Xanthomonas campestris* pv. *campestris* and general flagellin 22 (flg22) and general flagellin 28 (flgII-28) peptides designed from *Pseudomonas aeruginosa* to measure ROS production in each genotypes. BS disease was artificially developed in plants by spray inoculation of *Xanthomonas perforans*, race T4. Foliar disease severity was measured using Horsfall-Barratt (HB) scale and fruit disease incidence was measured on ten severely infested fruits

from a single plant of a genotype. Pearson correlation analysis on combined data showed a significant negative correlation of -0.25 and -0.27 between total and maximum ROS production, when *xcc22* was used to generate ROS, and foliar disease score whereas non-significant negative correlation of -0.22 and -0.17 was found between total and maximum ROS production, when *xcc22* was used to generate ROS, and disease score on fruits. Similar negative correlation was also observed, -0.23 and -0.17, between total and maximum ROS production when flg22 was used to generate ROS and fruit disease score and these correlations were also not significant. The significant correlation between foliar disease score and ROS production when *xcc22* was used infers that ROS assay can be used in screening tomato lines for bacterial spot disease resistance and can be useful for breeding programs.

INTRODUCTION

Foliar bacterial plant pathogens enter inside plant tissue through stomata, hydathodes or injuries. Unlike animals, plants do not have mobile defender cells and a somatic adaptive immune system (Jones and Dangl, 2006). That is why they depend upon performed defenses and on induced or systemic responses to infection (Dangl and Jones, 2001). Pathogen associated molecular patterns (PAMPs), also known as microbe associated molecular patterns (MAMPs) are highly conserved molecules, which are essential for survival, in entire class of microbes but are absent in the host and with which pathogens are able to infect the hosts. Plants can perceive PAMPs from various structural characteristics of microorganisms like flagellin (FLG), lipopolysaccharides (LPS), bacterial cold-shock protein (CSP), and elongation factor thermal unstable (EF-Tu) from bacteria, cell wall polysaccharides chitin

and the fungal sterol ergosterol from true fungi (Nurnberger et al., 2004; Zipfel and Felix, 2005), cell wall β-glucan, the pep13 epitope conserved in cell wall transglutaminases, and secreted lipotransfer proteins termed elicitins (Nurnberger et al., 2004). Bacterial flagellins are one of those prevalent PAMPs which are recognized by the innate immune systems of plants (Gomez-Gomez and Boller, 2002). Plants use pattern recognition receptors (PRRs) present in plasma membranes to recognize these PAMPs. Identification of PAMPs by PRRs initiates a large number of signaling molecules to restrict the growth of microbial pathogens in the host. In *Arabidopsis thaliana*, a transmembrane receptor kinase with a leucine-rich repeat (LRR) extracellular domain, FLAGELLIN SENSING2 (FLS2), is required for flagellin perception and flagellin elicited defense activation (Gomez-Gomez and Boller, 2000). FLS2 binds the flagellin (Chinchilla et al., 2006) and activates the downstream defense responses. Perception of PAMPs from pathogen by host cells leads to rapid activation of defense mechanisms such as reinforcement of cell walls by callose deposition, production of reactive oxygen species (ROS), and induction of numerous defense-related genes (Zipfel et al., 2006). ROS are generated from excitation or incomplete reduction of molecular oxygen during cellular metabolism in aerobic organisms (Halliwell, 2006). These highly reactive intermediates are produced when molecular dioxygen (O_2) accepts electrons released from different reactions inside cells. Superoxide ($O_2^{\cdot -}$) is formed when O_2 molecules are reduced. $O_2^{\cdot -}$ has a short half-life of 2 to 4 μs. $O_2^{\cdot -}$ and leads to the formation of hydrogen peroxide (H_2O_2) which is relatively stable and has a half-life of 1 ms. $O_2^{\cdot -}$ and H_2O_2 are two major ROS found in tomato. Apoplastic enzymes like plasmalemma-bound NAD(P)H oxidases and cellwall-associated peroxidases are the main producers of $O_2^{\cdot -}$ and H_2O_2 (Sagi

and Fluhr, 2006) in the extra cellular matrix. ROS generated due to apoplastic enzymes take part in oxidative bursts as a part of the HR to pathogens but also regulates cell growth, development and cell death (Foreman et al., 2003; Gapper and Dolan, 2006; Gechev and Hille, 2005; Sagi and Fluhr, 2006; Torres et al., 2002). Identification of PAMP recognition specificity across plant families can lead to a way to develop resistance to a wide range of pathogens. Lacombe et al. (2010) reported an increase in resistance to a range of phytopathogenic bacteria, from different genera, in solanaceous crops such as *Nicotiana benthamiana* and *Solanum lycopersicum* after transfer of EFR, a PRR from the cruciferous plant *Arabidopsis thaliana* which confers responsiveness to bacterial EF-Tu. Exploiting PRR and associated host defense signaling pathways has several advantages over current practices of managing disease and methods of resistance development. Utilizing the immune system of plants in controlling the pathogen could lead to less use of pesticides in crop management and financial, health and environmental costs associated management of diseases (Lacombe et al., 2010). Classical breeding, which predominantly targets R-gene mediated resistance, can be "broken down" often rapidly by evolving pathogens like *Xanthomonas.* In contrast, it may be difficult for pathogens to modify PAMPs-mediated resistance, since the targeted molecule recognized by PRRs is frequently associated with the fitness of the pathogen. Therefore, resistance through PRRs is hypothesized to last longer than through classical breeding methods that tend to rely on use of R genes. Rapid transfer of new PRRs to elite varieties and into crops through transgenic technologies can expedite resistance breeding (Lacombe et al., 2010). Successful pathogens are able to avoid recognition by PRRs by evolving new or modified effectors but PTI activation is still able to reduce pathogen

populations and this decreases the probability of emergence of new virulent pathogens and break down of the resistance (Brun et al., 2010). Lacombe et al. (2010) proposed that combinations of several PRRs as well as PRRs along with R proteins are able to enable broad spectrum disease resistance against multiple genera of plant pathogens with promising potential for durability under field conditions, by recognizing widely distributed effectors. Detailed protocols for measuring various PTI-associated phenotypes, induction of reporter genes, callose deposition, activation of mitogen-activated protein kinases (MAPKs), and a luciferase-based reporter system has been established (Nguyen et al., 2010). Despite being conserved across the plant kingdom, PTI, specific responses and underlying molecular mechanisms may differ between species. For instance, tomato recognizes a 15 amino acid flagellin peptide from *Escherichia coli* but *Arabidopsis* does not (Felix et al., 1999; Meindl et al., 2000). Flagellin present in bacteria is perceived by both *Solanaceae* and *Brassicaceae* families but the response to different peptides of the protein is different (Nguyen et al., 2010). The *xcc22* region of *Xanthomonas* is known for the production of detectable elicitation of *Arabidopsis* defense responses (Sun et al., 2006). Elicitation of ROS by host cells after recognition of PAMP from microbes is known as the oxidative burst. Oxidative burst can be artificially induced by using synthesized forms of PAMP and increase in ROS can be measured. Polymorphisms in *xcc22* has been reported (Sun et al., 2006) and the sequence from the one that elicited ROS production, reported by Felix et al. (1999), was used in our experiment. Oxidative bursts are rapid and can be measured using high-throughput assay, to characterize PAMP responsiveness in diverse accessions and mapping populations (Lloyd et al., 2014). Quantitative assessment of ROS production between *Arabidopsis* leaf

tissue and live *Pseudomonas syringae* pv *tomato* strains as well as synthesized peptides sequence has been previously done (Smith and Heese, 2014; Sun et al., 2006). Since PAMPs triggered responses are mostly Pathogen-specific (Wan et al., 2008; Zipfel et al., 2006) and have been reported not to necessarily induce microbe-specific immunity (Ferrari et al., 2007; Zipfel et al., 2004), Lloyd et al. (2014) mentioned the possibility of PAMPs being used to correlate the extent of PTI responsiveness to quantitative resistance to distinct pathogens. Here we assess resistance in diverse tomato lines and compare the BS resistance with ROS production, one of the PTI responses, using three different flagellin peptides: flg22, flgII-28 and *xcc22* as a source of ROS induction in tomato.

MATERIALS AND METHODS

Plant materials and plant growth

Sixty three tomato genotypes including lines from tomato breeding programs of North Carolina State University and University of Florida, heirlooms and wild lines were sown in 4P soil mixture (Fafard®, Florida, USA) in 24-cell flat trays for a greenhouse experiment in March, 2013 at the Method Road Greenhouse, North Carolina State University, Raleigh, NC. Six plants per genotype were planted in three replications in a completely randomized design. Plants in the greenhouse study were fertilized using a 20:20:20 ratio of nitrogen, phosphorus and potassium respectively. Standard greenhouse spraying for insects and fungal diseases (powdery mildew) were followed whereas copper was not applied. For the field experiment, sixty three genotypes were sown in flat bed metal trays in a standard seeding mix (2:2:1 (v/v/v) peat moss:pine bark:vermiculite with macro- and micro-nutrients (Van Wingerden International Inc., Mills River, NC) on May, 2013. After 10 days, seedlings were

transplanted to 72-cell flats (56 cm X 28cm). After four weeks these plants were transplanted to the field at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina. Six plants per plot were planted with plant to plant spacing of 45 cm and 150 cm distance between rows in two replications in a randomized complete block design. The recommended management practices for fertilization, insect management and management of foliar fungal diseases were done according to standard recommendations (Ivors and Louws, 2013). Copper was not sprayed on the crop.

Oxidative burst/Reactive oxygen species (ROS)/Pathogen associated molecular pattern (PAMP) assay

ROS measurement was performed by using luminometer based assay as described by Felix et al. (1999) and Sainsbury lab, Norwich, UK.

1. PREPARATION OF REAGENTS

Peptides sequences designed from flagellin 22 (flg22) of *Pseudomonas aeruginosa*, flagellin 28 (flgII-28) designed from *Pseudomonas aeruginosa* and *Xanthomonas* flagellin 22 (*xcc22*) designed from *Xanthomonas campestris* pv. *campestris* were used in this experiment (Felix et al., 1999). The amino acid sequence of *xcc22* peptide used was QRLSSGLRINSAKDDAAGLAIS (EZBiolabs, Carmel, IN, USA), sequence of flg22 was QRLSTGSRINSAKDDAAGLQIA (EZBiolabs, Carmel, IN, USA) and sequence of flgII-28 was ESTNILQRMRELAVQSRNDSNSSTDRDA (EZBiolabs, Carmel, IN, USA). Luminol (Sigma Lifescience, Saint Louis, Missouri, USA) was dissolved in Dimethyl Sulfoxide (DMSO) at a concentration of 17 mg/ml. Horseradish peroxidase (Sigma Type VI-A, Saint Louis, Missouri, USA) was dissolved in dH_2O at the concentration of 10 mg/ml.

2. PREPARATION OF SAMPLES

Four mm diameter leaf discs were sampled from the completely open leaf of each plant, second from the top, by using a leaf corer and were incubated with the adaxial surface facing upward in 200 µl of dH_2O in Lumitrac 200 medium 96 well micro plate (Greiner bio-one, Product #: 655075) for 12 - 16 hours. The samples in 96 micro-well plate were covered with aluminum foil and kept at room temperature. Four leaf cores from each tomato line were sampled. Samples for the greenhouse experiment were sampled after 30 days of planting through 45 days after planting. ROS measurement from individual plant was done three times.

3. PROCESSING OF SAMPLES

The dH_2O used to incubate the samples was removed from each well of the 96 micro-well plates. A mixture of 12 µl of peptide, 24 µl of HR peroxidase and 24 µl of luminol was mixed well in 1200 µl of dH_2O. 100 µl of the solution was added to each sample. Measurement of the photons in the form of relative light units was done after adding these reagents.

4. MEASUREMENT OF ROS PRODUCTION

Production of ROS in the samples was measured for the greenhouse experiment using a luminometer (Glomax™ 96 microplate, Promega Corporation, WI, USA). For the field experiment, a different luminometer (Biotek Multi Detection Microplate Reader Synergy™ 2) was used to measure ROS production. ROS was measured for 15 cycles or 60 minutes ie. each well was read at ~4 minutes intervals.

5. CONTROL

Negative controls for the experiment were designed by sampling leaf tissues from random lines and incubated in 200 µl of dH_2O in the 96 micro-well plates and production of ROS without using any peptides were measured. After 16 hours of incubation, the dH_2O was removed and 100 µl of assay solution including 2 µl of luminol and horseradish peroxidase each but no peptides. Each plate was read in the luminometer (Glomax™ 96 microplate, Promega Corporation, WI, USA) for 60 minutes.

Inoculum preparation and inoculation

Plants in both the greenhouse and field studies were artificially inoculated with Isolate 9 (Figure 2.2), isolated from infected tissue in a field in North Carolina and characterized as *X. perforans* race T4 (by Dr. Jefferey B. Jones lab, University of Florida, Gainsville, Florida). The description of characterized isolates is given in Table 2.2. The strain was obtained in pure culture and stored at -80°C. Bacteria stored at -80°C was grown in Yeast Dextrose Chalk (YDC) agar medium (Lelliott and and Stead, 1987) for 24-48 hours at 28°C. Distilled water was poured over the culture plates and bacterial cells were suspended. Bacterial suspension was standardized at 0.3 optical density by using a LKB Biochrom Ultrospec II spectrophotometer (American Laboratory Training, USA) absorbance at 600 nm which is approximately $2\text{-}5\times10^8$ CFU/ml, as previously used by Hutton et al. (2010) and immediately used for inoculations.

For greenhouse inoculations, humidity in the immediate vicinity of the plants was maintained by using V5100NS humidifiers (Vicks Ultrasonic Humidifiers, NY, USA) from 24 hours prior to the inoculation to 48 hours after inoculation and covering plants with white plastic.

Spray inoculation to runoff from the foliage was performed on the plants 30 days after sowing using a hand sprayer. A second inoculation was done 15 days after the first inoculation and a third inoculation was done after another 15 days following the same procedure. The first and second scorings were done after 15 and 21 days of the first inoculation and a scoring was done 30 days after the third inoculation.

In the field, spray inoculation was done uniformly on all plants using a backpack sprayer 30 days after transplanting. Plants were scored beginning 10 days post inoculation and every week for five weeks.

Disease evaluation

Greenhouse plants were scored for foliage symptoms using the most severely infected leaves of the plant through a modified use of the Horsfall-Barratt scale (1945) where 0% = 1, 1-3% = 2, 3-6% = 3, 6-12% = 4, 12-25% = 5, 25-50% = 6, 50-75% = 7, 75-87% = 8, 87-94% = 9, 94-97% = 10, 97-100% = 11 and 100% dead tissue = 12 (Figure 2.3).

Plants were scored in the field using the same scale as used in the greenhouse, except complete foliage was rated rather than the severity of infected leaves. Disease severity on the fruit was rated one time after the last foliar disease rating. Fruit disease incidence was rated by counting the number of BS lesions on ten severely infected fruits of a most infected plant among the six plants of a genotype.

Statistical analyses

For each genotype and peptide used for ROS measurement, four leaf cores were taken from each genotype. Total ROS production for a tomato line was calculated by adding all relative light units (RLUs) measured by the luminometer in the entire cycle of sixty minutes. The

peak was used as maximum values for data analysis. For each peptide four leaf cores from each genotype were used. Averages of total and maximum RLUs from four leaf cores were calculated. Averages of total and maximum RLUs from three sets of data and three replications were calculated and least squares means of each were used for further analysis. Analysis of ROS was conducted separately for greenhouse and field experiments and for complete analysis, combined analysis of greenhouse and field experiments was also done and was presented.

For ROS analysis, RLUs produced from the non-responsive leaf samples were deleted according to the protocol designed by Sainsbury laboratory, Norwich, UK. Determination of non-responsive samples for each experiment was done by studying the nature of RLUs production and background noise of machine. The standard nature of the RLUs production is skewed bell curve but samples not producing the skewed bell curves were not used in the analysis. Also leaf cores producing RLUs below 100 in their cycles in the greenhouse experiment and 20 in the field experiment were deleted because these were identified as background noise of the luminometers used from the control experiments and in addition didn't produce the skewed bell curve.

Area under the disease progress curve (AUDPC) was calculated and used in further analysis. Analyses of variance were performed on ROS and AUDPC disease scores from the greenhouse and the field data using GLM procedure from SAS 9.3 (SAS Institute, Inc., Cary, NC). Least Squares Means were calculated and were separated using LSD at $P = 0.05$. Correlation analysis was performed using the Pearson method.

RESULTS

Total and maximum ROS productions for each tomato line were used for data analysis. In the greenhouse experiment, analysis of variance (ANOVA) of all traits (Table 3.1) showed that total and maximum ROS production was not affected (P<0.05), when flg22 (P=0.9004 and 0.9063) was used, whereas total and maximum ROS productions were increased (P< 0.05) when flgII-28 (P=0.0405 and 0.0151; total and maximum ROS production respectively) and *xcc22* (P=0.0403 and 0.0265; total and maximum ROS production respectively) were used as peptides. AUDPC scores for BS (P<0.0001) was impacted by different tomato lines (P< 0.0001). Simple statistics of the above mentioned traits are given in Table 3.2. Least square (LS) means of these traits were calculated and used for correlation analysis (Table 3.3). Maximum correlation between AUDPC and ROS production occurred when *xcc22* was used. Pearson correlation between total and maximum ROS production and AUDPC when the *xcc22* peptide was used was 0.13 and 0.15 respectively (Table 3.3) but these correlations were not significant (P<0.05) (P=0.322 and 0.248 for total and maximum ROS production respectively).

Using plant tissue from the field experiment, ANOVA of all traits (Table 3.1) showed that total and maximum ROS production was significantly impacted, when flg22 (P<0.0001 for both total and maximum ROS production), flgII-28 (P<0.0001 for both total and maximum ROS production) and *xcc22* (P= 0.0015 and 0.0003 for total and maximum ROS production respectively) were used. Differences in tomato lines based on AUDPC (P< 0.0001) and fruit spot severity (P< 0.0001) were significant (at P=0.0001 or 0.01). Simple statistics of these traits are given in Table 3.4. LS means of these traits were calculated and used for correlation

analysis (Table 3.5). Maximum correlation between ROS production and the AUDPC disease score occurred when *xcc22* was used for ROS generation (r= -0.16 and -0.16) which was not significant (*P*= 0.224 and 0.213). Maximum negative correlation between tomato fruit scores and total and maximum ROS production was also obtained when *xcc22* was used (r = -0.20 and -0.16 respectively; *P*=0.1229 and 0.2263). Correlation between fruit scores of tomato genotypes and total and maximum ROS production, when flg22 was used, was also negative but less than values obtained when *xcc22* was used (Table 3.5) but none of these values were significant. Correlation between ROS production when flgII-28 was used and fruit disease score was high and significant but positive (Table 3.5).

Combined data analysis of greenhouse and field experiments showed no significant differences between tomato lines in ANOVA analysis (Table 3.1). Simple statistics of all ROS production and disease traits are given in Table 3.6. Pearson correlation analysis showed a significant negative correlation between Total and maximum ROS production, when *xcc22* was used, and AUDPC score (r = -0.25 and -0.27 for total and maximum ROS production respectively and *P*= 0.0488 and 0.031 respectively) (Table 3.7). Negative, but not significant, correlation was found between total and maximum ROS production and spot disease score on tomato fruits when flg22 and *xcc22* peptides were used for ROS generation (Table 3.7).

Total and maximum ROS production with and without use of peptide is shown in Figure 3.1 and Figure 3.2 respectively. Figure 3.3 and Figure 3.4 shows total and maximum ROS production when blank wells and wells with leaf samples containing luminol and peroxidase were read in luminometer.

DISCUSSION

Previous result has shown that oxidative burst can be used to characterize PAMP responsiveness in different genotypes and species (Lloyd et al., 2014). Veluchamy et al. (2014) reported that there was no clear correlation between bacterial speck (*Pseudomonas syringae* pv. *tomato*) from field experiment and ROS production when flg22, flgII-28 and Cold Shock Protein (*csp22*) peptides were used, in heirloom tomato genotypes but some genotypes showing high disease score produced low ROS and vice-versa. We selected three different peptides *xcc22* and flg22 and flgII-28, designed from *Xanthomonas campestris* pv *campestris* and *Pseudomonas aeruginosa*, respectively (Felix et al., 1999). These 22 amino-acid peptides are conserved sequences present in N- terminal domain of flagellin protein in bacterial flagella. Production of ROS using these peptides reached at maximum point within 5 – 15 minutes, depending upon the tomato line and peptide used, after initiating the assay and commencing reads in the luminometer. ROS production was measured up to 60 minutes until production completely subsided or was very low. The Luminometer measured photons, converted from ROS in the presence of luminol and horseradish peroxidase, into relative light units (RLUs).

In control experiments, where no peptides were used, there was very low or no ROS production in comparison to the assays when peptides were used. The photon counts generated in the control experiments were due to background noise of the machine which was confirmed by running blank wells with luminol, horseradish peroxidase and dH_2O but no leaf discs or peptides. Total and maximum ROS production from the controls with blanks

wells and wells with leaf samples containing luminol, horseradish peroxidase but no peptides confirmed that ROS production occurred only when peptides started the elicitation process. In the greenhouse experiment, total and maximum ROS production using *xcc22* peptide and AUDPC score was correlated but were not significant. All correlations were positive which was interesting. ANOVA showed that total and maximum ROS production using flg22 was not significant whereas it was significant when flgII-28 and *xcc22* were used for ROS generation. AUDPC was also significant in the greenhouse experiment. In field, there was additional disease trait, disease score on fruit, which was also significant along with ROS production when flg22 and flgII-28 was used. Total and maximum ROS production, when *xcc22* was used, was significant. It means that tomato lines were significantly different from each other for all these traits. Negative correlation between disease scores (AUDPC and fruit disease score) and ROS production, when flg22 and *xcc22* was used, indicates that higher the ROS production lower is the disease incidence and vice versa. In correlation analysis of combined data from greenhouse and field, ROS production using *xcc22* was significantly correlated with AUDPC scores. Significant correlation of AUDPC score and ROS production using *xcc22* indicates that PAMP designed from *Xanthomonas campestris* pv *campestris* is able to correlate the ROS response and disease resistance in BS. The low correlation is due to variation in ROS production between the leaf discs within the same tomato line and use of high number of tomato lines in the experiment. It has also to do with the quantitative nature of the resistance for BS in tomato. Hence, use of *xcc22* peptide can be used to generate ROS production and screen tomato lines for BS resistance using oxidative burst assay. Therefore,

with more studies on application of this assay in other crops, it can be used as a technique to screen different lines of individual crops for disease resistance.

REFERENCES

Brun, H., A. Chevre, B.D.L. Fitt, S. Powers, A. Besnard, M. Ermel, V. Huteau, B. Marquer, F. Eber, M. Renard, and D. Andrivon. 2010. Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. New Phytol. 185(1):285-299.

Chinchilla, D., Z. Bauer, M. Regenass, T. Boller, and G. Felix. 2006. The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18(2):465-476.

Dangl, J.L. and J.D.G. Jones. 2001. Plant pathogens and integrated defence responses to infection. Nature (London) 411(6839):826-833.

Felix, G., J. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 18(3):265-276.

Ferrari, S., R. Galletti, C. Denoux, G. De Lorenzo, F.M. Ausubel, and J. Dewdney. 2007. Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiol. 144(1):367-379.

Foreman, J., V. Demidchik, J. Bothwell, P. Mylona, H. Miedema, M. Torres, P. Linstead, S. Costa, C. Brownlee, J. Jones, J. Davies, and L. Dolan. 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature 422(6930):442-446.

Gapper, C. and L. Dolan. 2006. Control of plant development by reactive oxygen species. Plant Physiol. 141(2):341-345.

Gechev, T. and J. Hille. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. J. Cell Biol. 168(1):17-20.

Gomez-Gomez, L. and T. Boller. 2000. FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell 5(6):1003-1011.

Gomez-Gomez, L. and T. Boller. 2002. Flagellin perception: a paradigm for innate immunity. Trends Plant Sci. 7(6):251-256.

Halliwell, B. 2006. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 141(2):312-322.

Horsfall, J. G., and R. W. Barratt. 1945. An improved grading system for measuring plant diseases. Phytopathology 35: 655.

Hutton, S.F., J.W. Scott, and J.B. Jones. 2010. Inheritance of resistance to bacterial spot race T4 from three tomato breeding lines with differing resistance backgrounds. J. Am. Soc. Hort. Sci. 135(2):150-158.

Ivors K. L. and Louws F. J. 2013. 2013 North Carolina Agricultural Chemicals Manual. College of Agriculture and Life Sciences, North Carolina State University.

Jones, J.D.G. and J.L. Dangl. 2006. The plant immune system. Nature (London) 444(7117):323-329.

Lacombe, S., A. Rougon-Cardoso, E. Sherwood, N. Peeters, D. Dahlbeck, H.P. van Esse, M. Smoker, G. Rallapalli, B.P.H.J. Thomma, B. Staskawicz, J.D.G. Jones, and C. Zipfel. 2010. Interfamily transfer of a plant pattern-recognition receptor confers broad-spectrum bacterial resistance. Nat. Biotechnol. 28(4):365-U94.

Lelliott, R.A. and D.E. and Stead. 1987. Methods for the diagnosis of bacterial diseases of plants. In: Methods in Plant Pathology. Vol. 2 ed. T.F. Preece Series, British Society of Plant Pathology, Blackwell Scientific Publications, Oxford.

Lloyd, S.R., H. Schoonbeek, M. Trick, C. Zipfel, and C.J. Ridout. 2014. Methods to Study PAMP-Triggered Immunity in Brassica Species. Mol. Plant-Microbe Interact. 27(3):286-295.

Meindl, T., T. Boller, and G. Felix. 2000. The bacterial elicitor flagellin activates its receptor in tomato cells according to the address-message concept. Plant Cell 12(9):1783-1794.

Nguyen, H.P., S. Chakravarthy, A.C. Velasquez, H.L. McLane, L. Zeng, H. Nakayashiki, D. Park, A. Collmer, and G.B. Martin. 2010. Methods to Study PAMP-Triggered Immunity Using Tomato and *Nicotiana benthamiana*. Mol. Plant-Microbe Interact. 23(8):991-999.

Nurnberger, T., F. Brunner, B. Kemmerling, and L. Piater. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198:249-266.

Sagi, M. and R. Fluhr. 2006. Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol. 141(2):336-340.

Smith, J.M. and A. Heese. 2014. Rapid bioassay to measure early reactive oxygen species production in *Arabidopsis* leave tissue in response to living *Pseudomonas syringae*. Plant Methods 10:6.

Sun, W., F. Dunning, C. Pfund, R. Weingarten, and A. Bent. 2006. Within-species flagellin polymorphism in *Xanthomonas campestris* pv *vesicatoria* and its impact on elicitation of *Arabidopsis* FLAGELLIN SENSING2-dependent defenses. Plant Cell 18(3):764-779.

Torres, M.A., J.D.G. Jones, and J.L. Dangl. 2002. *Arabidopsis* gp91phox homologues AtrbohD and AtrobohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. U. S. A. 99(1):517-522.

Veluchamy, S., S.R. Hind, D.M. Dunham, G.B. Martin, and D.R. Panthee. 2014. Natural Variation for Responsiveness to flg22, flgII-28, and csp22 and *Pseudomonas syringae* pv. *tomato* in Heirloom Tomatoes. PLoS One 9(9):n/a-e106119. <http://search.proquest.com.prox.lib.ncsu.edu/docview/1559041054?accountid=12725>.

Wan, J., X. Zhang, D. Neece, K.M. Ramonell, S. Clough, S. Kim, M.G. Stacey, and G. Stacey. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. Plant Cell 20(2):471-481.

Zipfel, C. and G. Felix. 2005. Plants and animals: a different taste for microbes? Curr. Opin. Plant Biol. 8(4):353-360.

Zipfel, C., G. Kunze, D. Chinchilla, A. Caniard, J. Jones, T. Boller, and G. Felix. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. Cell 125(4):749-760.

Zipfel, C., S. Robatzek, L. Navarro, E. Oakeley, J. Jones, G. Felix, and T. Boller. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428(6984):764-767.

Table 3.1. Analysis of Variance (ANOVA) of total and maximum photon counts produced in reactive oxygen species (ROS) assay using flg22, flgII-28 and *xcc22* peptides and disease traits for field, greenhouse experiments and combining both experiments together in 2013

Source of variation	Mean sum of squares		
	Field	Greenhouse	Combined
Total photon count traits			
flg22Total	308078850***	71424613^{NS}	81441360 ^{NS}
flgII-28Total	4057485576.8***	128291499*	971595772.9 ^{NS}
*xcc22*Total	483286840**	81646816*	124385898 ^{NS}
Maximum photon count traits			
flgII-28Max	88257269***	1907005.3*	21084632 ^{NS}
flg22Max	18693746***	1226246.5^{NS}	4797373.4 ^{NS}
*xcc22*Max	25192641**	1468241.1*	6437010.8 ^{NS}
Disease traits			
AUDPC	14.95***	75.93***	20.32^{NS}
BS on fruits	404.21***		

^{NS}, *, ** and *** are non- significant, significant at P-value <0.05, 0.01 and 0.0001.
AUDPC Area under disease progress curve
BS Bacterial spot

Table 3.2. Simple statistics of total and maximum photon count produced in reactive oxygen species (ROS) assay using flg22, flgII-28 and *xcc22* peptides and area under disease progress curve for greenhouse experiment in 2013

Variable	N	Mean[z]	Std Dev	Minimum	Maximum
Total photon count					
flg22Total	189	8463	9366	420.50	54946
flgII-28Total	188	8140	10082	350.25	60864
*xcc22*Total	189	7766	8040	398.50	48830
Maximum photon count					
flg22Max	189	1426	1231	73.25	6682
flgII-28Max	188	1276	1198	56.25	6963
*xcc22*Max	189	1301	1066	65.50	6307
Disease score					
AUDPC	188	27.61	6.62	6.57	46.00

[z]Total Photon counts measured by Luminometer during ROS production

Table 3.3. Correlation analysis of total and maximum photon count in reactive oxygen species (ROS) using flg22, flgII-28 and *xcc22* peptides production and area under disease progress curve for greenhouse experiment in 2013

Pearson Correlation Coefficients, N = 63							
Prob > |r| under H0: Rho=0							
	flg22Total	flg22Max	flgII-28Total	flgII-28Max	*xcc22*Total	*xcc22*Max	AUDPC
flg22Total	1						
flg22Max	0.91***	1					
flgII-28Total	0.36**	0.45**	1				
flgII-28Max	0.30*	0.47***	0.95***	1			
*xcc22*Total	0.62***	0.60***	0.56***	0.49***	1		
*xcc22*Max	0.57***	0.64***	0.61***	0.61***	0.93***	1	
AUDPC	0.10^{NS}	0.12^{NS}	0.05^{NS}	0.08^{NS}	0.13^{NS}	0.15^{NS}	1

^{NS}, *, ** and *** are non- significant, significant at $P<0.05$, 0.01 and 0.0001.
AUDPC Area under disease progress curve
BS Bacterial spot

Table 3.4. Simple statistics of total and maximum photon count produced in reactive oxygen species (ROS) assay using flg22, flgII-28 and *xcc22* peptides and area under disease progress curve and bacterial spot disease score on tomato fruits for field experiment in 2013

Variable	N	Mean[z]	Std Dev	Minimum	Maximum
Total photon count					
flg22Total	121	10768	14317	63.75	90847
flgII-28Total	112	29512	52651	145	296488
*xcc22*Total	114	6697	11553	103	94510
Maximum photon count					
flgII-28Max	112	4857	7772	14	43036
flg22Max	121	3132	3517	9.25	19242
*xcc22*Max	114	1954	3122	18	24366
Disease score					
AUDPC	126	25.97	2.96	14.8	32.67
BS on fruits	126	15.88	16.22	0	84

[z] Total photon counts measured by Luminometer during ROS production

Table 3.5. Correlation analysis of total and maximum photon count in reactive oxygen species (ROS) production using flg22, flgII-28 and *xcc22* peptides and area under disease progress curve and bacterial spot disease score on tomato fruits for field experiment in 2013

	AUDPC	flg22Total	flg22Max	flgII-28Total	flgII-28Max	*xcc22*Total	*xcc22*Max
AUDPC	1						
flg22Total	-0.0004^{NS}	1					
flg22Max	-0.05 ^{NS}	0.98 ^{NS}	1				
flgII-28Total	0.06 ^{NS}	0.14 ^{NS}	0.14 ^{NS}	1			
flgII-28Max	0.02 ^{NS}	0.14 ^{NS}	0.15 ^{NS}	0.97***	1		
*xcc22*Total	-0.16 ^{NS}	0.44**	0.45**	0.03 ^{NS}	-0.006 ^{NS}	1	
*xcc22*Max	-0.16 ^{NS}	0.40**	0.43**	0.02 ^{NS}	-0.001 ^{NS}	0.97***	1
BS on fruits	-0.11 ^{NS}	-0.15 ^{NS}	-0.13 ^{NS}	0.24 ^{NS}	0.30*	-0.20 ^{NS}	-0.15 ^{NS}

^{NS}, *, ** and *** are non- significant, significant at P-value <0.05, 0.01 and 0.0001 respectively.
AUDPC Area under disease progress curve
BS Bacterial spot

Table 3.6. Simple statistics of total and maximum photon count produced in reactive oxygen species (ROS) assay using flg22, flgII-28 and *xcc22* peptides and area under disease progress curve for combined data including greenhouse and field experiments in 2013

Variable	N	Mean[z]	Std Dev	Minimum	Maximum
Total photon count					
flg22Total	63	9549	6381	2813	39303
*xcc22*Total	63	10583	7886	1982	49085
flgII-28Total	63	17350	22186	843.75	144336
Maximum photon count					
flgII-28Max	63	2825	3271	298.17	21499
flg22Max	63	2262	1549	720.10	8592
*xcc22*Max	63	2497	1794	340.81	9880
Disease score					
AUDPC	63	26.7919	3.18753	18.52	34.92

[z]Total photon counts measured by Luminometer during ROS production

Table 3.7. Correlation analysis between total and maximum photon count produced in reactive oxygen species (ROS) assay using flg22, flgII-28 and *xcc22* peptides and area under disease progress curve for combined data including greenhouse and field experiments in 2013

Pearson Correlation Coefficients, N = 63							
Prob > |r| under H0: Rho=0							
	AUDPC	flg22Total	flg22Max	flgII-28Total	flgII-28Max	*xcc22*Total	*xcc22*Max
AUDPC	1						
flg22Total	-0.04 ^{NS}	1					
flg22Max	-0.10 ^{NS}	0.96***	1				
flgII-28Total	0.13 ^{NS}	0.13 ^{NS}	0.15 ^{NS}	1			
flgII-28Max	0.15 ^{NS}	0.11 ^{NS}	0.15 ^{NS}	0.97***	1		
*xcc22*Total	-0.25**	0.41**	0.42**	0.04 ^{NS}	-0.002 ^{NS}	1	
*xcc22*Max	-0.27**	0.37**	0.43**	0.03 ^{NS}	0.01 ^{NS}	0.95***	1

^{NS}, *, ** and *** are non- significant, significant at P-value <0.05, 0.01 and 0.0001 respectively.
AUDPC Area under disease progress curve
BS Bacterial spot

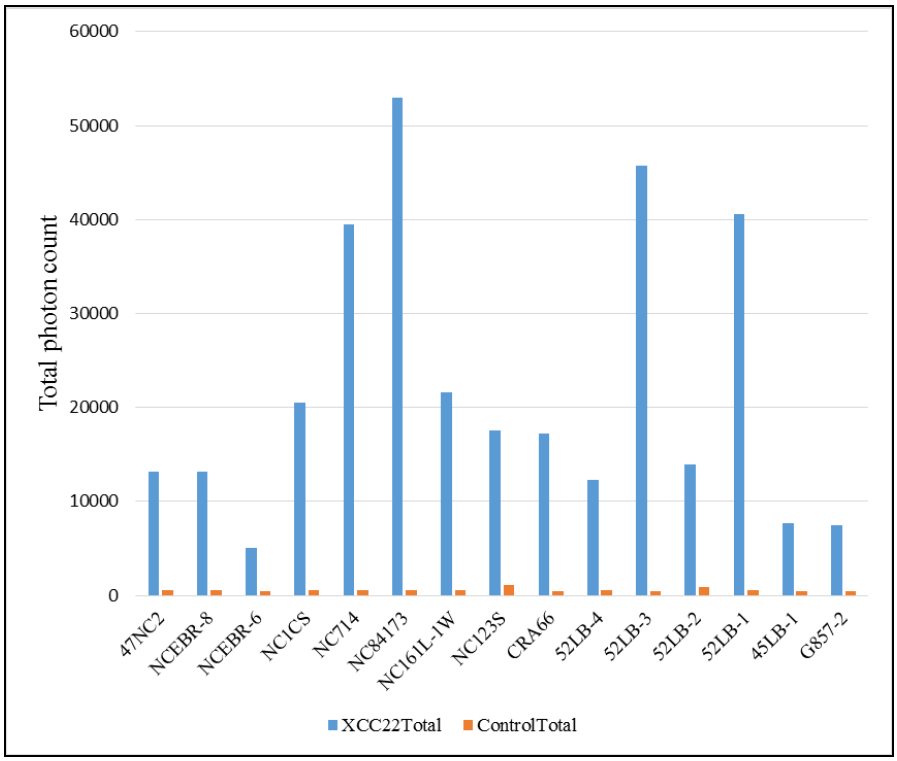


Figure 3.1. Total photon count in reactive oxygen species (ROS) assay using *xcc22* peptide and dH2O

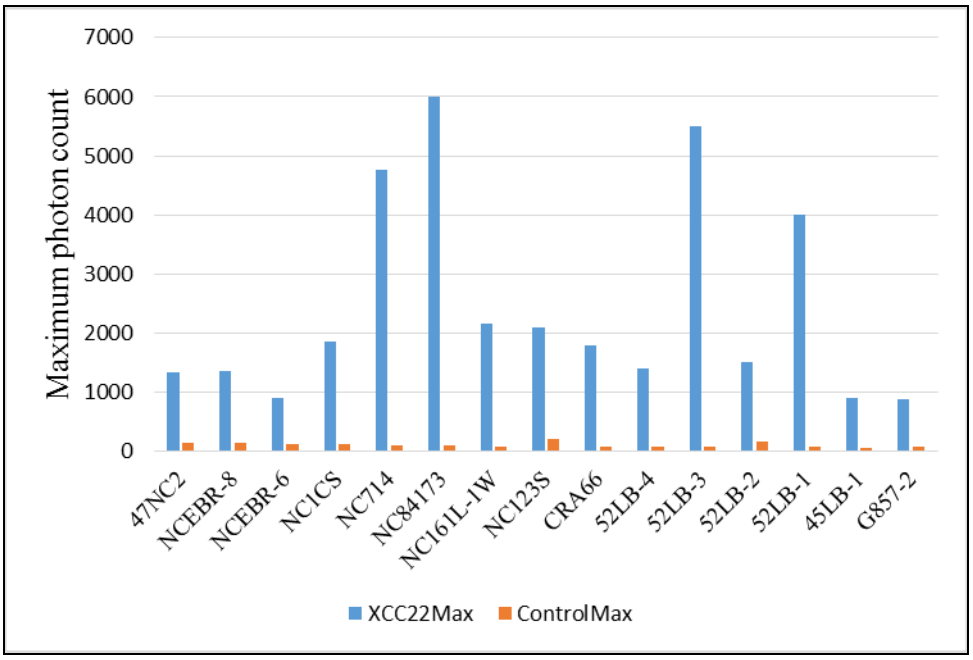


Figure 3.2. Maximum photon count in reactive oxygen species (ROS) assay using *xcc22* peptide and dH2O

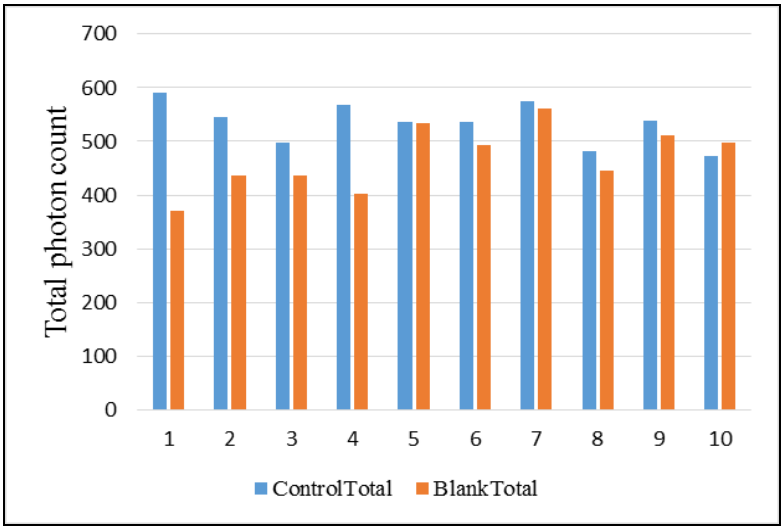


Figure 3.3. Total photon count in reactive oxygen species (ROS) assay using control wells with leaf discs, horseradish peroxidase and luminol and blank wells with horseradish peroxidase, luminol and dH2O but no leaf sample

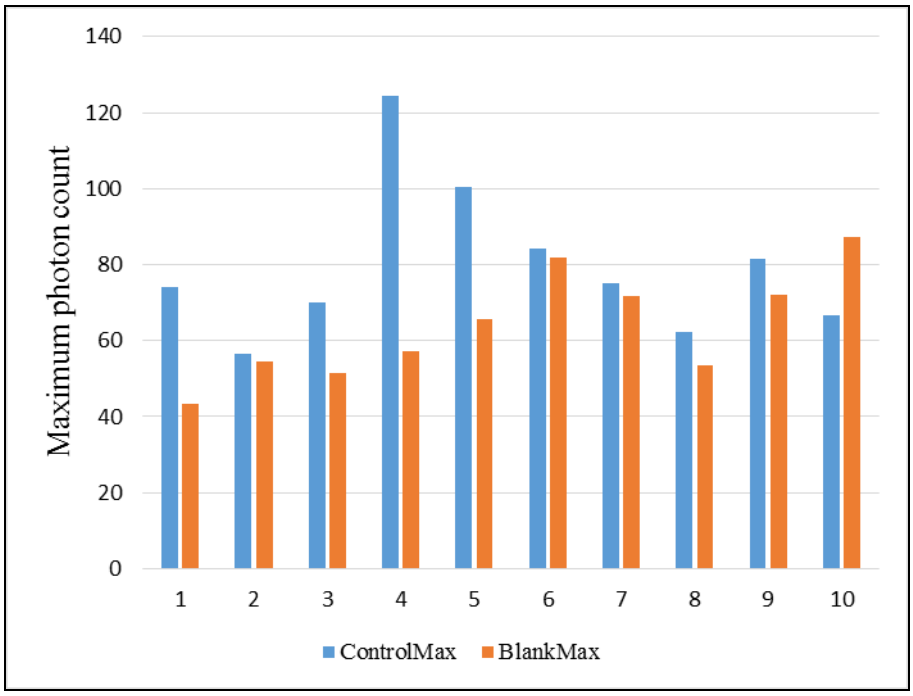


Figure 3.4. Maximum photon count in reactive oxygen species (ROS) assay using control wells with leaf discs, horseradish peroxidase and luminol and blank wells with horseradish peroxidase, luminol and dH2O but no leaf sample

CHAPTER 4 PRINCIPAL COMPONENT AND CLUSTER ANALYSIS OF TOMATO (*SOLANUM LYCOPERSICUM* L.) GENOTYPES

ABSTRACT

Tomato is one of the most economically important vegetable crops in the world. Determination of genetic diversity of tomato is useful for tomato breeding programs in order to develop desired varieties that can fulfill the needs of the world today. Cluster analysis and principal component analysis (PCA) has been used to study genetic diversity in tomato using phenotypic, biochemical and molecular information. Seventy one tomato genotypes were planted at the Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, NC, in two replications and randomized complete block design. Genotypes, including wild, heirloom and advanced lines, were evaluated for eight vegetative and reproductive traits using a scale from tomato descriptor published by the international plant genetic resource institute (IPGRI) with some modifications. Hierarchical cluster analysis produced six clusters based on Average-linkage method and was presented in the form of a dendrogram. These clusters were clearly separated from each other particularly differentiating the fruit traits. Five principal components explained more than 92% of the phenotypic variation in the genotypes based upon the traits included in PCA. Fruit size, shape and category and maturity were included in PC1, height and growth types were included in PC2 and leaf characteristics, color and type were included in PC3. Clusters produced in this analysis can be of importance for breeding programs in order to develop specific fruit types depending upon consumer's demand or production enhancement.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is the second most important vegetable in production in the world (FAOSTAT, 2008). United States ranks second among all countries in tomato production by volume (FAO, 2011). Despite being second largest producer, US was the leading importer of tomatoes in the whole world in 2010 (http://www.unctad.info/en/Infocomm/AACP-Products/COMMODITY-PROFILE---Tomato/). This clarifies that there exists a huge market for tomato, grown inside the country, in the US.

Cultivated tomato is reported to be in existence for only about 400 years (Boswell, 1937). It was introduced to Europe from Central and Southern America, considered to be center of origin of tomato. The first cultigens grown in the US came from England and France and represent a narrow range of the genetic diversity due to bottlenecking in the cultivated tomato germplasm that occurred in Europe (Rick, 1976) and by subsequent selection. Genetic variation in wild species has been the source of traits for crop improvement in quality and disease and insect resistance in modern breeding programs (Rick and Chetelat, 1995).

One of the major goals that has garnered considerable effort and resources in tomato breeding programs in the US has been to increase yield. Success has been achieved but there remains a huge demand to enhance production efficiency and to meet domestic demand along with improving the quality and health benefitting factors of the produce.

Tomato breeding program of North Carolina State University has been focused on increasing fruit size, increasing marketable yield, improving fruit quality and advancing disease resistance (Panthee and Gardner, 2011). Growth habit, shelf life, fruit smoothness and fruit

firmness have also been improved in recent years (Gardner and Panthee, 2010; Gardner, 1990; Gardner, 2000).

Knowledge and data about the levels and patterns of genetic diversity can be a significant aid in plant breeding for various applications (Mohammadi and Prasanna, 2003) like analysis of genetic variability in cultivars (Cox et al., 1986; Smith, 1988), identification of diverse parental combinations for creating segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998) and introgression of desirable genes from diverse germplasm into the available genetic base (Thompson and Nelson, 1998). In order to develop desired tomato varieties, it is important to catalogue the genetic diversity within the germplasm (Islam, 2004). Morphological traits have been used in estimating genetic diversity and cultivar development since they provide a simple way of quantifying genetic variation (Fufa et al., 2005). It is necessary to select parent for hybridization appropriately in order to enhance the genetic recombination for potential yield increase (Islam, 2004). The principle of clustering is to join genotypes into groups, so that there is uniformity within and heterogeneity among groups. Here we present a cluster analysis of seventy one tomato lines by means of hierarchical cluster analysis based upon eight vegetative and reproductive traits.

MATERIALS AND METHODS

Plant material

Seventy one genotypes including advanced breeding lines, heirlooms and wild genotypes genotypes were sown in flat bed metal trays in a standard seeding mix (2:2:1 (v/v/v) peat moss:pine bark:vermiculite with macro- and micro-nutrients (Van Wingerden International

Inc., Mills River, NC) on May, 2013. After 10 days, seedlings were transplanted to 72-cell flats (56 cm x 28cm). After four weeks these plants were transplanted to the field at the Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina. Six plants per plot were planted with plant to plant spacing of 45 cm and 150 cm distance between rows in two replications in a randomized complete block design. The recommended management practices for fertilization, insect management and management of foliar diseases were done according to standard recommendations (Ivors et al., 2013).

Data collection

Eight vegetative and reproductive traits of tomato lines were measured using the ratings on tomato descriptor, published by International plant genetic resources institute (IPGRI; http://www.bioversityinternational.org/e-library/publications/detail/descriptors-for-tomato-emlycopersiconem-spp/), with some modifications in scale. Traits measured were growth type, height, leaf type, leaf color, fruit shape, fruit size, maturity and fruit category (cherry, grape, plum or large fruited). All traits were measured by visual observations.

Statistical analysis

Data analysis was conducted using SAS Software version 9.3 (SAS Institute, Inc. Cary, NC, USA). Analysis of variance (ANOVA) was done to identify if genotypes were significantly different from each other for those traits. Correlation analysis was done using the Pearson product-moment correlation coefficient (Pearson, 1985). Principal component analysis (PCA) and cluster analysis was performed on the data sets. Cluster analysis was done using the Average-linkage method. Clustering results are presented graphically by means of dendrograms showing clusters.

RESULTS AND DISCUSSION

The list of tomato lines from diverse genetic background used in the study is given (Table 4.1). All vegetative and reproductive traits considered to have a direct or indirect relationship with yield were selected and phenotyped for this experiment. Analysis of variance (ANOVA) of the traits showed that genotypes were significantly different for all traits (P<0.0001) (Table 4.2). Growth habit and height of tomato plant helps in explaining length of production period. Determinate lines are easy to manage but have few fruit clusters in comparison to indeterminate lines. Leaf type and leaf color explain about the chlorophyll content, surface area and orientation of leaves in plants that leads to production and conversion of photosynthesized substrates into fruits. Fruit shape and size are directly correlated with the yield where increment in fruit size leads to increase in yield. Categories of tomato fruits, which differentiates type of fruit into plum, cherry, grape and beef tomato, contribute differently in tomato fruit production. Cultivation of tomato with different fruit traits also depends upon the preference of farmer and consumer. A matrix of Pearson correlation coefficients between all traits recorded is given (Table 4.3). Correlation above 0.4 are discussed but all significant correlation coefficients are flagged. Significant correlation, (r = 0.97, P< 0.0001), was observed between growth type and height of tomato plants (Table 4.1). Significant correlations were obtained between leaf type and three other traits, leaf color (r = 0.65, P< 0.0001), fruit size (r = 0.49, P< 0.0001) and fruit category (r = 0.42, P = 0.0002) (Table 4.3). Fruit size was significantly correlated with fruit shape (r = 0.51, P < 0.0001) (Table 4.3). Significant negative correlation (r = -0.37, P= 0.002) was observed between fruit size and maturity (Table 4.3). Generally small sized tomato matures earlier

than larger sized tomato and vice versa. Fruit size was also significantly correlated with fruit category (r = 0.41, P= 0.0004) (Table 4.3). Maturity was found to be negatively correlated with fruit category (r = -0.61, P< 0.0001) which explains maturity of grape earlier than beef or plum type tomato (Table 4.3).

Cluster analysis based on the Average linkage method grouped seventy one tomato genotypes into six clusters (Figure 4.1). Tomato genotypes grouped in each cluster are presented in table 4.2. Cluster 1 consisted of one genotype, cluster II consisted of two genotypes, cluster III consisted of 7 genotypes, cluster IV consisted 50 genotypes, cluster V consisted of 8 and cluster VI consisted of 3 genotypes.

CLUSTER I

Only one tomato genotype was included in this cluster (Table 4.4). This is an indeterminate genotype. Fruits of this genotype are large, have a unique shape, which resembles with bell pepper, are late ripening and turn yellow when ripe. Fruits are partially hollow inside. This genotype can be used by breeding programs for unique fruit shape for particular niche markets.

CLUSTER II

This cluster included two genotypes (Table 4.4) that are indeterminate and tall. Fruits of these genotypes are small. Yellow pear has yellow, small, pear-shaped fruit where as NC22L-1(2008) is a red colored grape tomato. Genotypes from this cluster are important in fresh market as salad tomatoes.

Cluster III

This cluster includes seven genotypes (Table 4.4). All of the genotypes included in this cluster have plum-shaped fruits. Leaf color of these genotypes is relatively dark green. Maturity ranges from early to late in this cluster. Growth type of these genotypes ranges from determinate to indeterminate and plant height also ranges from short to tall. These genotypes can be used in breeding plum shaped tomatoes by breeding programs.

Cluster IV

This cluster includes 50 genotypes (Table 4.4) of which 49 are large fruited and one is plum shaped. These genotypes have a standard tomato leaf type. This is a large cluster as it represents a priority in tomato breeding programs to develop large fruited tomato cultivars. This cluster contains heirloom and advanced genotypes from breeding programs. These genotypes can be of importance in breeding for large fruited tomatoes.

Cluster V

This cluster includes eight genotypes (Table 4.4). Fruits of these genotypes are medium size and round. They are mostly early maturing except CRA66. These lines range from semi-determinate to an indeterminate growth habit. This cluster is not very important for desired fruit characteristics. However, some lines like CRA 66 and HI 7997, and HI 7981 and HI 7998 and are reported to have resistance genes for bacterial wilt (Sonoda and Augustine, 1978) and bacterial spot diseases (Somodi et al., 1994; Scott et al., 1995). So, these can be used in breeding programs for disease resistance.

CLUSTER VI

This cluster includes three genotypes (Table 4.4). Two of these genotypes PI114490-1-1 and PI134417 are wild lines (Table 4.4). Genotypes in this cluster are indeterminate and tall. They have small leaves with serrated margins. Leaves of PI134417 have obvious long leaf trichomes on the leaf surface. They have very small fruits and resemble cherry tomato. Fruits of PI134417 have significantly long fruit-hairs and do not drop even after fruits mature. Fruits of this genotype are late maturing and always stay green.

Principal component analysis (PCA)

Two dimensional graphical representation of component patterns based on PC1 and PC2 is shown in Figure 4.2. PCA showed that five principal components explained more than 92% of the total variation among the genotypes (Table 4.5). Scree plot and explanation of variance of individual PCs are shown in Figure 4.3. Rotated factor pattern indicated the fruit traits including fruit size, fruit shape, fruit category and maturity were included in principal component (PC) 1; height and growth type in PC2 and leaf type and leaf color in PC3 (Table 4.6). Scatter plot and 95% prediction ellipse based upon PC1 and PC2 are given in Figure 4.4 and 4.5 respectively.

Diversification in tomato fruits depending upon demand and consumption has been developed by breeding programs all over the world. Generally for fresh market, large fruited tomato is preferred, which also includes heirloom varieties. Along with large fruited lines, plum tomato are also used very frequently. Grape and cherry tomatoes are often consumed raw e.g. in salads because of high sugar and acid content as compared to use of large size fruits (McGillivray and Clemente, 1956). An inverse relationship between yield and soluble

solids has been shown by Stevens and Rudich (1978). With increasing demand of production, breeding programs need to set their goals accordingly. Recent trends direct the breeding programs objectives towards developing high yielding disease resistance varieties. As a result, large fruited high yielding tomato varieties are a major focus. This can also be seen in the number of large fruited advanced lines in the cluster analysis (Table 4.4). As depicted by the cluster analysis, genotypes from the clusters can be used for developing lines of particular type for meeting the objective and demand in future by breeding programs.

REFERENCES

Barrett, B.A. and K.K. Kidwell. 1998. AFLP-based genetic diversity assessment among wheat cultivars from the Pacific Northwest. Crop Sci. 38(5):1261-1271.

Boswell, V. R. 1937. Improvement and genetics of tomatoes, peppers and eggplant. U.S. Dept. Agr. Yearbook 1937: 176206.

Cox, T., J. Murphy, and D. Rodgers. 1986. Changes in Genetic Diversity in the Red Winter-Wheat Regions of the United-States. Proc. Natl. Acad. Sci. U. S. A. 83(15):5583-5586.

FAO, 2011. http://faostat.fao.org/site/339/default.aspx

FAOSTAT. 2008. FAO statistical databases. Food and agriculture organization of the United Nations, Statistics Division. 8 Apr. 2012. <http://faostat.fao.org/site/339/default.aspx>

Fufa, H., P. Baenziger, B. Beecher, I. Dweikat, R. Graybosch, and K. Eskridge. 2005. Comparison of phenotypic and molecular marker-based classifications of hard red winter wheat cultivars. Euphytica 145(1-2):133-146.

Gardner, R.G. and D.R. Panthee. 2010. 'Plum Regal' Fresh-market Plum Tomato Hybrid and Its Parents, NC 25P and NC 30P. HortScience 45(5):824-825.

Gardner, R. 1990. Mountain Delight Tomato - NC-8288 Tomato Breeding Line. HortScience 25(8):989-990.

Gardner, R. 2000. 'Carolina Gold", a hybrid tomato, and its parents, NC1Y and NC2Y. HortScience 35(5):966-967.

Islam M.R. 2004. Genetic diversity in irrigated rice. Pak J Biol Sci 2:226-229.

Ivors K. L. and Louws F. J. 2013. 2013 North Carolina Agricultural Chemicals Manual. College of Agriculture and Life Sciences, North Carolina State University.

Karl Pearson. 1895. Notes on regression and inheritance in the case of two parents. Proceedings of the Royal Society of London. 58: 240–242.

McGillivray, J.H. and L.J. Clemente. 1956. Effect of tomato size on solids content. Proc. Amer. Soc. Hort. Sci. 68:466-469.

Mohammadi, S. and B. Prasanna. 2003. Analysis of genetic diversity in crop plants - Salient statistical tools and considerations. Crop Sci. 43(4):1235-1248.

Panthee, D.R. and R.G. Gardner. 2011. Genetic Improvement of Fresh Market Tomatoes for Yield and Fruit Quality Over 35 Years in North Carolina: A Review. Taylor & Francis Ltd.

Rick, C. and R. Chetelat. 1995. Utilization of related wild species for tomato improvement. First International Symposium on Solanacea for Fresh Market (412):21-38.

Rick C. M. 1976. Tomato, *Lycopersicon esculentum* (Solanaceae). In: Simmonds, N.W. (Ed.), Evolution of crop plants. Longman group, London, UK, pp. 268-273.

Scott, J., J. Jones, G. Somodi, and R. Stall. 1995. Screening Tomato Accessions for Resistance to *Xanthomonas-campestris* pv. *vesicatoria,* Race T3. HortScience 30(3):579-581.

Smith, J. 1988. Diversity of United-States Hybrid Maize Germplasm - Isozymic and Chromatographic Evidence. Crop Sci. 28(1):63-69.

Somodi, G., J. Jones, J. Scott, and J. Jones. 1994. Screening Tomato Seedlings for Resistance to Bacterial Spot. HortScience 29(6):680-682

Sonoda, R. M. and J. J. Augustine. 1978. Reaction of bacterial wilt resistant tomato lines to *Pseudomonas solanacearum* in Florida. Plant Dis. Rptr. 62:464-466.

Stevens, M.A. and J. Rudich. 1978. Genetic potential for overcoming physiological limitations on adaptability, yield, and quality in the tomato. HortScience. 13(6):673-677.

Thompson, J. and R. Nelson. 1998. Core set of primers to evaluate genetic diversity in soybean. Crop Sci. 38(5):1356-1362.

Table 4.1. List of tomato lines planted in the fields of Mountain Horticultural Crops Research and Extension Center (MHCREC), Mills River, NC in 2013

Genotypes	Scientific name	Comment	Source
NC714	*Solanum lycopersicum*	Breeding line	NCSU
081-12-1X-gsms	*S. lycopersicum*	Breeding line	NCSU
15BC-4(94)	*S. lycopersicum*	Breeding line	NCSU
16BC-1(94)	*S. lycopersicum*	Breeding line	NCSU
16BC-2(94)	*S. lycopersicum*	Breeding line	NCSU
17BC-1(94)	*S. lycopersicum*	Breeding line	NCSU
30LB-1W(95)	*S. lycopersicum*	Breeding line	NCSU
31LB-1W(95)	*S. lycopersicum*	Breeding line	NCSU
38BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
38BC-2R(96)	*S. lycopersicum*	Breeding line	NCSU
39BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
45LB-1	*S. lycopersicum*	Breeding line	NCSU
46BC-2R(96)	*S. lycopersicum*	Breeding line	NCSU
47NC2	*S. lycopersicum*	Breeding line	NCSU
48BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-1R(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-3R(96)	*S. lycopersicum*	Breeding line	NCSU
48BC-4R(96)	*S. lycopersicum*	Breeding line	NCSU
52LB-1	*S. lycopersicum*	Breeding line	NCSU
52LB-2	*S. lycopersicum*	Breeding line	NCSU
52LB-3	*S. lycopersicum*	Breeding line	NCSU
52LB-4	*S. lycopersicum*	Breeding line	NCSU
71BC-1(96)	*S. lycopersicum*	Breeding line	NCSU
72E-1(96)	*S. lycopersicum*	Breeding line	NCSU
74L-1W(2008)	*S. lycopersicum*	Breeding line	NCSU
87E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
89E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-1W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-2W(95)	*S. lycopersicum*	Breeding line	NCSU
97E-3W(95)	*S. lycopersicum*	Breeding line	NCSU
AkersWestVirginia	*S. lycopersicum*	Heirloom	
BlackfromTula	*S. lycopersicum*	Heirloom	
Brandywine	*S. lycopersicum*	Heirloom	
CherokeePurple	*S. lycopersicum*	Heirloom	
CRA66	*S. lycopersicum*	Breeding line	France

Table 4.1 Continued

Favorite	*S. lycopersicum*	Breeding line	
FD502-3-BK	*S. lycopersicum*	Breeding line	NCSU
Fla7600	*S. lycopersicum*	Breeding line	UFL
Fla8000	*S. lycopersicum*	Breeding line	UFL
Fla8233	*S. lycopersicum*	Breeding line	UFL
G357-1(2011)	*S. lycopersicum*	Breeding line	
G357-2(2011)	*S. lycopersicum*	Breeding line	
HI7981	*S. lycopersicum*	Breeding line	UFL
HI7997	*S. lycopersicum*	Breeding line	UFL
HI7998	*S. lycopersicum*	Breeding line	UFL
IRAT-L3	*S. lycopersicum*	Breeding line	France
Moneymaker	*S. lycopersicum*	Heirloom	
NC109	*S. lycopersicum*	Breeding line	NCSU
NC123S	*S. lycopersicum*	Breeding line	NCSU
NC161L-1W(2007)	*S. lycopersicum*	Breeding line	NCSU
NC1CELBR	*S. lycopersicum*	Breeding line	NCSU
NC1CS	*S. lycopersicum*	Breeding line	NCSU
NC22L-1(2008)	*S. lycopersicum*	Breeding line	NCSU
NC2CELBR	*S. lycopersicum*	Breeding line	NCSU
NC50-7	*S. lycopersicum*	Breeding line	NCSU
NC84173	*S. lycopersicum*	Breeding line	NCSU
NCEBR-6	*S. lycopersicum*	Breeding line	NCSU
NCEBR-8	*S. lycopersicum*	Breeding line	NCSU
OrangeStrawberry	*S. lycopersicum*	Heirloom	
Oxheart	*S. lycopersicum*	Heirloom	
PI114490-1-1	*S. lycopersicum var. cerasiforme*	Wild	USDA
PI134417	*S. habrochaites*	Wild	USDA
Rutgers	*S. lycopersicum*	Breeding line	Rutgers
Stupice	*S. lycopersicum*	Heirloom	
YellowPear	*S. lycopersicum*	Heirloom	
YellowStuffer	*S. lycopersicum*	Heirloom	
918-4B(2007)-9-11	*S. lycopersicum*	Breeding line	NCSU
918-4B(2007)-9-12	*S. lycopersicum*	Breeding line	NCSU
918-4B(2007)-9-13	*S. lycopersicum*	Breeding line	NCSU
NC30P	*S. lycopersicum*	Breeding line	NCSU

Table 4.2. Analysis of Variance of traits used in cluster analysis

Source of variation	Means of squares
Growth	0.691***
Height	2.075***
Leaftype	0.717***
Leafcolor	0.207***
Fruitsize	2.228***
Fruitshape	6.453***
Maturity	0.893***
Fruitcategory	5.608***

*** Significant at probability of <0.0001

Table 4.3. Pearson correlation coefficients table showing the correlation coefficients between traits used in cluster analysis

Pearson Correlation Coefficients, N = 71								
Prob > |r| under H0: Rho=0								
	growthtype	Height	Leaftype	leafcolor	fruitsize	fruitshape	maturity	fruitcategory
Growthtype	1.00							
Height	0.97***	1.00						
Leaftype	0.24*	0.21	1.00					
leafcolor	0.10	0.09	0.65***	1.00				
Fruitsize	0.25*	0.24*	0.49***	0.32*	1.00			
fruitshape	0.10	0.12	0.007	-0.05	0.51***	1.00		
Maturity	-0.10	-0.04	-0.20	-0.14	-0.37*	-0.17	1.00	
Fruitcategory	0.16	0.11	0.42**	0.25*	0.41**	0.19	-0.61***	1.00

*, ** and *** are significant at probability <0.05, 0.001 and 0.0001 respectively.

Table 4.4. Seventy one tomato genotypes grouped into six clusters based upon eight morphological traits

Cluster	Frequency	tomato genotypes
I	1	Yellow stuffer
II	2	Yellow pear, NC22L-1W(2008)
III	7	NCEBR-6, NCEBR-8, NC30P, 918-4B(2007)-9-13, 918-4B(2007)-9-12, 918-4B(2007)-9-11, 78L-1W(2008)
IV	50	Fla7600, Fla8000, Fla8233, NC1CELBR, Brandywine, Black from Tula, Orange strawberry, Aker's west virginia, NC161L-1W(2007), 47NC2, NC109, 72E-1(96), 48BC-1R(96), 97E-3W(95), 39BC-1(96), 97E-1W(95), 48BC-1(96), 31LB-1W(95), Rutgers, 45LB-1(98), 16BC-2(94), NC123S, NC84173, NC50-7, 46BC-2R(96), NC1CS, 89E-1W(95), 38BC-1(96), NC714, NC2CELBR, 87E-1W(95), 71BC-1(95), 16BC-1(94), Stupice, Moneymaker, Cherokee purple, 48BC-3R(96), 97E-2W(95), FD502-3-Bk, 48BC-4R(96), 30LB-1W(95), Oxheart, IRAT-L3, 17BC-1(94), G357-2(2011), G357-1(2011), 74L-1W(2008), Favorite, 38BC-2R(96), 15BC-4(94)
V	8	CRA66, HI7997, HI7981, HI7998, 52LB-4(98), 52LB-3(98), 52LB-2(98), 52LB-1(98)
VI	3	PI134417, PI114490-1-1, 081-12-1X-gsms

Table 4.5. Prior communality estimates, eigenvalues

Eigenvalues of the Correlation Matrix: Total = 8 Average = 1				
	Eigenvalue	Difference	Proportion	Cumulative
1	2.93	1.21	0.3658	0.3658
2	1.71	0.38	0.2141	0.5798
3	1.33	0.36	0.1665	0.7463
4	0.98	0.56	0.122	0.8683
5	0.41	0.05	0.0518	0.9201
6	0.40	0.13	0.0462	0.9663
7	0.24	0.21	0.0299	0.9962
8	0.03		0.0038	1

Table 4.6. Rotated factor pattern

	Factor1	Factor2	Factor3
Growth	9	97*	10
Height	6	98*	6
Leaftype	25	17	85*
Leafcolor	7	3	87*
Fruitsize	74*	22	28
fruitshape	71*	13	-33
Maturity	-72*	6	-17
fruitcategory	69*	1	37

Printed values are multiplied by 100 and rounded to the nearest integer. Values greater than 40 are flagged by an '*'

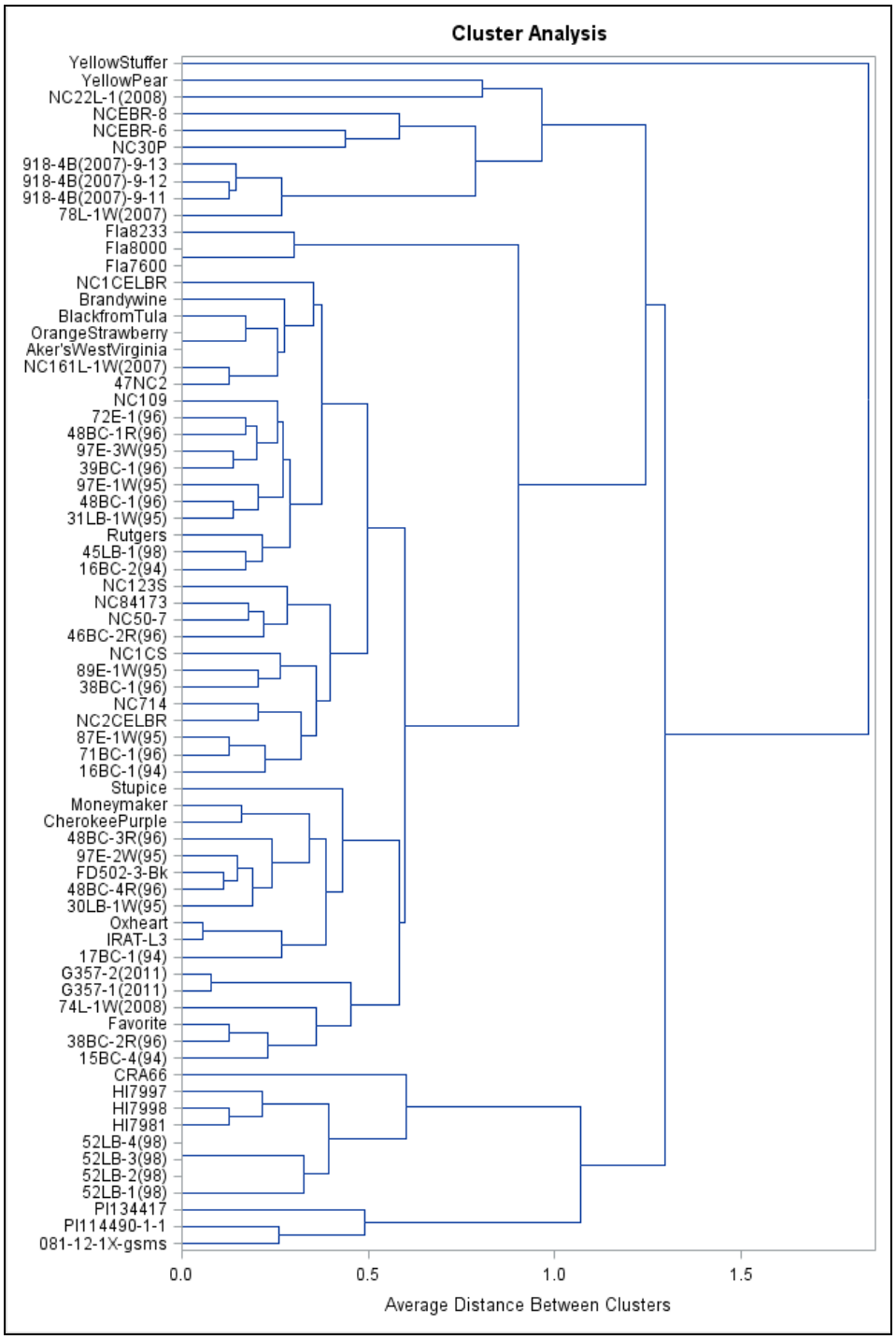


Figure 4.6. Dendrogram based on cluster analysis of tomato lines based upon phenotypic traits

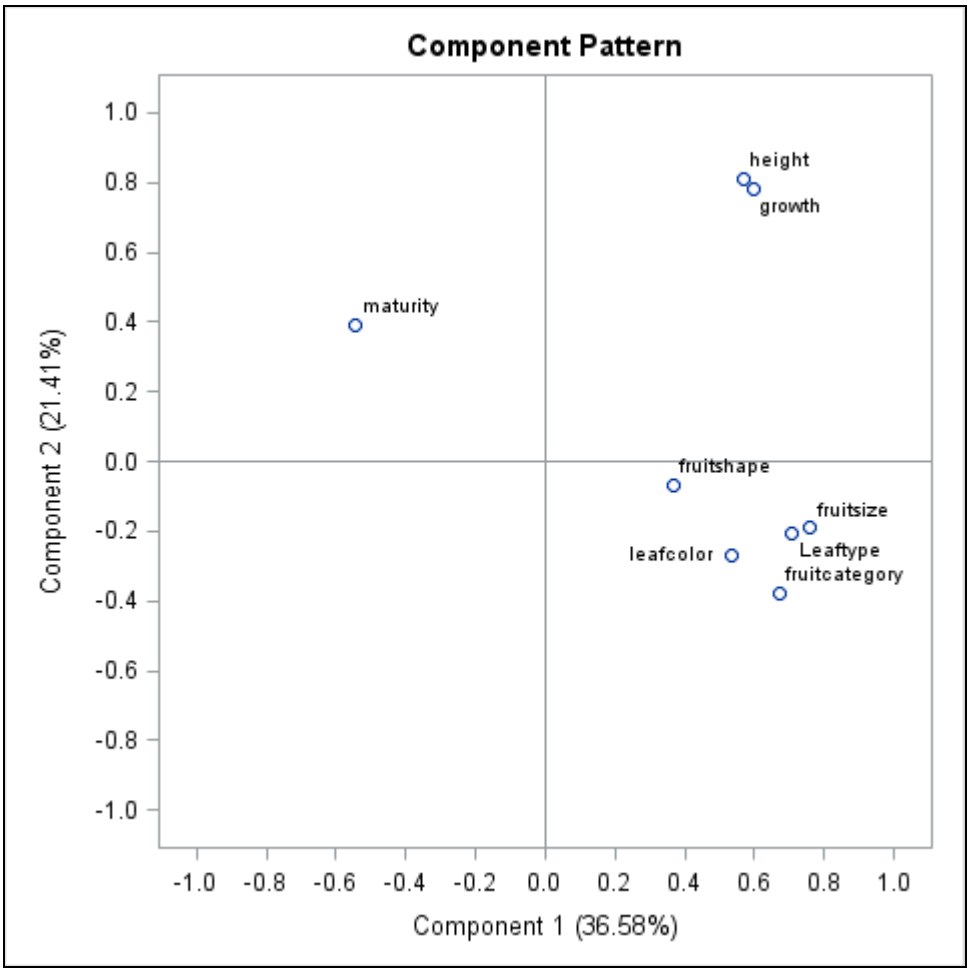


Figure 4.7. Component pattern based upon principal components 1 and 2

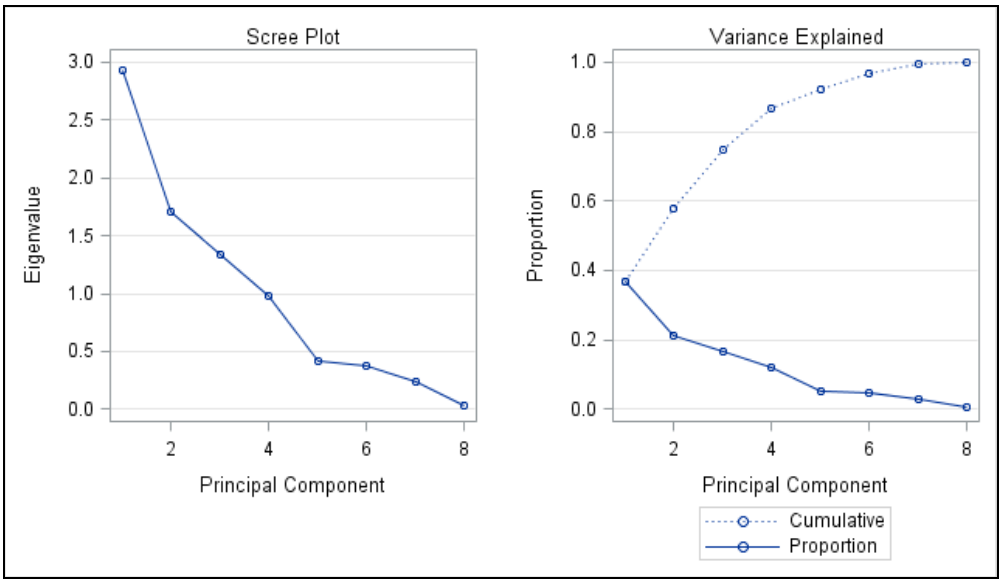


Figure 4.8. Scree plot and explanation of variance by principal components

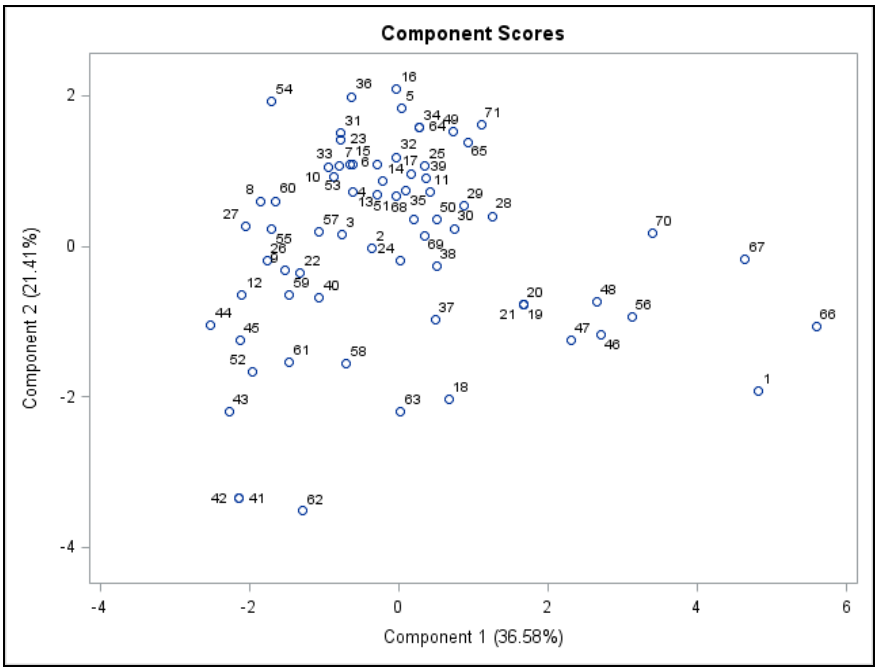


Figure 4.9. Component scores of genotypes based upon of Principal component 1 and 2

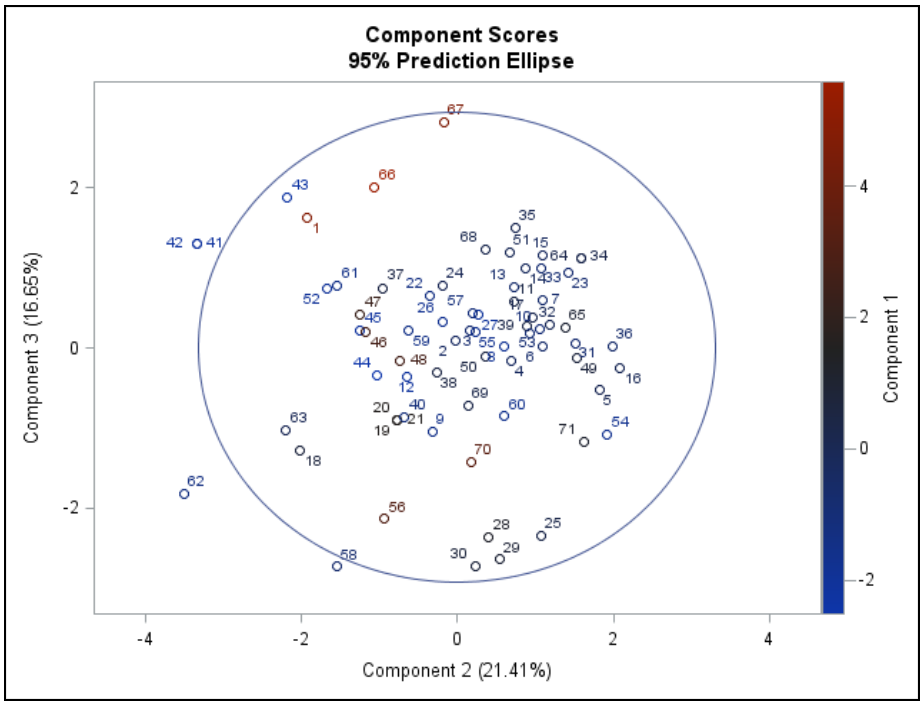


Figure 4.10. Component scores 95% prediction ellipse based upon principal components 1, 2 and 3

CHAPTER 5 FUTURE PERSPECTIVE OF THIS RESEARCH

DEVELOPING BACTERIAL SPOT RESISTANT TOMATO VARIETIES

This is the first report to characterize *Xanthomonas perforans*, race T4 to cause bacterial spot (BS) in tomato in North Carolina. While it was not screened in greenhouse and field conditions, source of resistance for BS, caused by *X. perforans* race T4, was *Solanum pimpinellifolium* L3707 based on pedigree studies. This line is believed to have resistance against BS cause by *X. perforans*, race T3 in Canada (R. G. Gardner, personal communication). In order to investigate further into resistance, mapping population could be developed. Inheritance analysis and mapping genes for BS resistance followed by market assisted selection would lead to gene pyramiding and development of resistant varieties.

In yet another variety trial, tomato breeding line NC109 was found to show least bacterial disease symptoms. Sources of resistance for BS in *S. pimpinellifolium* L3707 and NC109 are different. Therefore, combining the resistance from these two diverse sources would strengthen the resistance against the disease in new varieties.

Difference in BS disease severities in foliar and fruit tissues of tomato plants was interesting. We hypothesize that there are different modes of infection of *Xanthomonas* spp. in foliar and fruit tissues. Hence, mode of infection in these tissues should be studied in a structured experiment. We observed a low correlation between BS disease on tomato foliage and fruits. Further experiment is required to establish this information as a fact.

USE OF MICROBE ASSOCIATED MOLECULAR PATTERNS IN RESISTANCE BREEDING PROGRAMS

Correlation, low but significant, between foliar BS disease severity and ROS production, when *Xanthomonas*-specific flagellin 22 (*xcc22*) peptide was used, indicates the feasibility of applying luminol/peroxidase based ROS assay in screening tomato genotypes for the foliar disease resistance. However, low and non-significant correlation between BS disease on tomato fruits and ROS production, when *Xanthomonas*-specific flagellin 22 (*xcc22*) peptide was used, is interesting. Further research is required in understanding this difference in responses. Higher correlation between ROS production and disease severity can lead to use of this technique in screening tomato lines for BS in tomato breeding programs.

Practical challenge in conducting luminol/peroxidase based ROS assay is addressing the variation in photon count within a genotype. Increasing the number of samples per genotype can be one of the ways to decrease the standard deviation. Further research is required in modification of the protocol to decrease the variation within a genotype. If the variation can be addressed, and through molecular approach pattern recognition receptors (PRRs) that identifies the pathogen associated molecular patterns (PAMPs) from bacterial pathogen can be identified, it has been reported that transfer of PRRs can offer a novel potential biotechnological approach to engineer disease resistant crop plants.

DIVERSITY ANALYSIS OF TOMATO LINES IN NORTH CAROLINA TOMATO BREEDING PROGRAM

Large number of tomato lines clustering in a group of large fruit category indicates the focus of the tomato breeding program in increasing production and productivity. In addition,

resistance for different diseases has also been incorporated in the lines that have been developed from the breeding program. Regarding fruit quality, as explained by the cluster analysis, various fruit categories have been developed for instance large fruited, plum shaped and grape tomatoes with various specific characteristics.

In addition to the phenotypic observation, on which basis diversity analysis was conducted, molecular data and pedigree analysis can lead to the sources through which genes present in the germplasm can be further identified and used in future. Furthermore, gene pyramiding of essential genes into promising representative lines of each cluster can result in outstanding tomato varieties with desired characteristics which is one of the major goal of tomato breeding programs.