ABSTRACT

INGRAM, THOMAS WILLIAM. Grafted Tomatoes for Disease and Low-Disease Fields and Differentiating *Verticillium dahliae* from Around the World using Whole Genome Resequencing (Under the direction of Drs. Frank J. Louws and Ralph Dean).

Verticillium dahliae is a soil-borne fungus that causes disease on over 200 plant species from around the world. Most modern tomato (*Solanum lycopersicum* L.) cultivars have resistance to race 1 *V. dahliae*, however, consistent non-race 1 resistance has been elusive. Non-race 1 strains of *V. dahliae* are widespread in Western North Carolina. In 2017 and 2018 several tomato rootstocks with reported non-race 1 resistance and/or vigorous growth characteristics were planted in a non-fumigated *V. dahliae* infested field. The rootstocks were planted by themselves to determine their relative resistance level, or they had the non-race 1 susceptible scion 'Red Defender' (RD) grafted onto them to determine if they provide any protective benefit. Yield and disease ratings data showed that in both years many resistant rootstocks were identified. However, only 'Maxifort' managed to significantly increase yield in the susceptible RD scion. 'Maxifort' itself is only moderately resistant to non-race 1 *V. dahliae*; other tomato lines such as 'SG-06-220', 'Hawaii 7998', 'NC-7GEM', 'NC-6GEM', had significantly higher resistance than 'Maxifort', but this increased resistance did not protect the scion or improve yield. In both years 'NC-7GEM' had the lowest incidence and severity of *V. dahliae* foliar symptoms. In 2018 'NC-7GEM' was grafted onto the susceptible RD ('NC-7GEM'-RD). The AUDPC of 'NC-7GEM'-RD was statistically similar to non-grafted 'NC-7GEM'. Additionally, RD-'NC-7GEM' and RD had statistically higher AUDPC than 'NC-7GEM' and 'NC-7GEM'-RD, indicating that this non-race 1 *V. dahliae* resistance is more prevalent in above ground portion of a plant. Given these results using these sources of resistance in rootstocks alone is unlikely to provide an economic benefit sufficient enough for growers to utilize them in the field.

However, using a moderately resistant/highly vigorous rootstock such as ‘Maxifort’ may be economically beneficial, especially when combined with a resistant scion such as ‘NC-7GEM’.

A new source of qualitative resistance from the tomato rootstock ‘Aibou’ has been shown to prevent race 2 *V. dahliae* (Vd2) from causing disease in tomatoes in Japan. However, when 13 single-spore isolates from 6 different fields in North Carolina were inoculated onto ‘Aibou’, each isolate was able to be isolated from 2nd leaf. These results indicate that NC strains of *V. dahliae* are most likely non-race 1 or 2 (aka VdN or race 3). Only one isolate from NC, and one isolate from CA were unable to cause disease on Aibou and can referred to as Vd2. Multi-locus sequence analysis of 6 gene regions totaling 2400 bp revealed only 2 nucleotides differences. In order to more comprehensively describe these *V. dahliae* isolates, the genomes of 10 North Carolina, 4 California, and 6 Japanese isolates were sequenced. Using these lines and the already sequenced reference genome isolate (VdLs17) a genome wide study of secreted effectors was undertaken. 201 secreted effector like genes were found out of ~10,000 genes. Only one gene was found that had a SNP in the Vd2 isolates to distinguish them from VdN isolates. The gene is currently the most likely candidate effector responsible for the shift from race 2 to VdN.

Grafted Tomatoes for Disease and Low-Disease Fields and Differentiating *Verticillium dahliae* from Japan, California, and North Carolina using Whole Genome Resequencing

by
Thomas William Ingram

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Plant Pathology

Raleigh, North Carolina

2020

APPROVED BY:

Dr. Frank J. Louws Committee Co-Chair	Dr. Ralph Dean Committee Co-Chair
Dr. Chris Gunter	Dr. Ricardo Hernandez

DEDICATION

I would like to dedicate this dissertation to my parents. My father Dr. David Ingram, and my mother Angela Cross. No child could ask for more loving and diligent parents. My parents laid a strong foundational education and a stable loving environment, as well as all the nature documentaries and Dr. Who episodes we could digest. My parents also supported my journey through the Boys Scouts, which instilled me with a lifelong love of nature. They also took on the challenge of home-schooling me and my siblings, which greatly contributed to my early life education. My parents have also provided a great deal of support after I left home, and without that support it is unlikely I would be where I am today.

I would also like to dedicate this research to my fiancé Dr. Mengying Wang, and my friends Keunrak Kang, Dr. Heather Zimmerman, and Peter Malinski. All of these people have been there for me in the good and bad times. Their friendship and support are the things I treasure the most.

BIOGRAPHY

Thomas W. Ingram grew up in Athens Ohio, nestled in the foothills of the Appalachian Mountains. Thomas studied plants at Ohio University where he received his BS in Plant Biology, with a minor in Psychology. During his time at OU Thomas developed a love for Mycology, Plant Pathology facilitated by his undergraduate advisor and teacher Dr. Arthur Trese, and his teacher Dr. Sarah Wyatt. Thomas attended University of Georgia where he studied Pecan Anthracnose and received his MS in Plant Pathology under the advisement of Dr. Timothy Brenneman. Thomas also had the opportunity to hunt for pecan truffles with canines. Thomas worked as a contractor research technician for US Department of Agriculture where he again had the opportunity to work with dogs, but this time hunting for citrus greening and citrus canker. Soon the desire to perform his own research overcame his desire for a decent wage and reasonable hours, and Thomas eventually found himself back in back in graduate school at North Carolina State University. Thomas' lifelong pursuit of knowledge has led him down many paths, but a love for exploring the wonders of nature, and supportive family and friends sustained him on his journey!

ACKNOWLEDGMENTS

I would like to thank my advisors Dr. Frank Louws and Dr. Ralph Dean. Dr. Louws recruited me to my job as a contractor at the USDA, then transitioned to my advisor when I came to NC State. Both Dr. Louws and Dr. Dean deserve a great deal of credit for being outstanding mentors. I would also like to include Dr. Chris Gunter, Dr. Ricardo Hernandez, Dr. Tika Adhakari, Dr. Yeon Yee Oh, and Dr. Penelope Perkins-Veazie for their many contributions to the planning, execution, and post-processing of my various projects.

I am very grateful for my fellow graduate students, especially Dr. Jonathan Kressin who endured many long and arduous trips to the mountains with me. Jonathan was very proactive in the first 2 years of my program and taught me a great deal about tomato production and science in general.

I also need to thank Inga Meadows, Suzette Sharpe, and Marlee Trandel, as well as Jeff Chandler and Ryan Loudy, and the staff at the Mountain Horticultural Crops Research and Extension Center for their help with managing my on-station tomato field trials in 2017 and 2018. Without their diligence and hard work much of my field work would not have been possible.

The best field work happens in grower fields, and Kirby Johnson and his partners at Flavor First Growers and Packers donated a large amount of field space to my projects. In addition to space they also tended to the everyday maintenance of the tomatoes we planted. Without this level of cooperation from growers the best type of research in plant pathology we would not be possible.

I would also like to thank my former advisors and supervisors who were instrumental in bringing me to this point in my life. Thank you Dr. Arthur Trese my undergraduate advisor who

inspired me to pursue a career in plant pathology. Thank you, Dr. Sarah Wyatt, for being one the best teachers I have had, without your plant genetics class I am not sure I would have changed majors to plant biology. Thank you Dr. Tim Brenneman, my MS advisor, I appreciate the hard work and dedication you put into your career, you were a great role model for me, and I hope I gained some wisdom from you. Thank you, Dr. Steven Rideout, without your kind words I am not sure I would have returned to get a PhD. Thank you, Dr. Tim Gottwald, I learned so much working at the USDA, thank you for being such an inspiration for me. There are many more men and women who I have not listed who deserve credit for helping me get to where I am today. It truly does take a village to raise a PhD.

TABLE OF CONTENTS

LIST OF TABLES viii

LIST OF FIGURES x

CHAPTER 1: INTRODUCTION 1
Pathogen Biology 1
Resistance to *V. dahliae* 2
Disease Cycle and Management 4
Grafting Tomatoes for Yield and Disease Resistance 5
Research Objectives 5
References 7
Figures 13

CHAPTER 2: PRUNING REDUCES YIELDS IN GRAFTED TOMATOES PLANTED IN THE FIELD 16
Abstract 16
Introduction 16
Materials and Methods 18
Results and Discussion 19
Impacts of pruning on grafted tomato yields and fruit sizes 19
Impact of spacing on yield and fruit size 20
Comparison between grafted and non-grafted MFP and TL yields and fruit sizes 20
Conclusions 21
Acknowledgements 21
References 22
Tables 24
Figures 29

CHAPTER 3: VIGOROUS TOMATO ROOTSTOCKS IMPROVE YIELDS AND INCREASE FRUIT SIZES IN GRAFTED FRESH MARKET TOMATOES 31
Abstract 31
Introduction 33
Materials and Methods 35
Field Setting and Experimental Design 35
Rootstocks and scions 36
Pinching 36
Harvest 36
Fruit quality characteristics 37
Statistical Analysis 38
Results 38
Rootstock effects on yield 38
Pinching effect on yield 38
Plant spacing effect on yield 39
Rootstock effect on fruit quality 39
Discussion 39

Conclusions 41
Acknowledgements 42
References 42
Tables 47
Figures 51
Supplemental Tables 57

CHAPTER 4: *VERTICILLIUM* WILT AND BACTERIAL SPOT RESISTANT TOMATO SCION WITH VIGOROUS ROOTSTOCKS INCREASES YIELDS IN GRAFTED TOMATOES IN NON-FUMIGATED FIELDS 60
Abstract 60
Introduction 62
Materials and Methods 64
Fields conditions 64
Rootstock scion combinations 65
Disease ratings 66
Yield and fruit ratings 66
Vigor ratings 66
Nutrient analysis 67
Confirmation of VdN in field 68
Results 68
Effects of self-grafting and fumigation on VW disease development and yield 68
Impact of grafting on VdN disease progression 69
Impact of grafting on bacterial spot disease progression 69
Vigor comparisons 69
Yield and fruit sizing 69
Nutrient analysis 70
Discussion 70
References 73
Tables 77
Figures 83
Supplemental Table 95
Supplemental Figures 96

CHAPTER 5: WHOLE GENOME RESEQUENCING AND MACHINE LEARNING REVEAL CANDIDATE GENE FOR VERTICILLIUM DAHLIAE RACE 2 101
Abstract 102
Introduction 103
Materials and Methods 107
Fungal Isolation 107
DNA Extraction 107
Whole Genome Sequencing 108
Coding Sequence Extraction 108
Secreted Effector Discovery 108
Phylogenetic Analysis 109
Unassembled reads effector discovery 109

Pathogenicity assay 109

Results 110
Comparison of coding sequences of the 18 *V. dahliae* isolates 110
Race designation of *V. dahliae* isolates 111
Genetic diversity in *V. dahliae* 112
Effector analysis of 18 *V. dahliae* isolates and the reference genome VdLs17 113
De novo Assembly of Unmapped Reads 114

Discussion 115
Acknowledgements 119
Data availability 119
References 121
Figures 127
Tables 131
Supplementary figures 135
Supplementary tables 144

LIST OF TABLES

Table 2.1: Combinations of main effects for 2017 and 2018 field trials 24

Table 2.2: Marketable fruit harvested from pruned and non-pruned plants 25

Table 2.3: Marketable fruit harvested from tomato plants..... 26

Table 2.4: Total or marketable yields (kg/ha) of Tasti-lee grafted on to 'Beaufort' 27

Table 2.5: Total or marketable yields of 'Mountain Fresh Plus' grafted on 'Beaufort' 28

Table 3.1. Significance of main effects and interactions on marketable yield..... 47

Table 3.2: Marketable fruit (kg/hectare) from grafted and non-grafted tomato plants..... 48

Table 3.3: Marketable fruit from pinched and non-pinched grafted plants 49

Table 3.4: Fruit quality characteristics of fruit harvested from grafted 'Tasti-Lee' 50

Table S3.1: List of rootstock, spacing, pinching, and pruning treatment combinations..... 57

Table S3.2: Yields (kg/ha) of Tasti-lee grafted on to different rootstocks 58

Table S3.3: Yields (kg/ha) of 'Mountain Fresh Plus' grafted on to different rootstocks 59

Table 4.1: AUDPC scores developed from *V. dahliae* chlorosis/necrosis symptoms 2017 77

Table 4.2: AUDPC scores developed from *V. dahliae* chlorosis/necrosis symptoms 2018 78

Table 4.3: AUDPC and AUGPC scores from grafted and non-grafted tomatoes..... 79

Table 4.4: Specific fruit sizes, marketable, and total yields from 2019 field trial..... 80

Table 4.5: Nutrient analysis of foliar tissue (1/2) 81

Table 4.6: Nutrient analysis of foliar tissue (2/2) 82

Table S4.1: Cultivars used in 2017, 2018, and 2019 field trials 95

Table 5.1: 201 unique effector-like regions..... 131

Table 5.2: Unique effector-like regions..... 132

Table 5.3: Summary of candidate resistance genes 133

Table 5.4. Start Codon and location of each race 2 candidate gene on the JR2 chromosome.. 134

Table S5.1: Total, assembled, and unassembled paired reads to the VdLs17 RG..... 144

Table S5.2: Total number of secreted coding sequence regions and secreted effectors..... 145

Table S5.3: Total, assembled, and unassembled paired reads to the JR2 reference genome ... 146

Table S5.4: Total number of secreted coding sequence regions and secreted effectors..... 147

Table S5.5: Numbers of secreted effectors from 19 genomes 148

Table S5.6: Numbers of secreted effectors from 18 genomes. 149

LIST OF FIGURES

Figure 1.1: Verticillate conidiophore of *V. dahliae* on potato dextrose agar..... 13

Figure 1.2: Microscope image of melanized microsclerotia from *V. dahliae* 14

Figure 1.3: Chlorotic necrotic lesion caused by *V. dahliae* on tomatoes in Mills River NC..... 15

Figure 2.1: Fruit category distribution in pruning pinching combinations from MFP 29

Figure 2.2: Fruit category distribution in pruning pinching combinations from TL 30

Figure 3.1: Fruit category distribution from pruned, non-pinched, and grafted MFP 51

Figure 3.2: Fruit category distribution from pruned, non-pinched, and grafted TL 52

Figure 3.3: Mean fruit category distribution of all pinched and non-pinched MFP 2017 53

Figure 3.4: Mean fruit category distribution of all pinched and non-pinched MFP 2018 54

Figure 3.5: Mean fruit category distribution of all pinched and non-pinched TL 2017 55

Figure 3.6. Mean fruit category distribution of all pinched and non-pinched TL 2018 56

Figure 4.1: AUDPC scores from 'Red Defender' grafted combinations planted in 2017..... 83

Figure 4.2: AUDPC scores from 'Red Defender' grafted combinations planted in 2018..... 84

Figure 4.3: Marketable yields from 'Red Defender' grafted combinations planted in 2017..... 85

Figure 4.4: Total yields from 'Red Defender' grafted combinations planted in 2017 86

Figure 4.5: Marketable yields from 'Red Defender' grafted combinations planted in 2018..... 87

Figure 4.6: Total yields from 'Red Defender' grafted combinations planted in 2018 88

Figure 4.7: 2017 marketable yields from grafted 'Red Defender' plants..... 89

Figure 4.8: 2017 total yields from grafted 'Red Defender' plants..... 90

Figure 4.9: Extra-large fruit yields harvested from 2017 RD grafted plants..... 91

Figure 4.10: 2018 marketable yields (no culls) from grafted 'Red Defender' plants..... 92

Figure 4.11: 2018 total yields from grafted 'Red Defender' plants..... 93

Figure 4.12: Extra-large fruit yields harvested from 2017 RD grafted plants 94

Figure S4.1: Red Defender plants on September 19th 2019 (91 days post-planting) 96

Figure S4.2: 'NC-7GEM' plants on September 19th 2019 (91 days post-planting) 97

Figure S4.3: RD-'Maxifort' plants on September 19th 2019 (91 days post-planting) 98

Figure S4.4: 'NC-7GEM'-'Maxifort' plants on September 19th 2019 (91 days post-planting) . 99

Figure S4.5: RD-'NC-7GEM' plants on September 19th 2019 (91 days post-planting) 100

Figure S4.6: 'NC-7GEM'-RD plants on September 19th 2019 (91 days post-planting) 101

Figure 5.1: Area under the disease pressure curve scores (AUDPC) scores of 'Aibou' 127

Figure 5.2: Neighbor-joining consensus tree of reads mapped to the VdLs17 RG 128

Figure 5.3: Single nucleotide differences matrix with representative isolates 129

Figure 5.4: Phylogenetic tree developed from 64 concatenated protein sequences 130

Figure S5.1. Comparative analysis of nucleic acid differences between isolates 135

Figure S5.2. Comparison of nucleic acid differences (%) between isolates 136

Figure S5.3: 'Aibou' seedlings inoculated with race 1, 2, and 3 137

Figure S5.4: 'Bowman' seedlings inoculated with race 1, 2, and 3 138

Figure S5.5: 'Ganbarune-Karis' seedlings inoculated with race 1, 2, and 3 139

Figure S5.6: 'Bowman' seedling 45 days after having its roots dipped in water 140

Figure S5.7: 'Bowman' seedling 45 days after being inoculated with Ca36 (race 2) 141

Figure S5.8: 'Bowman' seedling 45 days after being inoculated with KJ14a (race 3) 142

Figure S5.9: 'Bowman' seedling 45 days after being inoculated with NC86 (race 3) 143

CHAPTER 1

INTRODUCTION

Pathogen Biology

Verticillium dahliae Kleb. is a soil-borne ascomycete fungus that infects hundreds of plant species around the world (Pegg and Brady 2002; Klosterman et al. 2009; Agrios 2005). The genus *Verticillium* contains fungi with verticillate conidiophores, which refers to the whorled arrangement of conidia bearing hyphae (Fig. 1). The original observation of *Verticillium* like fungi was made in 1816 CE by Christian G. D. Nees von Esenbeck. The *Verticillium* genus is diverse and contains fungi that can parasitize a wide array of hosts including plants, fungi, insects and nematodes (Pegg and Brady 2002; Kerry et al. 1993). Currently the species within *Verticillium* are grouped based on their verticillate conidiophores and genetic similarity, not necessarily by lifestyle (Zare, Gams, and Schroers 2004; Zare and Gams 2008; Bidochka and Gowanlockl 1999).

Tomato (*Solanum* spp. L.) can be infected by *V. longisporum*, V. *albo-atrum*, and *V. dahliae* (Bewley 1922; Bhat and Subbarao 1999). In North Carolina the dominant species that infects tomatoes is *V. dahliae* (Bender and Shoemaker 1984). The most insidious feature of *V. dahliae* is not necessarily the severity of the disease, but rather the ability of the fungus to survive for over a decade in infested soil.

V. dahliae creates microsclerotia that are asexually produced survival structures made up of melanized hyphae approximately 100 µm long and 40 µm wide (Fig. 2) (Xiao et al. 1998; Green 1980). *V. dahliae* creates true microsclerotia, whereas *V. albo-atrum* does not, however both are haploid. *V. dahliae* microsclerotia are elongated and its conidia are short (3.5-5.5 µm), as opposed to *V. longisporum* which has rounded microsclerotia and long conidia (7.1-8.8 µm)

(Karapapa, Bainbridge, and Heale 1997). Interestingly *V. longisporum* is a diploid hybrid made from the parasexual combining of *V. dahliae* and a cryptic *Verticillium* species (designated A1). Even more interesting is that the fusion of *V. dahliae* and this cryptic species has occurred 3 separate times, creating 3 unique lineages (A1/D1, A1/D2, and A1/D3) of *V. longisporum* (Inderbitzin et al. 2011). Each of these *V. longisporum* lineages have varied virulence and pathogenicity, but none of them seem excessively aggressive on tomato (Novakazi et al. 2015; Karapapa, Bainbridge, and Heale 1997). The majority of *V. dahliae* that infects tomato belong to lineage D3, although its ITS region is identical to that of *V. longisporum* A1/D3 which may lead to misdiagnosis in the absence of morphological observations (Inderbitzin and Subbarao 2014).

Although some strains of *V. dahliae* form appressoria to penetrate host roots, most strains do not, relying more on natural openings to infect the cortex (Tzima et al. 2010; Buchner, Nachmias, and Burstein 1982; Pegg and Brady 2002). The primary infection peg can form from asexually produced conidia or microsclerotia. Although mating types exist in, no sexual structures have been reported in *V. dahliae*. In tomato plants *V. dahliae* hyphae grow acropetally through the xylem from the roots into the main stem of the plant and infecting the foliage. Symptoms of *V. dahliae* include wilting or flagging foliage, and v-shaped necrotic lesions with yellow chlorotic halos (Fig. 3).

Resistance to *V. dahliae*

The most well characterized resistance to *V. dahliae* is conferred by the race 1 resistance gene *Ve1* (Diwan et al. 1999). Race 1 resistance was first discovered in the tomato line ‘Peru Wild’, and was eventually introgressed into commercial tomato lines in the 1950’s (Schaible, Cannon, and Waddoups 1951). The *Ve1* gene only confers resistance to race 1 isolates of *V. dahliae* which are defined by the presence of a single gene the *VdAve1* which codes for a

secreted effector protein. *VdAve1* homologs can be found in closely related fungal pathogens such as *Colletotrichum higginsianum*, *Cercospora beticola*, and *Fusarium oxysporum* f. sp. *lycopersici*, as well as the bacterial causal agent of citrus canker *Xanthomonas axonopodis*, and the *Ve1* gene can confer resistance to these homologs (Jonge et al. 2012). The *Ve1* gene encodes a cell surface-like receptor which can recognize the *VdAve1* effector and induce a defense response (Kawchuk et al. 2001). When Beefsteak (*Ve1*+) and Early Pak (*Ve1*-) were inoculated with race 1 and non-race 1 strains of *V. dahliae* their were hundreds of differentially expressed proteins in each interaction when the proteomes and metabolomes were sampled (Hu et al. 2019).

Non-race 1, (initially referred to as race 2), *V. dahliae* strains are defined by the absence of a functional copy of the *VdAve1* gene (Jonge et al. 2012). Strains with a functional copy of *VdAve1* are able to cause more damage to host plant lacking Ve1 than non-race 1 strains, suggesting VdAve1 is also a virulence factor. In tomato there are no known resistance genes characterized to defend against non-race 1 *V. dahliae* strains. In cotton *GbaNA1* confers resistance to non-race 1 *V. dahliae*, however there is no functional homolog in tomato (Li et al. 2018). In 2016 Usami et al. discovered that two hybrid tomato lines ‘Aibou’ and ‘Ganbarune-Karis’ had nearly complete resistance to non-race 1 *V. dahliae*. Strains pathogenic on ‘Aibou’ and ‘Ganbarune-Karis’ were termed race 3 (VdN), while strains unable to cause disease or colonize those lines were named race 2 (Vd2). When ‘Aibou’ and ‘Ganbarune-Karis’ were selfed and challenged with race 2 strains the ratio of resistant to susceptible plants was 3:1, indicating the resistance is conferred by a single dominant gene (Usami et al. 2017). Race 3 can be derived from some race 1 strains by silencing the *VdAve1* gene, indicating some race 3 isolates may have evolved directly from some lineages of race 1 (Kano and Usami 2019).

Disease Cycle and Management

V. dahliae is primarily only a pest in the temperate parts of the world, although the disease exists in sub-tropical regions of the world to a lesser degree too. The primary methods of controlling *Verticillium* wilt is through the use of resistant cultivars, crop rotation with non-hosts, and fumigation (Pegg and Brady 2002). Very little can be done to reduce inoculum distribution after *V. dahliae* has infested a field. The multi-year survival of microsclerotia, and *V. dahliae*'s wide host range make crop rotation difficult. The rapid emergence of non-race 1 strains has made using resistant varieties uncertain (Bender and Shoemaker 1984, 2).

In the past methyl-bromide (bromomethane) was an important and powerful tool for growers to radically reduce the inoculum density of many plant pathogens (Ristaino and Thomas 1997). Along with other greenhouse gas destroying chemicals such as CFCs the import and production of methyl-bromide ended in 2005, and stockpiles have dwindled from 9,974 metric tons in 2005, to 158 in 2014 (US EPA 2015). Pesticides such as chloropicrin and 1,3-dichloropropene (Telone) can control a number of soil-borne pathogens, including *V. dahliae*. However, these fumigants are not as effective as methyl-bromide (Duniway 2002). Chloropicrin makes a zone of exclusion that provides plants a short amount of time before the root system grows out of the sterile zone into *V. dahliae* infested soil that escaped the fumigation. Chloropicrin may be more effective in sandy soils over thicker clay soils (Gullino, Minuto, and Garibaldi 2002). Anaerobic soil disinfestation (ASD) involves pumping water and cheap/locally available carbon source, such as molasses or wheat bran, and covering the soil with plastic to seal out oxygen. This process creates an anaerobic environment that may be hostile to many plant pathogenic agents (Goud et al. 2004). ASD may also reduce pathogen density by inducing early germination of microsclerotia.

Grafting Tomatoes for Yield and Disease Resistance

Grafting tomatoes has been an effective strategy for growers in dealing with a wide range of plant pathogens. Highly resistant rootstocks that completely protect, and often have added vigor, are available for *Fusarium oxysporum* f. sp. *lycopersici* (FOL) races 1-3, *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), and bacterial wilt (*Ralstonia solanacearum*) (Hibar et al. 2006; Ozbay and Newman 2004; Louws, Rivard, and Kubota 2010; C. L. Rivard and Louws 2008; C. Rivard et al. 2012; Suchoff et al. 2014). Moderate to high resistance against root knot nematodes can be achieved by rootstocks containing the Mi gene (Lopez-Perez et al. 2006; Verdejo-Lucas et al. 2013). Resistance against Vd1 in tomatoes is controlled by the *Ve1* locus (Diwan et al. 1999, 1; Jonge et al. 2012). Vd1 isolates are more aggressive than Vd2 or VdN (Song and Thomma 2018). Although rootstocks with race 1 resistance can prevent wilting, this resistance does not completely protect the scion (Mackey 2013; Nazar et al. 2018). Race 2 resistance in 'Aibou' and 'Ganbarune-Karis' also appears to provide a high level of resistance against Vd2 isolates, however the pathogen can occasionally be found above the cotyledon in plants 30 days post-inoculation (Usami et al. 2017).

Research Objectives

The first objective of this study was to evaluate the optimal conditions of growing grafted tomatoes in Western North Carolina. To accomplish this objective, tomatoes were grown in low disease conditions in a field with no recent history of tomato cultivation. Horticultural practices such as pruning, pinching, and spacing was used in a large field trial with 3 rootstocks and 2 scion combinations. This trial was conducted over the course of two summers in 2017 and 2018. Marketable and Total yield, along with fruit sizing data, were analyzed.

The second objective of this study was to survey many potential sources of Vd2 and VdN resistance. To accomplish this we planted grafted and non-grafted plants in 2017, 2018, and 2019. Resistance against VdN was rated, and the ability of each rootstock to increase yield in the susceptible scions was determined. This survey also tested the effects of fumigation and self-grafting on disease and yield.

The third objective of this study was to analyze Vd1, Vd2, and VdN isolates using whole genome sequencing and machine learning algorithms. Isolates will be screened on race 1 and race 2 resistance tomato varieties to confirm their race type. The genomes of race 1, 2, and 3 isolates was sequenced, and the secreted effector like genes were analyzed for any variations which may explain the race 2 phenotype in *V. dahliae*.

REFERENCES

1. Agrios, George N. 2005. *Plant Pathology- 5th Ed.* Academic Press,. http://thuvienso.vanlanguni.edu.vn/handle/Vanlang_TV/8539.
2. Bender, C. G., and P. B. Shoemaker. 1984. "Prevalence of Verticillium Wilt of Tomato and Virulence of Verticillium Dahliae Race 1 and Race 2 Isolates in Western North Carolina." *Plant Disease* 68 (4): 305–9.
3. Bewley, W. F. 1922. "'Sleepy Disease' of the Tomato1." *Annals of Applied Biology* 9 (2): 116–34. https://doi.org/10.1111/j.1744-7348.1922.tb05942.x.
4. Bhat, R. G., and K. V. Subbarao. 1999. "Host Range Specificity in *Verticillium Dahliae*." *Phytopathology* 89 (12): 1218–25. https://doi.org/10.1094/PHYTO.1999.89.12.1218.
5. Bidochka, Michael J, and Karen Gowanlockl. 1999. "Nuclear RDNA Phylogeny in the Fungal Genus Verticillium and Its Relationship to Insect and Plant Virulence, Extracellular Proteases and Carbohydrases," 9.
6. Buchner, Virginia, Abraham Nachmias, and Yigal Burstein. 1982. "Isolation and Partial Characterization of a Phytotoxic Glycopeptide from a Protein—Lipopolysaccharide Complex Produced by a Potato Isolate of Verticillium Dahliae." *FEBS Letters* 138 (2): 261–64. https://doi.org/10.1016/0014-5793(82)80456-0.
7. Diwan, N., R. Fluhr, Y. Eshed, D. Zamir, and S. D. Tanksley. 1999. "Mapping of Ve in Tomato: A Gene Conferring Resistance to the Broad-Spectrum Pathogen, Verticillium Dahliae Race 1." *Theoretical and Applied Genetics* 98 (2): 315–19. https://doi.org/10.1007/s001220051075.

8. Duniway, J. M. 2002. “Status of Chemical Alternatives to Methyl Bromide for Pre-Plant Fumigation of Soil.” *Phytopathology* 92 (12): 1337–43. https://doi.org/10.1094/PHYTO.2002.92.12.1337.
9. Goud, Jan-Kees C., Aad J. Termorshuizen, Wim J. Blok, and Ariena H. C. van Bruggen. 2004. “Long-Term Effect of Biological Soil Disinfestation on Verticillium Wilt.” *Plant Disease* 88 (7): 688–94. https://doi.org/10.1094/PDIS.2004.88.7.688.
10. Green, R. J. 1980. “Soil Factors Affecting Survival of Microsclerotia of Verticillium Dahliae.” *Phytopathology* 70 (4): 353–55.
11. Gullino, M. L., A. Minuto, and A. Garibaldi. 2002. “Soil Fumigation with Chloropicrin in Italy: Experimental Results on Melon, Eggplant and Tomato.” *Mededelingen (Rijksuniversiteit Te Gent. Fakulteit van de Landbouwkundige En Toegepaste Biologische Wetenschappen)* 67 (2): 171–80.
12. Hibar, Khaled, Mejda Daami-Remadi, Hayfa Jabnoun-Khiareddine, and Mohamed El Mahjoub. 2006. “Control of Fusarium Crown and Root Rot of Tomato, Caused by Fusarium Oxysporum f. Sp. Radicis-Lycopersici by Grafting onto Resistant Rootstocks.” *Plant Pathology Journal* 5 (2): 161–65.
13. Hu, Xiaoping, Krishna D. Puri, Suraj Gurung, Steven J. Klosterman, Christopher M. Wallis, Monica Britton, Blythe Durbin-Johnson, et al. 2019. “Proteome and Metabolome Analyses Reveal Differential Responses in Tomato -Verticillium Dahliae-Interactions.” *Journal of Proteomics* 207 (September): 103449. https://doi.org/10.1016/j.jprot.2019.103449.
14. Inderbitzin, Patrik, R. Michael Davis, Richard M. Bostock, and Krishna V. Subbarao. 2011. “The Ascomycete Verticillium Longisporum Is a Hybrid and a Plant Pathogen with

an Expanded Host Range." *PLOS ONE* 6 (3): e18260. https://doi.org/10.1371/journal.pone.0018260.

15. Inderbitzin, Patrik, and Krishna V. Subbarao. 2014. "Verticillium Systematics and Evolution: How Confusion Impedes Verticillium Wilt Management and How to Resolve It." *Phytopathology* 104 (6): 564–74. https://doi.org/10.1094/PHYTO-11-13-0315-IA.
16. Jonge, Ronnie de, H. Peter van Esse, Karunakaran Maruthachalam, Melvin D. Bolton, Parthasarathy Santhanam, Mojtaba Keykha Saber, Zhao Zhang, et al. 2012. "Tomato Immune Receptor Ve1 Recognizes Effector of Multiple Fungal Pathogens Uncovered by Genome and RNA Sequencing." *Proceedings of the National Academy of Sciences* 109 (13): 5110–15. https://doi.org/10.1073/pnas.1119623109.
17. Karapapa, V. K., B. W. Bainbridge, and J. B. Heale. 1997. "Morphological and Molecular Characterization of Verticillium Longisporum Comb, Nov., Pathogenic to Oilseed Rape." *Mycological Research* 101 (11): 1281–94. https://doi.org/10.1017/S0953756297003985.
18. Kawchuk, Lawrence M., John Hachey, Dermot R. Lynch, Frank Kulcsar, Gijs van Rooijen, Doug R. Waterer, Albert Robertson, et al. 2001. "Tomato Ve Disease Resistance Genes Encode Cell Surface-like Receptors." *Proceedings of the National Academy of Sciences* 98 (11): 6511–15. https://doi.org/10.1073/pnas.091114198.
19. Kerry, B. R., I. A. Kirkwood, F. A. A. M. de Leij, J. Barba, M. B. Leijdens, and P. C. Brookes. 1993. "Growth and Survival of Verticillium Chlamydosporium Goddard, a Parasite of Nematodes, in Soil." *Biocontrol Science and Technology* 3 (3): 355–65. https://doi.org/10.1080/09583159309355290.

20. Kano, K., and Usami, T. 2019. Race 3 tomato Verticillium wilt pathogen ptoentially derives from race 1 isolate. Soil Microorg. 73:71–78.

21. Klosterman, Steven J., Zahi K. Atallah, Gary E. Vallad, and Krishna V. Subbarao. 2009. "Diversity, Pathogenicity, and Management of Verticillium Species." *Annual Review of Phytopathology* 47 (1): 39–62. https://doi.org/10.1146/annurev-phyto-080508-081748.

22. Li, Nan-Yang, Xue-Feng Ma, Dylan P. G. Short, Ting-Gang Li, Lei Zhou, Yue-Jing Gui, Zhi-Qiang Kong, et al. 2018. "The Island Cotton NBS-LRR Gene GbaNA1 Confers Resistance to the Non-Race 1 Verticillium Dahliae Isolate Vd991." *Molecular Plant Pathology* 19 (6): 1466–79. https://doi.org/10.1111/mpp.12630.

23. Lopez-Perez, Jose-Antonio, Michelle Le Strange, Isgouhi Kaloshian, and Antoon T. Ploeg. 2006. "Differential Response of Mi Gene-Resistant Tomato Rootstocks to Root-Knot Nematodes (Meloidogyne Incognita)." *Crop Protection* 25 (4): 382–88.

24. Louws, Frank J, Cary L Rivard, and Chieri Kubota. 2010. "Grafting Fruiting Vegetables to Manage Soilborne Pathogens, Foliar Pathogens, Arthropods and Weeds." *Scientia Horticulturae* 127 (2): 127–46.

25. Mackey, Melora. 2013. "Resistance to Verticillium in Tomatoes: The Root-Stem Controversy."

26. Nazar, Ross N, Xin Xu, Josefa Blaya Fernandez, Hakeem Shittu, Alexander Kurosky, and Jane Robb. 2018. "Defence Cascade in Verticillium-Infected Grafted Tomato." *Plant Signaling & Behavior* 13 (6): e1475807.

27. Novakazi, Fluturë, Patrik Inderbitzin, German Sandoya, Ryan J. Hayes, Andreas von Tiedemann, and Krishna V. Subbarao. 2015. "The Three Lineages of the Diploid Hybrid

Verticillium Longisporum Differ in Virulence and Pathogenicity." *Phytopathology* 105 (5): 662–73. https://doi.org/10.1094/PHYTO-10-14-0265-R.

28. Ozbay, Nusret, and Steven E Newman. 2004. "Fusarium Crown and Root Rot of Tomato and Control Methods." *Plant Pathol J* 3: 9–18.
29. Pegg, G. F., and B. L. Brady. 2002. *Verticillium Wilts*. CABI.
30. Ristaino, Jean Beagle, and William Thomas. 1997. "Agriculture, Methyl Bromide, and the Ozone Hole: Can We Fill the Gaps?" *Plant Disease* 81 (9): 964–77. https://doi.org/10.1094/PDIS.1997.81.9.964.
31. Rivard, Cary L, and Frank J Louws. 2008. "Grafting to Manage Soilborne Diseases in Heirloom Tomato Production." *HortScience* 43 (7): 2104–11.
32. Rivard, CL, S O'connell, MM Peet, RM Welker, and FJ Louws. 2012. "Grafting Tomato to Manage Bacterial Wilt Caused by Ralstonia Solanacearum in the Southeastern United States." *Plant Disease* 96 (7): 973–78.
33. Schaible, L., O. S. Cannon, and V. Waddoups. 1951. "Inheritance of resistance to Verticillium wilt in a Tomato cross." *Phytopathology* 41 (11). https://www.cabdirect.org/cabdirect/abstract/19521100756.
34. Song, Yin, and Bart PHJ Thomma. 2018. "Host-induced Gene Silencing Compromises Verticillium Wilt in Tomato and Arabidopsis." *Molecular Plant Pathology* 19 (1): 77–89.
35. Suchoff, D, C Gunter, J Schultheis, and FJ Louws. 2014. "On-Farm Grafted Tomato Trial to Manage Bacterial Wilt." In , 119–27.
36. Tzima, Aliki, Epaminondas J. Paplomatas, Payungsak Rauyaree, and Seogchan Kang. 2010. "Roles of the Catalytic Subunit of CAMP-Dependent Protein Kinase A in

Virulence and Development of the Soilborne Plant Pathogen Verticillium Dahliae." *Fungal Genetics and Biology* 47 (5): 406–15. https://doi.org/10.1016/j.fgb.2010.01.007.

37. US EPA, OAR. 2015. "Methyl Bromide." Policies and Guidance. US EPA. July 23, 2015. https://www.epa.gov/ods-phaseout/methyl-bromide.
38. Usami, T., N. Momma, S. Kikuchi, H. Watanabe, A. Hayashi, M. Mizukawa, K. Yoshino, and Y. Ohmori. 2017. "Race 2 of Verticillium Dahliae Infecting Tomato in Japan Can Be Split into Two Races with Differential Pathogenicity on Resistant Rootstocks." *Plant Pathology* 66 (2): 230–38. https://doi.org/10.1111/ppa.12576.
39. Verdejo-Lucas, Soledad, María Blanco, Laura Cortada, and F Javier Sorribas. 2013. "Resistance of Tomato Rootstocks to Meloidogyne Arenaria and Meloidogyne Javanica under Intermittent Elevated Soil Temperatures above 28 C." *Crop Protection* 46: 57–62.
40. Xiao, C. L., K. V. Subbarao, K. F. Schulbach, and S. T. Koike. 1998. "Effects of Crop Rotation and Irrigation on *Verticillium Dahliae* Microsclerotia in Soil and Wilt in Cauliflower." *Phytopathology* 88 (10): 1046–55. https://doi.org/10.1094/PHYTO.1998.88.10.1046.
41. Zare, Rasoul, and Walter Gams. 2008. "A Revision of the Verticillium Fungicola Species Complex and Its Affinity with the Genus Lecanicillium." *Mycological Research* 112 (7): 811–24. https://doi.org/10.1016/j.mycres.2008.01.019.
42. Zare, Rasoul, Walter Gams, and Hans-Josef Schroers. 2004. "The Type Species of Verticillium Is Not Congeneric with the Plant-Pathogenic Species Placed in Verticillium and It Is Not the Anamorph of 'Nectria' Inventa." *Mycological Research* 108 (5): 576–82. https://doi.org/10.1017/S0953756204009839.

FIGURES

Figure 1.1: Verticillate conidiophore of *V. dahliae* on potato dextrose agar (Difco 39g/L) at 200x magnification.

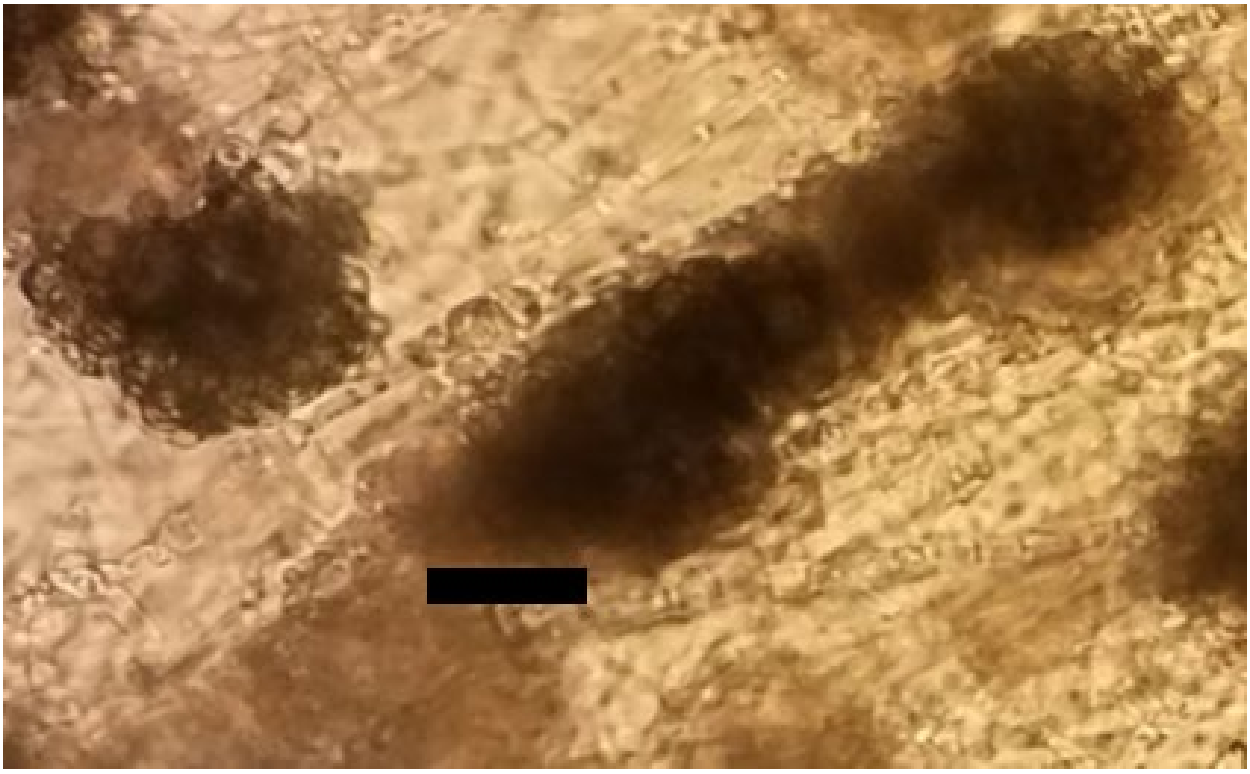

Figure 1.2: Microscope image of melanized microsclerotia from *V. dahliae* on Sorenson's NP10 media at 200x magnification. Band = 20 µm.

Figure 1.3: Chlorotic necrotic v-shaped lesion caused by *V. dahliae* on tomatoes in Mills River NC.

CHAPTER 2

PRUNING REDUCES YIELDS IN GRAFTED TOMATOES PLANTED IN THE FIELD

ABSTRACT

In 2017 and 2018, field trials were conducted with tomatoes grafted onto the rootstock 'Beaufort' or not grafted to evaluate the effects of various cultural practices: pruning side shoots (up to the first flower cluster), pinching the main stem before planting, spacing (56 vs. 61 cm), and scion variety ('Tasti-Lee' and 'Mountain Fresh Plus' [MFP]). Fruits were harvested five times in each year and marketable yield per acre was calculated for each treatment group. Both scion varieties grafted onto 'Beaufort' had consistently higher yields than non-grafted tomatoes. Pruning the grafted plants, regardless of scion variety, reduced yields over the non-pruned plants in both years. On 'Tasti-Lee', marketable yields improved by 32% and 57% when grafted plants were pruned or not pruned, respectively, over the non-grafted plants. In 'MFP', yields improved by 35% and 47% when grafted plants were pruned or not pruned, respectively, over the non-grafted plants. Fruit size also increased when either variety was grafted over the non-grafted plants, regardless of spacing, pruning or pinching. Spacing among the grafted plants had no effect on yield or fruit size; both 56 and 61 cm spacings produced statistically similar yields per acre. Growers may be able to offset the higher cost of grafted plants by planting fewer plants per acre, and not pruning those grafted plants. Pinching significantly increased yields in pruned plants, but the same increase was not present in non-pruned plants.

INTRODUCTION

Grafting tomatoes (*Solanum lycopersicum* L.) onto vigorous rootstocks is an effective way of reducing diseases and increasing fruit yields (Grieneisen et al. 2018; Kubota et al. 2008). 'Tasti-lee' (TL) and 'Mountain Fresh Plus' (MFP) represent two fresh market tomato varieties

that may benefit from the vigorous root systems certain rootstocks provide. ‘Beaufort’ is a highly vigorous rootstock that also has a high level of resistance to several soilborne pathogens (Leonardi and Giuffrida 2006; Romano and Paratore 2000). Most research on management of grafted plants has been conducted in greenhouse vegetable production, and there is limited information on proper cultural management practices in the open field (Grieneisen et al. 2018). More information is needed on what the optimal cultural practices are to maximize yields and fruit sizes in staked fresh market tomato production systems widely used in the United States.

Pruning side shoots is a common practice on non-grafted tomato plants in the open field to reduce vegetative growth and increase fruit size and yield. The number of shoots to prune varies by variety (Kanyomeka and Shivute 2005; Davis and Estes 1993; Maboko et al. 2011) and some varieties do not respond to pruning (Davis and Estes 1993). However, pruning grafted tomatoes may not respond in the same manner as when not grafted, as grafting onto vigorous rootstock often makes grafted plants overly vegetative.

Pinching, the removal of the meristem to instigate axillary shoots to extend to form two main stems, has been promoted to increase yield per plant and potentially reduce the number of transplants a grower would need to plant per hectare while maintaining or improving yield (Rahmatian et al. 2014). However, there is limited research available on this practice.

Spacing may play a key role in determining if pinched or non-pruned plants can fill an area effectively with foliage, thus increasing the photosynthetic potential of the plant. It remains unclear exactly how much space grafted plants need to maximize yields while minimizing the number of transplants planted per hectare to reduce costs. Some studies suggest the largest spacing a grower can use without reducing yield per hectare is around 61 cm (Suchoff et al. 2014; Turhan et al. 2011). The recommended spacing for grafted plants is currently 56-61 cm

(Tri-Hishtil, personal communication), and more studies are needed to elucidate any subtle effects between these spacings, if any exist.

The objective of this study is to examine the effects of pruning, pinching, and spacing on TL and MFP tomato plants grafted onto the rootstock 'Beaufort' or not grafted. Examining these different cultural practices will help to understand how they affect the yields of grafted plants in the field.

MATERIALS AND METHODS

In 2017 and 2018, field trials were conducted at the Mountain Horticultural Crops Research and Extension Center (MHCREC) in Mills River, North Carolina. Commercial determinate tomato cultivars 'Tasti-lee'® (Bejo Seed, Oceana, CA, USA [TL]) and 'Mountain Fresh Plus' (HMClause Seed Company, Davis, CA, USA [MFP]) were grafted onto rootstock 'Beaufort' (DeRuiter Seeds, St. Louis, MO, USA) or not grafted. Grafted transplants were grown and grafted by Tri-Hishtil (Mills River, NC, USA) using the splice grafting method. Plants were either pinched (asceptic removal of the apical meristem) three to five days before transplanting or not pinched. Non-grafted plants were seeded in potting media and grown in a greenhouse for six weeks at MHCREC before being transplanted in the field. All transplants were planted on 8 June 2017 and 6 June 2018 in raised beds that had been covered in black plastic and spaced 1.5 m from center to center with a 1 m fallow section between the plastic. The field had no history of solanaceous vegetable production and disease pressure was expected to be minimal. In 2017 the field was not fumigated. In 2018, the field was fumigated with 90 kg/ha Pic-Chlor 60 six weeks prior to planting. Non-grafted plants were spaced 46 cm apart within the row according to standard practice, while grafted plants were spaced either 56 or 61 cm apart within the row.

Four weeks after planting plants were either pruned by removing the suckers up to one sucker below the first flower cluster or not pruned. Table 1 provides a summary of the treatments examined in this study. Standard disease and insect control and nutrition practices were employed in both years.

Plots were harvested five times each year on 14, 23, 30 Aug. and 13, 25 Sep. in 2017 and 14, 23, 29 Aug. and 6, 13 Sep. in 2018. Fruit were sorted by USDA standards into unmarketable, mediums, larges, and extra-large (XLPlus). Total marketable yields were analyzed using proc GLM in SAS (SAS Institute, Cary, NC, USA). Fruit categories were analyzed using GraphPad Prism v8.0.2 (GraphPad Software, La Jolla, CA, USA).

RESULTS AND DISCUSSION

Impacts of pruning on grafted tomato yields and fruit sizes

Pruning: Pruning the grafted plants significantly reduced yields by 8% in 2017 and 10% in 2018 compared to non-pruned plants (Table 2). This effect is not unique to grafted plants, more likely the explanation is that determinate tomato varieties do not always require pruning (Davis and Estes 1993). When examining fruit sizes pruned and non-pruned plants have statistically similar distributions of fruit sizes (Figure 1 and 2).

Pinching: Pinching increased yields in pruned tomatoes, but not non-pruned tomatoes. This trend was consistent in both years (Table 3). This indicates that while pinching increases yields, at 56 and 61 cm spacings non-pruned plants appear unaffected by pinching. No consistent significant differences were found in fruit sizes when comparing pinched and non-pinched yields (Figure 1 and 2) Future research should focus on increasing spacings past 61 cm with pinched grafted plants, to determine what the optimal maximum spacing would be. In this study it is hard to recommend pinching grafted plants, unless future research could show they increased or

maintained the same yields per hectare at larger spacings. Pinching is an added cost of $0.10-0.12 (Tri-Hishtil, personal communication) and more research is needed to justify using this technique outside the greenhouse.

Impact of spacing on yield and fruit size

Spacing at 56 or 61 cm had no effects on marketable yields, or fruit size. Spacing also had no interactions with any other effects (variety, pruning, pinching) (Table 4 and 5). Grafted plants represent a significant increase in price (50-75% more per plant). As yields were similar between 56 and 61 cm it is recommended to plant at 61 cm to cut down on input costs. This research was also conducted using only two different scions (TL and MFP), different scions may be more or less responsive to increases in spacings.

Comparison between grafted and non-grafted MFP and TL yields and fruit sizes

Every single grafted tomato treatment had numerically higher marketable and total yield averages than the non-grafted controls. Except for two pruned MFP treatments in 2017, and two pruned TL treatments in 2018 in both cultivars the increase in marketable and total yield was significant (P=0.05) (Table 4 and 5). Grafted plants had significantly fewer culls. In 2017 in TL the marketable yield per hectare increase due to grafting was as high as 81.55% in non-pruned non-pinched plants (Table 4). In both years in TL grafted non-pruned treatments marketable yields were all significantly higher compared to the non-grafted control (Table 4 and 5). TL plants grafted onto Beaufort yielded significantly more extra-large fruit than non-grafted standards (Figure 2). Overall MFP received less of a benefit from grafting when comparing fruit sizes and overall yields of grafted MFP to non-grafted MFP. In MFP the yield per hectare increase due to grafting was 35-60%, compared to the non-grafted control (Table 5). Numerically both large and extra-large were found in higher quantities in the grafted plants.

However, the quantity of extra-large fruit was only significantly higher in the pinched, non-pruned, compared to non-grafted MFP.

CONCLUSIONS

Grafting represents a powerful tool that growers can use to increase yields per hectare. In this study, grafting increased yields in non-pruned plants. While pinching increased yields in pruned plants, non-pruned plants had statistically similar yields, indicating that in this system pinching is unnecessary. Spacing at 61 cm yielded similar results as 56 cm, so planting at 61 cm is recommended to save on input costs. Both MFP and TL responded to the aforementioned effects. Some treatments of TL were similar to non-grafted plants, and some had nearly double the yields. MFP had lower maximum increases in yields, and did not benefit as much from grafting.

ACKNOWLEDGEMENTS

The authors would like to thank the station crew at the Mountain Horticultural Crops Research and Extension Center in Mills River, North Carolina. Funding for this project were provided by the Agriculture Foundation, and a USDA-NIFA SCRI grant. Tri-Hishtil provided all the grafted plants for this project, and Bejo Seeds provided seeds.

REFERENCES

1. Davis, J.M., and Estes, E.A. (1993). Spacing and Pruning Affect Growth, Yield, and Economic Returns of Staked Fresh-Market Tomatoes. Journal of the American Society for Horticultural Science *118*, 719–25 https://dx.doi.org/10.21273/JASHS.118.6.719
2. Grieneisen, M.L., Aegerter, B.J., Stoddard, C.S., and Zhang, M. (2018). Yield and Fruit Quality of Grafted Tomatoes, and Their Potential for Soil Fumigant Use Reduction. A Meta-Analysis. Agronomy for Sustainable Development *38*, 29 https://dx.doi.org/10.1007/s13593-018-0507-5
3. Kanyomeka, L., and Shivute, B. (2005). Influence of Pruning on Tomato Production under Controlled Environments. Agricultura Tropica et Subtropica *38*, 79–83.
4. Kubota, C., McClure, M.A., Kokalis-Burelle, N., Bausher, M.G., and Rosskopf, E.N. (2008) Vegetable grafting: History, use, and current technology status in North America. HortScience *43*, 1664-1669 https://dx.doi.org/10.21273/HORTSCI.43.6.1664
5. Leonardi, C. and Giuffrida, F. (2006). Variation of plant growth and macronutrient uptake in grafted tomatoes and eggplants on three different rootstocks. European Journal of Horticultural Science *71*, p.97.
6. Maboko, M.M., Du Plooy, C.P., and Chiloane, S. (2011). Effect of Plant Population, Fruit and Stem Pruning on Yield and Quality of Hydroponically Grown Tomato. African Journal of Agricultural Research *6*, 5144–48.
7. Rahmatian, A., Delshad, M., & Salehi, R. (2014). Effect of grafting on growth, yield and fruit quality of single and double stemmed tomato plants grown hydroponically. Horticulture, Environment, and Biotechnology *55*, 115-119 https://dx.doi.org/10.1007/s13580-014-0167-6

8. Romano, D. and Paratore, A. (2000), March. Effects of grafting on tomato and eggplant. In V International Symposium on Protected Cultivation in Mild Winter Climates: Current Trends for Sustainable Technologies *559*, 149-154 https://dx.doi.org/10.17660/ActaHortic.2001.559.21
9. Suchoff, D., Gunter C., Schultheis, J., and Louws, F. J. (2014). On-Farm Grafted Tomato Trial to Manage Bacterial Wilt. In I International Symposium on Vegetable Grafting *1086*, 119–27 https://dx.doi.org/10.17660/ActaHortic.2015.1086.14
10. Turhan, A., Ozmen N., Serbeci M.S., and Seniz V. (2011). Effects of Grafting on Different Rootstocks on Tomato Fruit Yield and Quality." Hortic. Sci *38*, 142–49 https://dx.doi.org/10.17221/51/2011-HORTSCI

TABLES

Table 2.1: Combinations of main effects for 2017 and 2018 field trials. Each treatment listed was randomized within each of the four replications.

No.	Scion	Rootstock[W]	Pruned[X]	Pinched[Y]	Spacing (cm)[Z]
1	MFP	Beaufort	Yes	Yes	61
2	MFP	Beaufort	Yes	No	61
3	MFP	Beaufort	No	Yes	61
4	MFP	Beaufort	No	No	61
5	MFP	Beaufort	Yes	Yes	56
6	MFP	Beaufort	Yes	No	56
7	MFP	Beaufort	No	Yes	56
8	MFP	Beaufort	No	No	56
9	MFP	Non-grafted	Yes	No	46
10	TL	Beaufort	Yes	No	61
11	TL	Beaufort	Yes	Yes	61
12	TL	Beaufort	No	No	61
13	TL	Beaufort	No	Yes	61
14	TL	Beaufort	Yes	No	56
15	TL	Beaufort	Yes	Yes	56
16	TL	Beaufort	No	No	56
17	TL	Beaufort	No	Yes	56
18	TL	Non-grafted	Yes	No	46

[W]MFP or TL was grafted onto the rootstock Beaufort or was non-grafted.
[X]plants were pruned up to the first flower cluster, or non-pruned.
[Y]plants had their main stem pinched before planting or were non-pinched.
[Z]spacing indicates distance between each tomato plant within plot.

Table 2.2: Marketable fruit harvested from pruned and non-pruned plants of Tasti-Lee and Mountain Fresh Plus (combined) all grafted onto 'Beaufort' in 2017 and 2018 trials conducted in Mills River, NC.

Pruning[y]	Marketable fruit ($kg \cdot ha^{-1}$)[z]	
	2017	2018
Pruned	86556 a	86090 a
Non-Pruned	93991 b	95058 b

[z]numbers followed by the same letter are not significantly different within a column as determined by Students t-test ($P>0.05$).

Table 2.3: Marketable fruit harvested from tomato plants ('Mountain Fresh Plus' and 'Tasti-Lee') grafted onto 'Beaufort'. Treatments were pinched or non-pinched and pruned or non-pruned. No cultivar interaction was detected ($P>0.05$), so reported yields from the two scion varieties are combined.

		Marketable ($kg \cdot ha^{-1}$)[z]	
Pruning[x]	Pinching[y]	2017	2018
Pruned	Non-pinched	82848 a	80467 a
Pruned	Pinched	90264 b	91712 b
Non-Pruned	Non-Pinched	94914 b	95377 b
Non-Pruned	Pinched	93068 b	94737 b

[Z]numbers followed by the same letter are not significantly different within a column as determined by Students t-test ($P>0.05$).

[Y]main stem of plant was pinched or non-pinched before planting.

[X]plant was non-pruned or pruned by removed all suckers up to the first flower cluster.

Table 2.4: Total or marketable (MRK) yields (kg/ha) of Tasti-lee grafted on to 'Beaufort' and submitted to different horticultural practices. Rootstock, spacing, pinching, pruning combinations reported. Letters indicate significance groupings determined by students t-test (P>0.05).

				Tasti-lee (kg/ha)			
				2017		2018	
Rootstock	**Spacing**	**Pinched**	**Pruned**	**Total**	**MRK**	**Total**	**MRK**
Beaufort	56 cm	Yes	Yes	111953 b	92775 ab	99585 b	92090 b
Beaufort	61 cm	Yes	Yes	107729 b	89363 ab	97788 bc	89023 bc
Beaufort	56 cm	No	Yes	116561 ab	98647 a	80665 cd	72276 d
Beaufort	61 cm	No	Yes	108684 b	91338 ab	81537 cd	74493 cd
Beaufort	56 cm	Yes	No	112917 b	95042 a	99528 b	90559 b
Beaufort	61 cm	Yes	No	102747 b	84678 b	103199 b	94508 ab
Beaufort	56 cm	No	No	114495 ab	97291 a	102324 b	94481 ab
Beaufort	61 cm	No	No	128121 a	100247 a	120359 a	108748 a
none	46 cm	No	Yes	83300 c	55216 c	81869 d	72216 d

Table 2.5: Total or marketable (MRK) yields (kg/ha) of 'Mountain Fresh Plus' grafted on to 'Beaufort' and submitted to different horticultural practices. Rootstock, spacing, pinching, pruning combinations reported. Letters indicate significance groupings determined by students t-test (P>0.05).

				Mountain Fresh Plus (kg/ha)			
				2017		2018	
Rootstock	**Spacing**	**Pinched**	**Pruned**	**Total**	**MRK**	**Total**	**MRK**
Beaufort	56 cm	Yes	Yes	105805 ab	84311 a	100160 a	87558 a
Beaufort	61 cm	Yes	Yes	104315 ab	82785 ab	109054 a	97900 a
Beaufort	56 cm	No	Yes	98171 ab	85717 a	103542 a	89596 a
Beaufort	61 cm	No	Yes	89466 b	75729 ab	100135 a	85260 a
Beaufort	56 cm	Yes	No	118627 a	97130 a	107382 a	95348 a
Beaufort	61 cm	Yes	No	106828 ab	88209 a	108721 a	98251 a
Beaufort	56 cm	No	No	105840 ab	93080 a	97945 a	87563 a
Beaufort	61 cm	No	No	108870 ab	94783 a	102735 a	90431 a
none	46 cm	No	Yes	102805 ab	60918 b	88499 a	63547 b

Figures

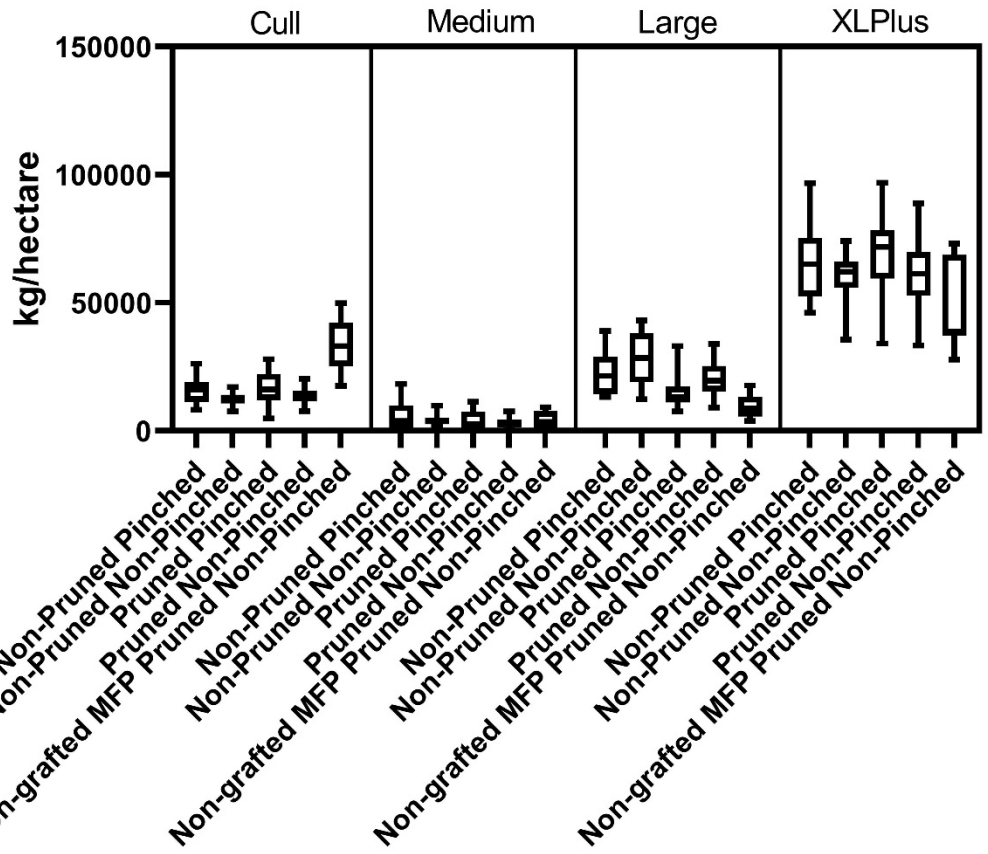

Figure 2.1: Fruit category distribution in pruning pinching combinations from MFP tomatoes grafted on 'Beaufort' compared to non-grafted MFP in 2017 and 2018. Error bars represent the 95% confidence intervals.

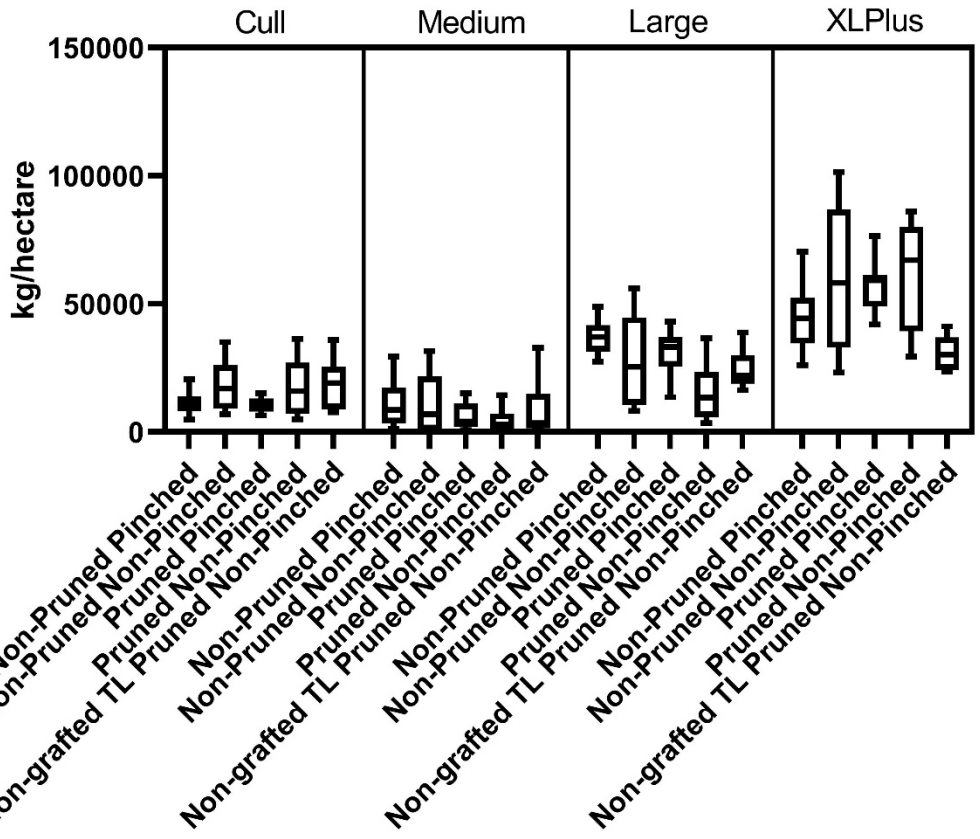

Figure 2.2: Fruit category distribution in pruning pinching combinations from TL tomatoes grafted on 'Beaufort' compared to non-grafted TL in 2017 and 2018. Error bars represent the 95% confidence intervals.

CHAPTER 3

VIGOROUS TOMATO ROOTSTOCKS IMPROVE YIELDS AND INCREASE FRUIT SIZES IN GRAFTED FRESH MARKET TOMATOES

ABSTRACT

Grafting high yielding tomato cultivars (*Solanum lycopersicum* L.) onto vigorous rootstocks can increase marketable yields, but questions remain regarding optimal cultural and growing conditions such as pinching and plant spacing. This study addressed some of the dynamics between grafted plants and cultural practices. Two scions, 'Tasti-Lee'(TL) and 'Mountain Fresh Plus' (MFP) were grafted onto each of three rootstocks, 'Beaufort', 'Arnold', and 'Shield'. Plants were pinched (removal of main shoot to induce both axillary shoots to grow) or non-pinched, and spaced at 56 or 61 cm. All 32 grafted treatments were compared to the grower standard: non-grafted TL and MFP spaced at 46 cm, and non-pinched. Fruit quality traits including soluble solids content, pH, lycopene concentration, and citric acid content were recorded for fruit harvested from tomatoes grafted onto 'Arnold', 'Beaufort', 'Shield' and non-grafted TL. The overall effect of grafting TL and MFP onto vigorous rootstocks 'Arnold', 'Beaufort', and 'Fortamino' increased marketable yields per hectare by 24-35% compared to non-grafted grower standards. The rootstock 'Shield' did not significantly increase yields with either scion. TL had a more positive response to grafting than MFP. 'Arnold', 'Bueafort', and 'Fortamino' significantly increased TL fruit size, but the fruit size results were not as significantly impacted by graft treatments for MFP. Plants spaced at 56 compared to 61 cm generated similar yields. Pinched plants significantly increased yields over non-pinched plants by 15% in 2018, but did not impact yield in 2017. No consistent difference was observed between pinched and non-pinched plants with regard to fruit size. Soluble solids content, pH, lycopene

concentration, and citric acid content were slightly different between grafted and non-grafted plants but unlikely to positively or negatively affect overall perception of fruit quality in tomatoes.

INTRODUCTION

In the United States and the world in general, the amount of arable land per capita has been decreasing for many decades (WorldBank 2015). Increased restrictions on the use of chemical fumigants, such as the phase-out of methyl-bromide, to manage soilborne pathogens has compounded this problem (Louws 2009). Growers are struggling to produce the same yields on lower quality and smaller areas of land (Qiao et al. 2015). Grafting presents a unique solution to this problem by allowing growers to select preferred vegetable varieties for grafting onto vigorous and/or disease resistant rootstocks (Louws et al. 2010). The hardiness, disease resistance, or vigor of rootstocks can improve yield of grafted scions, specifically fresh market tomato (*Solanum lycopersicum* Mill.) cultivars (Lee 1994; Grieneisen et al. 2018; Oztekin et al. 2009). The use of grafted plants is common in greenhouse vegetable production, but the use of grafted plants has not been adopted as readily in field production systems in the USA (Kuboto et al. 2008).

Although some tomato rootstocks can provide high levels of resistance or tolerance a wide variety of plant pathogens (Harrison and Burgess 1962; Grimault and Prior 1994; Ioannou 2001; Rivero, Ruiz, and Romero 2003; Louws et al. 2010), there is little information on grafting plants onto vigorous rootstocks to increase yields in fields where there is little to no soilborne pathogen pressure (Suchoff et al. 2019). In a recent meta-analysis, grafted plants yielded similarly or less than non-grafted or self-grafted plants 65% of the time. Grafting benefits differ under specific conditions, for example the authors noted that the rootstock ‘Maxifort’ has variable yield benefits depending on scion (Grieneisen et al. 2018). This indicates more research is needed to better document the parameters and practices under which grafting increases yields. As grafted plants cost appreciably more per plant than non-grafted, grafting nursery businesses

and growers must realize a positive return on investment to consider adopting the practice. Therefore, there is a need for more information on the optimal conditions under which grafted plants can be grown to increase yields (Rysin and Louws 2015).

Pinching, the removal of meristems to allow two axillary stems to grow, has been shown to increase yields in grafted and non-grafted tomatoes in hydroponic systems (Rahmatian et al. 2014). Hypothetically, two main stems with one large vigorous root system should allow growers to plant at a decreased density. However, in the field, pinching has not been sufficiently evaluated to understand if this practice has an effect on yield. Currently pinching is employed by growers without a thorough understanding of the exact parameters under which this practice should be used.

In standard tomato field production in North Carolina and surrounding states, non-grafted tomato plants are spaced at approximately 46 cm between plants. One study in the region suggested grafted plants can be spaced farther apart due to the increased vigor of the rootstocks and associated increase of fruit production (Suchoff et al. 2014). Grafted tomato plants are recommended to be spaced at 56-61 cm (Tri-Hishtil, personal communication), but limited data regarding yield at these spacings is available. Increased yield can be realized by harvesting more fruit or larger fruit. Spacing plants farther apart is a cost-saving move growers can adopt to offset the expense that grafting adds to the per-plant cost and to reduce over-all foliage density (Suchoff et al. 2014; Miguel 2002; Rivard et al. 2010b; Barrett et al. 2012).

Although increasing yield and fruit sizes is important, fruit quality must also be maintained or improved as compared to non-grafted tomato plants. The effect of grafting on fruit quality in solanaceous and cucurbitaceous plants has been mixed. Specific rootstock-scion combinations have resulted in significantly diluted fruit quality characteristics than non-grafted

or self-grafted tomatoes (Kyriacou et al. 2017; Pogonyi et al. 2005; Riga et al. 2016). Regardless, an evaluation of any positive or negative effects of grafting on fruit quality, and thus marketability, is warranted.

The impact of pinching, spacing and grafting has been studied either independently or in greenhouse systems, information on combinations of these cultural practices on fresh market tomato under field production is lacking. Therefore, the objectives of this study were to evaluate yield effects impacted by (1) grafted TL and MFP plants onto rootstocks 'Beaufort', 'Arnold', 'Shield', and 'Fortamino' and non-grafted; (2) pinched and non-pinched plants; (3) spacing [56, 61, or 46 cm (non-grafted only)]; and to (4) evaluate fruit quality including lycopene concentration, pH, citric acid, and soluble solids content (SSC) of fruit from grafted and non-grafted plants.

MATERIALS AND METHODS

Field Setting and Experimental Design. This study was conducted in 2017 and 2018 at the Mountain Horticultural Research and Extension Center (MHCREC) in Mills River, NC (35°25'36.5"N 82°33'33.0"W). Treatments were arranged in a randomized complete block design with four replicates. Treatment plots were 9.14 m long and separated by a 1 m fallow section. Grafted plants were planted at either 56 or 61 cm in-row spacing. Non-grafted controls were planted at the standard spacing of 46 cm. Seedlings were planted on 8 Jun 2017 and 6 Jun 2018 in raised beds, 30 cm high and 100 cm wide on 1.5 m row centers and covered with 1.5ml polyethylene black plastic. Because the field had not been planted in vegetables for over 20 years, soilborne pathogen pressure was expected to be low and the beds were not fumigated in 2017. In 2018, beds were fumigated with 90 kg/ha Pic-Clor 60 (37.1% 1,3 Dichloropropene, 56.7% Chloropicrin, 6.1% other ingredients) six weeks prior to planting. Beds were watered and

fertilized through drip irrigation and pesticide applications were consistent with commercial tomato production in North Carolina (Ivors 2010). Plants were trained using the stake-and-weave-system according to standard practices. Four weeks after planting all plants in this study were pruned by removing all the suckers up to the sucker below the first flower cluster. Plants grafted onto the rootstock 'Beaufort' also had identical non-grafted treatments, but the results from those plots are not reported in this study.

Rootstocks and scions. Grafting was conducted by Tri-Hishtil (Mills River, NC) using the splice grafting method. Commercial tomato (*Solanum lycopersicum* L.) cultivars 'Tasti-lee'® (Bejo Seed, Oceana, CA [TL]) and 'Mountain Fresh Plus' (HMClause Seed Company, Davis, CA [MFP]) were grafted onto rootstocks 'Arnold' (Siegers Seed Co., Holland, MI), 'Beaufort' (DeRuiter Seeds, St. Louis, MO), and 'Shield'(Rijk Zwaan, De Lier, Netherlands). 'Arnold' is a hybrid rootstock thought to confer vigor and has not been evaluated in previous reports. 'Beaufort' was chosen because it is a commonly used rootstock and also thought to be vigorous. 'Shield' was used as it is a common choice for its resistance to bacterial wilt (*Ralstonia solanacearum* [Smith] Yabuuchi, Kosako, Yano, Hotta & Nishiuchi), although it is not considered a vigorous rootstock. Non-grafted plants of both scion cultivars were used as controls and represented standard practice. Transplants of non-grafted plants were grown in a greenhouse at MHCREC under similar conditions as the grafted plants for six weeks until transplanted.

Pinching. All grafted plants were either pinched or not pinched (Supplemental Table 1). The shoot of each plant was aseptically removed 5-7 days before transplanting to allow the two axillary buds to sprout and produce two apical stems.

Harvest. Vine-ripe tomatoes were hand-harvested on 14, 23, 30 Aug and 13, 25 Sep 2017 and 14, 23, 29 August and 6, 13 September 2018. All plants in each plot were harvested. At the

final harvest both years, all the fruit on the plant was harvested (>2.5 cm diameter). All fruit was sorted into unmarketable and marketable, and the marketable fruit was sorted based on USDA size designations. Diameters of specific fruit sizes are: medium = 5.71 - 6.43 cm; large = 6.35 – 7.06 cm; extra-large (XLPlus) = >7 cm. Unmarketable fruit (culls) were determined by size (<5.71 cm) or fruit that had unmarketable fruit characteristics (zippering, cat-facing, rot). The weight and number of fruit in each category was recorded.

Fruit quality characteristics. In 2018 only, after fruit were sorted and weighed, a subsample of fruit collected from TL grafted onto 'Arnold', 'Shield', or 'Beaufort' rootstocks that had been pruned, but not pinched were compared to fruit from non-grafted TL at the second and third harvests to evaluate fruit quality characteristics. A total of 15 fruit were sampled from each rep, for a total of 60 fruit per treatment per harvest. Fruit were evaluated for soluble solids content (SSC%), pH, citric acid %, and lycopene concentration. Fruit were frozen for 2 days at –20 ºC, thawed, pureed with a blender, and further homogenized with a Polytron® PT 10/35 (Brinkmann, Midland, ON, Canada). An aliquot 0.5 mL of puree was placed on a digital refractometer (Atago Pocket Pal, Bellvue WA) and SSC was recorded. The pH of the undiluted puree was determined with a pH meter and a stainless-steel electrode (Hach, Loveland, CO). An aliquot of the concentrated puree was diluted (1:25) with deionized, distilled water and percent titratable acidity (as citric acid equivalent) was determined using a refractometer (Atago acid refractometer, Bellevue WA). Another aliquot of the concentrated puree was diluted (1:3) with deionized, distilled water and total lycopene content was determined using a colorimeter (HunterLab Ultra Pro Xenon, Columbia, MD) with absorbance at 560 and 750 nm. Total lycopene (µg/g fresh weight) was calculated using the formula where m is slope and DF is the dilution factor:

$$\text{Lycopene (ug/g fresh weight)} = (A_{560}\text{-}A_{750})\text{mDF}$$

Statistical Analysis. Yield and fruit quality data were analyzed using PROC GLM in SAS 9.4 (SAS Institute, Cary, NC). Means for both datasets were separated using Tukey's honestly significant difference test (P-value ≤ 0.05). Fruit size category data were analyzed using GraphPad Prism v8.0.2 (GraphPad Software, La Jolla, CA). Fruit category means were separated using 95% confidence intervals.

RESULTS

Rootstock effects on yield. Rootstock was the only effect to significantly increase yield in both years (Table 1). In 2017 and 2018, marketable yield (kg/ha) was significantly greater from both MFP and TL grafted onto the rootstocks 'Arnold' and 'Beaufort', compared to the non-grafted grower standard (Table 2). MFP and TL generated similar yields (P=0.05) so these data were combined (Table 2). 'Shield' did not increase yield in either year nor for each scion compared to non-grafted plants (Table 2). 'Fortamino', evaluated only in 2018, increased yield (kg/ha) by 33.5% in TL and 29.2% in MFP. MFP XLPlus and large fruit sizes were not significantly greater when MFP was grafted on any rootstock, compared to fruit from the non-grafted plants (Fig. 1). TL XLPlus and large fruit sizes were significantly greater when grafted on 'Arnold', 'Beaufort', and 'Fortamino', but not on Shield as compared to fruit from the non-grafted plants (Fig. 2). Bacterial spot and *Verticillium* wilt disease ratings were taken, but did not interact with any of the main effects, and had no significant impact on yield.

Pinching effect on yield. Pinching significantly increased marketable yields by 17.2% in 2018, however, the effect was not significant in 2017 (Table 3). Pinching interacted with both variety and rootstock in 2017, but this interaction was not repeated in 2018 (Table 1). For MFP both in 2017 and 2018 there was no significant difference between pinched and non-pinched fruit

sizes (Fig. 3 & 4). In contrast non-pinched TL plants had significantly more large and XLPlus fruit sizes in 2017 (Fig. 5). In 2018 the exact opposite effect was observed, with pinched TL having significantly more large and XLPlus fruit sizes than non-pinched TL (Fig. 6).

Plant spacing effect on yield. In 2017 56 cm spaced plants had slightly higher yields than grafted plants at 61 cm (81588 vs 77343 kg/ha) (P = 0.037) (Table 1). The marketable yield in plants spaced at 56 cm and 61 apart in 2018 were similar at 81620 and 82418 kg/ha, respectively (P = 0.744) (Table 1). Spacing did not interact with any other main factors.

Rootstock effect on fruit quality. The pH of fruit from TL grafted onto 'Arnold' differed significantly from non-grafted, 'Beaufort', and 'Shield' plants (Table 3). The SSC% of fruit from non-grafted plants was significantly higher than fruit from grafted plants (Table 4). Fruit from non-grafted TL plants had the highest titratable acidity, compared to fruit from grafted TL (Table 4). Total lycopene content of fruit from non-grafted TL and those grafted onto 'Arnold' (56 and 56.70 ug/g fwt, respectively) were statistically higher than 'Beaufort' and 'Shield' plants (52.9 and 53.1 ug/g fwt) (Table 4).

DISCUSSION

Grafting tomatoes onto the rootstocks 'Arnold' and 'Beaufort' consistently increased marketable yield of fresh market tomatoes produced in the field in this study. This fits into a larger body of evidence that shows certain rootstocks can provide yield and fruit sizing benefits to growers (Rivard et al. 2010a; Grieneisen et al. 2018) even in the absence of known negative abiotic and biotic constraints. The most significant gains in fruit size increase was in TL at the XLPlus size when grafted onto Arnold and Beaufort at 74% and 94% respectively for TL, and 30% and 25% for MFP. These data give a strong indication that grafting is a strong way to increase fruit size in low yielding, low disease resistance, but high fruit quality varieties. The

most likely explanation is that TL naturally has less vigor than MFP and therefore responded proportionately more to the beneficial impact of the RS treatments. Other studies have shown that MFP does not appear to benefit from grafting, specifically on the rootstock 'Maxifort' (Grieneisen et al. 2018; Lang and Nair 2019). The grafted tomatoes in our study were planted at a greater spacing than non-grafted plants (56 and 61 cm vs 46 cm) yet still produced significantly higher yields with the two rootstocks. In contrast, the rootstock 'Shield' did not confer an advantage in the absence of bacterial wilt pressure (Table 2).

Although the overall effect of spacing had no significant affect on yield, the highest yielding treatment were unpruned TL grafted onto 'Beaufort', non-pinched, planted at 61 cm spacing (128121 and 120358 total kg/ha for 2017 and 2018 respectively) (Supplemental Table 2). The identical treatment at 56 cm spacing had lower, but still significantly high total yields in 2017 and 2018 at 114495 and 102324 kg/ha (Supplemental Table 2). This indicates that there may be specific circumstances where a small increase in spacing could result in significant increases in yield, and more detailed research is needed it elucidate this. Research tailored for specific cultivars may define winning combinations of techniques. Also, when using 56 and 61 cm spacing, 18-25% fewer plants per hectare are needed for yields equivalent to 46 cm spacing. Because there were no consistent significant differences in overall yield or fruit sizes in 56 compared to 61 cm spaced grafted plants, it is recommended that growers plant grafted plants at 61 cm. This agrees with Suchoff et al. 2014 that show spacing grafted plants (non-pinched) tends to be optimized around 61 cm.

Pinching grafted tomatoes did not provide a consistent benefit at 56 and 61 cm across years. It is likely that the spacing differences were too small to show a significant benefit to pinching. It should also be mentioned that in while both pinching and not pruning plants

increases yields ~10%, the effect is not additive. Increasing the foliage with either technique may allow the plant to take advantage of the more vigorous root system of the tomato rootstock (Kanyomeka and Shivute 2005; J. M. Davis and Estes 1993; Gaytán-Mascorro et al. 2008). To see a more consistent effect of pinching or not pruning future studies should include wider spacings (>61 cm) to accommodate the additional foliage created by grafted pinched or non-pruned plants. Increasing spacing for pinched plants may be the key to offsetting the costs related to pinching. Also, not pruning pinched varieties may be an important component in maximizing foliage while minimizing planting costs. Pinching is an added cost for growers and the optimal parameters under which this practice is used needs to be studied further before widescale adoption by growers in the field. More details on the pruning and pinching interactions will be reported in a separate publication.

Despite some significant differences, overall changes in fruit quality from grafted and non-grafted plants may not result in perceptible differences in taste. Turhan et al. (2011) found that SSC was consistently higher in fruit from non-grafted plants compared to those from ‘Arnold’ and ‘Beaufort’, but was 0.4%, less than the 2% SSC generally thought to be needed for consumers to detect differences in sweetness. In contrast, other research studies have found that grafting has no significant positive or negative effect on overall tomato fruit quality (Davis et al. 2008; Pogonyi et al. 2005; Gaytán-Mascorro et al. 2008).

CONCLUSIONS

Rootstocks ‘Arnold’, Beaufort’, and ‘Fortamino’ all significantly improved yields and, sometimes, fruit size in ‘Tast-lee’ and ‘Mountain Fresh Plus’ grafted plants compared to the non-grafted plants. Of the grafted plants, scions grafted onto ‘Shield’ produced the lowest yields, on average, and were not significantly different than the non-grafted plants. The 61-cm spaced

plants had similar yields to 56-cm spaced plants, indicating that growers can plant fewer grafter plants and achieve similar yields and fruit sizes. Pinching grafted tomato plants increased yield in only one year, so more research ought to be conducted before recommending this practice. Some mixes of horticultural techniques and grafting produced higher yields than non-grafted plants. In the cultivar 'Tasti-lee', fruit quality characteristics such as lycopene, citric acid, SSC%, and pH differed in grafted and non-grafted plants.

ACKNOWLEDGEMENTS

This work was funded by the USDA-NIFA specialty crop research initiative under award number 2016-51181-25404. Additional sources of funding and/or resources include the NC Agricultural Foundation, Inc., NC Tomato Growers Association, Tri-Hishtil, and Bejo Seeds, Inc.. We would like to thank Ryan Loudy and all the staff at the Mountain Horticultural Crops Research and Extension Center for their assistance with executing this research.

REFERENCES

Barrett, C. E., Z. Xin, and A. W. Hodges 2012. "Cost Benefit Analysis of Using Grafted Transplants for Root-Knot Nematode Management in Organic Heirloom Tomato Production." HortTechnology 22 (2): 252–57.

Davis, Angela R., P. Perkins-Veazie, R. Hassell, A. Levi, S. R. King, and X. Zhang, 2008. "Grafting Effects on Vegetable Quality." HortScience 43 (6): 1670–72.

Davis, Jeanine M., and E. A. Estes. 1993. "Spacing and Pruning Affect Growth, Yield, and Economic Returns of Staked Fresh-Market Tomatoes." Journal of the American Society for Horticultural Science 118 (6): 719–25.

Gaytán-Mascorro, A., J. Zaragoza Castellanos-Ramos, S. Villalobos-Reyes, J. C. Díaz-Pérez, and F. Camacho-Ferre. 2008. "Response of Grafted Tomato Plants (*Lycopersicon Esculentum* Mill.) to Leaf Pruning and Nutrient Solution Concentration." Journal of Food, Agriculture and Environment 6: 269–77.

Grieneisen, M. L., B. J. Aegerter, C. S. Stoddard, and M. Zhang. 2018. "Yield and Fruit Quality of Grafted Tomatoes, and Their Potential for Soil Fumigant Use Reduction. A Meta-Analysis." Agronomy for Sustainable Development 38 (3): 29.

Grimault, V., and P. Prior. 1994. "Grafting Tomato Cultivars Resistant or Susceptible to Bacterial Wilt: Analysis of Resistance Mechanisms." Journal of Phytopathology 141 (3): 330–34.

Harrison, D. J., and P. G. Burgess. 1962. "Use of Rootstock Resistance for Controlling *Fusarium* Wilt of Tomatoes." Plant Pathology 11 (1): 23–25.

Ioannou, N. 2001. “Integrating Soil Solarization with Grafting on Resistant Rootstocks for Management of Soil-Borne Pathogens of Eggplant.” The Journal of Horticultural Science and Biotechnology 76 (4): 396–401.

Ivors, K. 2010. “Commercial Production of Staked Tomatoes in the Southeast.” North Carolina State Univ. Coop. Ext., Raleigh, NC.

Kanyomeka, L., and B. Shivute. 2005. “Influence of Pruning on Tomato Production under Controlled Environments.” Agricultura Tropica et Subtropica 38 (2): 79–83.

Kubota, C., M. A. McClure, N. K., Michael G. Bausher, and E. N. Rosskopf. "Vegetable grafting: History, use, and current technology status in North America." HortScience 43, no. 6 (2008): 1664-1669.

Kyriacou, M. C., Y. Rouphael, G. Colla, R. Zrenner, and D. Schwarz. 2017. “Vegetable Grafting: The Implications of a Growing Agronomic Imperative for Vegetable Fruit Quality and Nutritive Value.” Frontiers in Plant Science 8: 741.

Lang, K.M. and Nair, A., 2019. Effect of Tomato Rootstock on Hybrid and Heirloom Tomato Performance in a Midwest High Tunnel Production System. HortScience, *54*(5), pp.840-845.

Lee, J. 1994. “Cultivation of Grafted Vegetables I. Current Status, Grafting Methods, and Benefits.” HortScience 29 (4): 235–39.

Maboko, M. M., C. P. Du Plooy, and S. Chiloane. 2011. “Effect of Plant Population, Fruit and Stem Pruning on Yield and Quality of Hydroponically Grown Tomato.” African Journal of Agricultural Research 6 (22): 5144–48.

Miguel, A. 2002. “Grafting as a Non-Chemical Alternative to Methyl Bromide for Tomato in Spain.” In Proceedings of International Conference on Alternatives to Methyl Bromide. The Remaining Challenges, 283–84.

Oztekin, G. B., F. Giuffrida, Y. Tuzel, and C. Leonardi. 2009. “Is the Vigour of Grafted Tomato Plants Related to Root Characteristics.” Journal of Food, Agriculture and Environment 7: 364–68.

Pogonyi, Á., Z. Pék, L. Helyes, and A. Lugasi. 2005. “Effect of Grafting on the Tomato’s Yield, Quality and Main Fruit Components in Spring Forcing.” Acta Alimentaria 34 (4): 453–62.

Qiao, K., Wang, Z., Wei, M., Wang, H., Wang, Y., & Wang, K. (2015). Evaluation of chemical alternatives to methyl bromide in tomato crops in China. Crop Protection, *67*, 223–227.

Rahmatian, A., Delshad, M., & Salehi, R. (2014). Effect of grafting on growth, yield and fruit quality of single and double stemmed tomato plants grown hydroponically. Horticulture, Environment, and Biotechnology, 55(2), 115-119.

Riga, P., L. Benedicto, L. García-Flores, D. Villaño, S. Medina, and A. Gil-Izquierdo. 2016. “Rootstock Effect on Serotonin and Nutritional Quality of Tomatoes Produced under Low Temperature and Light Conditions.” Journal of Food Composition and Analysis 46: 50–59.

Rivard, C. L., S. O’Connell, M. M. Peet, and F. J. Louws. 2010. “Grafting Tomato with Interspecific Rootstock to Manage Diseases Caused by *Sclerotium Rolfsii* and Southern Root-Knot Nematode.” Plant Disease 94 (8): 1015–21.

Rivard, C. L., O. Sydorovych, S. O’Connell, M. M. Peet, and F. J. Louws. 2010. “An Economic Analysis of Two Grafted Tomato Transplant Production Systems in the United States.” HortTechnology 20 (4): 794–803.

Rivero, R. M., J. M. Ruiz, and L. Romero. 2003. “Role of Grafting in Horticultural Plants under Stress Conditions.” Journal of Food Agriculture and Environment 1: 70–74.

Rysin, O., and F. J. Louws. 2015. “Decision Tool for Growers to Evaluate Economic Impact of Grafting Technology Adoption: An Application to Open-Field Conventional Tomato Production.” HortTechnology 25 (1): 132–38.

Scott, J. W., E. A. Baldwin, H. J. Klee, J. K. Brecht, S. M. Olson, J. A. Bartz, and C. A. Sims. 2008. “Fla. 8153 Hybrid Tomato; Fla. 8059 and Fla. 7907 Breeding Lines.” HortScience 43 (7): 2228–30.

Suchoff, D., C. Gunter, J. Schultheis, and F. J. Louws. 2014. “On-Farm Grafted Tomato Trial to Manage Bacterial Wilt.” In I International Symposium on Vegetable Grafting 1086, 119–27.

Suchoff, David H., Christopher C. Gunter, Jonathan R. Schultheis, Richard L. Hassell, and Frank J. Louws. "The effect of grafting on nitrogen use in determinate field-grown tomatoes." The Journal of Horticultural Science and Biotechnology 94, no. 1 (2019): 102-109.

Turhan, A., N. Ozmen, M. S. Serbeci, and V. Seniz. 2011. “Effects of Grafting on Different Rootstocks on Tomato Fruit Yield and Quality.” Hortic. Sci 38: 142–49

World Bank. (2015). Arable land (hectares per person). Retrieved from https://data.worldbank.org/indicator/AG.LND.ARBL.HA.PC

Table 3.1. Significance of main effects and interactions, based on their ability to influence the marketable yield. Higher level interactions not displayed because none were significant (P>0.05)

	2017	2018
Effect	P value[z]	P value[z]
Variety	0.271	0.044
Rootstock	<0.0001	0.016
Pinched	0.07	<0.0001
Spacing	0.037	0.744
Rootstock x Variety	0.173	0.388
Rootstock x Spacing	0.745	0.498
Pinched x Rootstock	0.0057	0.163
Pinched x Variety	0.046	0.285
Pinched x Spacing	0.511	0.796
Variety x Spacing	0.413	0.886

[z]P values determined using the general linear model.

Table 3.2: Marketable fruit (kg/hectare) from grafted and non-grafted tomato plants from trials conducted in 2017 and 2018 in Mills River, NC.

Rootstock [z]	Marketable ($kg \cdot ha^{-1}$)[y]	
	2017	2018
Arnold	84883 a	78679 a
Beaufort	82848 a	80467 a
Fortamino	--	82798 a
Shield	60335 b	63437 b
Non-grafted	58111 b	67933 b

[z]Mountain Fresh Plus and Tasti-Lee were grafted onto rootstocks Arnold, Beaufort, Fortamino (2018 only), Shield or non-grafted.

[y]Mean $kg \cdot ha^{-1}$ followed by significance letter groupings determined by Tukey's HSD.

Table 3.3: Marketable fruit from pinched and non-pinched grafted plants from trials conducted in 2017 and 2018 in Mills River, NC. No cultivar or rootstock interaction detected ($P>0.05$); reported yields are combined from TL and MFP plants.

Pinching[y]	Marketable (kg/ha)[z]	
	2017	2018
Non-pinched	76021 a	74194 a
Pinched	80552 a	86979 b

[z]Mean kg/ha followed by significance letter groupings determined by Tukey's HSD at.

[y]Number of heads/leaders/main stems created after seedlings were pinched.

Table 3.4: Fruit quality characteristics of fruit harvested from TL grafted onto three rootstocks or non-grafted in 2018.

Rootstock	pH[z]	SSC%[z]	Citric Acid%[z]	Lycopene (µg/g)[z]
Non-Grafted Tasti-lee	4.35 b	4.9 a	0.50 a	56.7 a
Tasti-Lee/Arnold	4.39 a	4.6 b	0.48 ab	56 a
Tasti-Lee/Beaufort	4.35 b	4.5 b	0.47 b	53.1 b
Tasti-Lee/Shield	4.34 b	4.7 b	0.44 c	52.9 b

[z]Mean (n=120) followed by significance letter groupings determined by Tukey's HSD test.

[y]Percent soluble solids content.

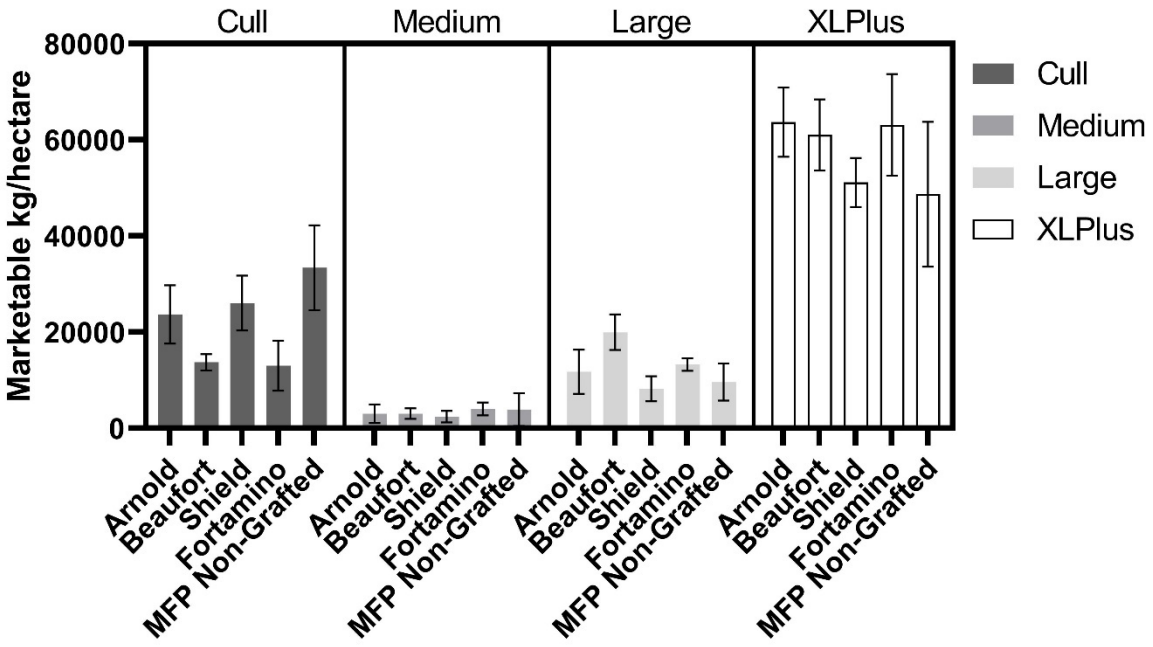

Figure 3.1: Fruit category distribution from pruned, non-pinched, grafted and non-grafted MFP tomatoes, 2017 and 2018 combined. Error bars represent the 95% confidence interval. Fortamino was only planted in 2018.

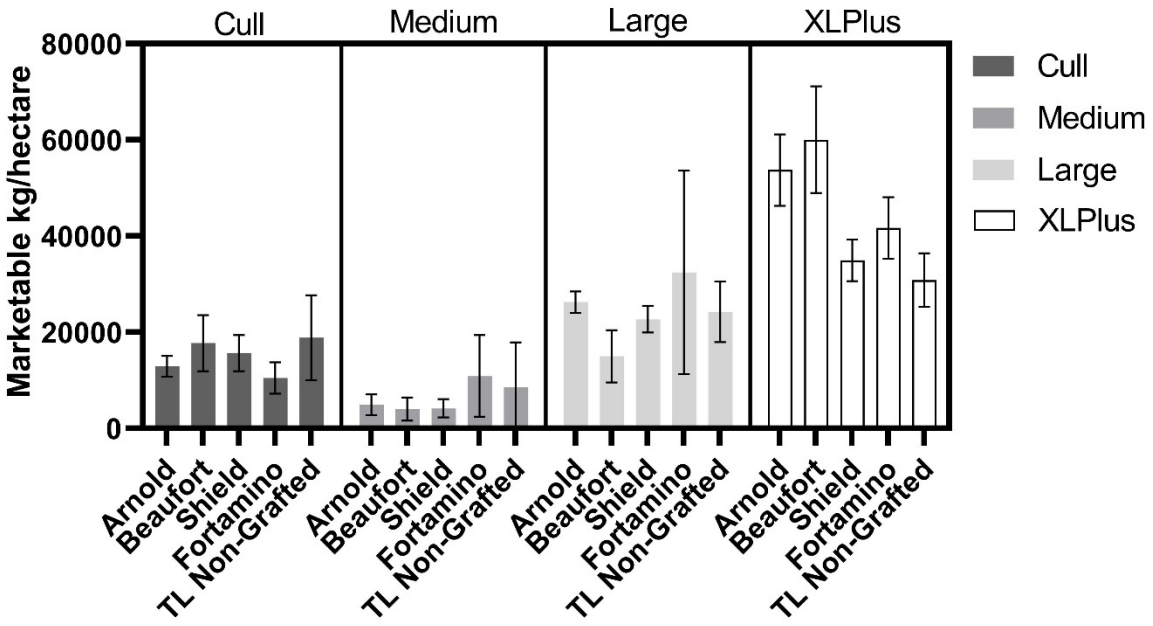

Figure 3.2: Fruit category distribution from pruned, non-pinched, grafted and non-grafted TL tomatoes, 2017 and 2018 combined. Error bars represent the 95% confidence interval. Fortamino was only planted in 2018.

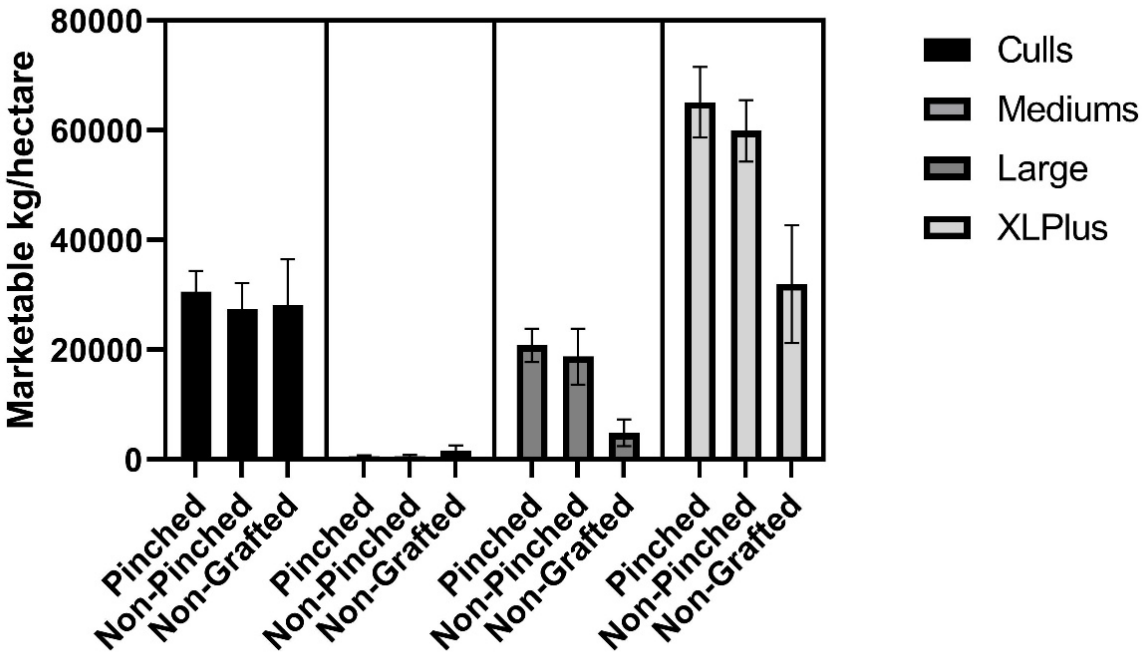

Figure 3.3: Mean fruit category distribution of all pinched and non-pinched MFP tomatoes in 2017. Error bars represent the 95% confidence interval.

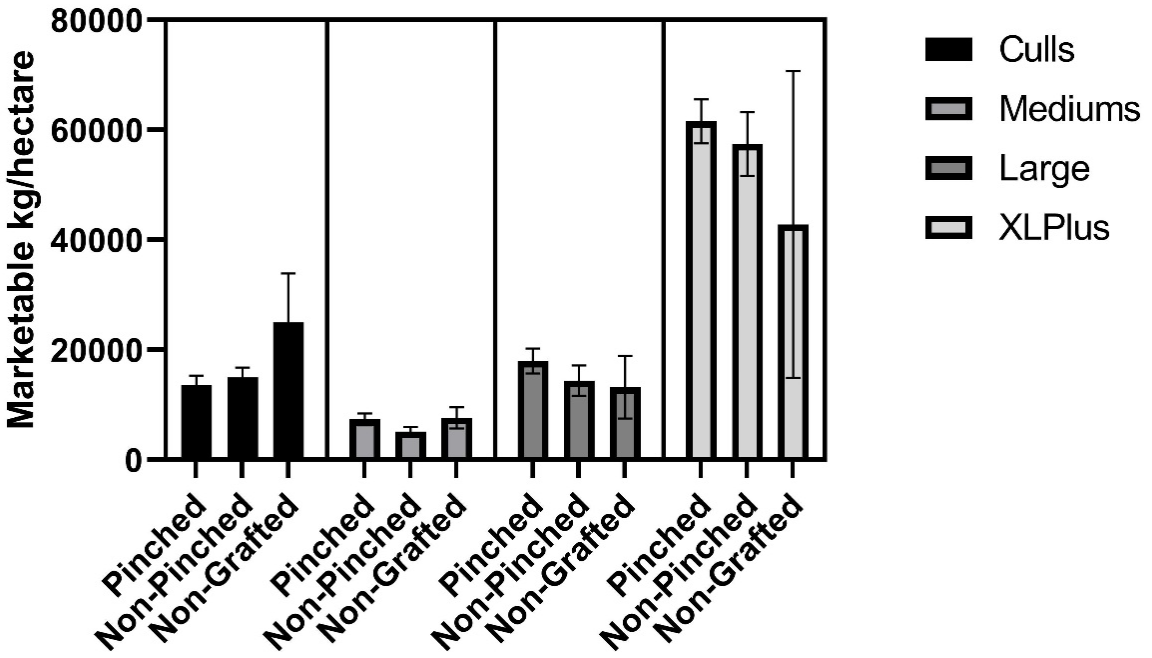

Figure 3.4: Mean fruit category distribution of all pinched and non-pinched MFP tomatoes in 2018. Error bars represent the 95% confidence interval.

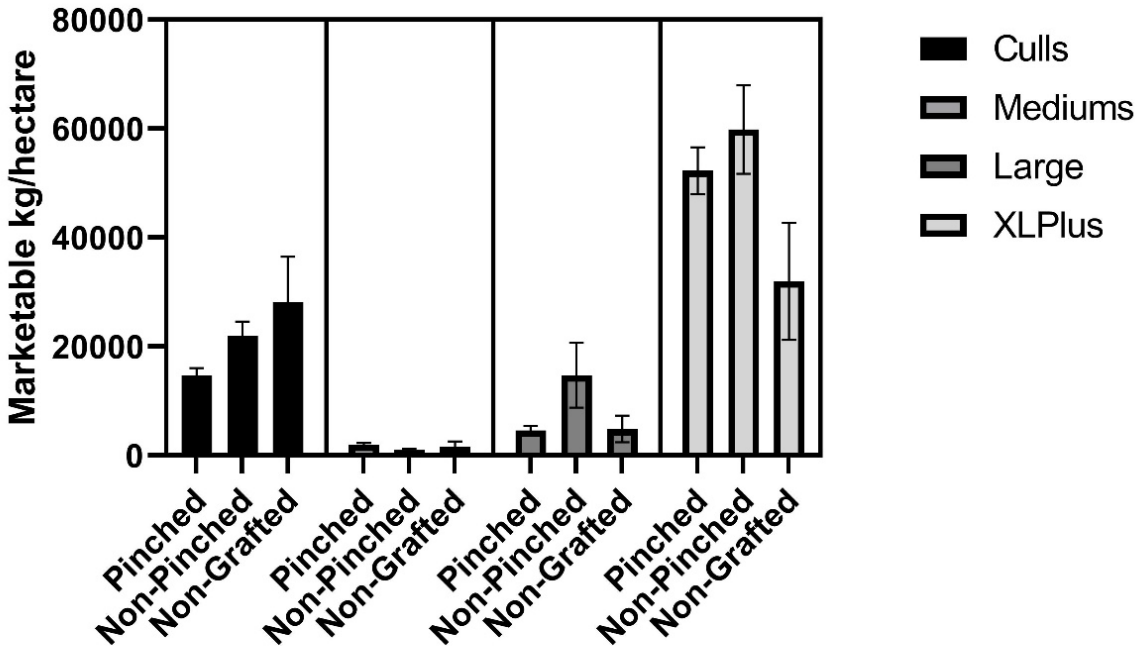

Figure 3.5: Mean fruit category distribution of all pinched and non-pinched TL tomatoes in 2017. Error bars represent the 95% confidence interval.

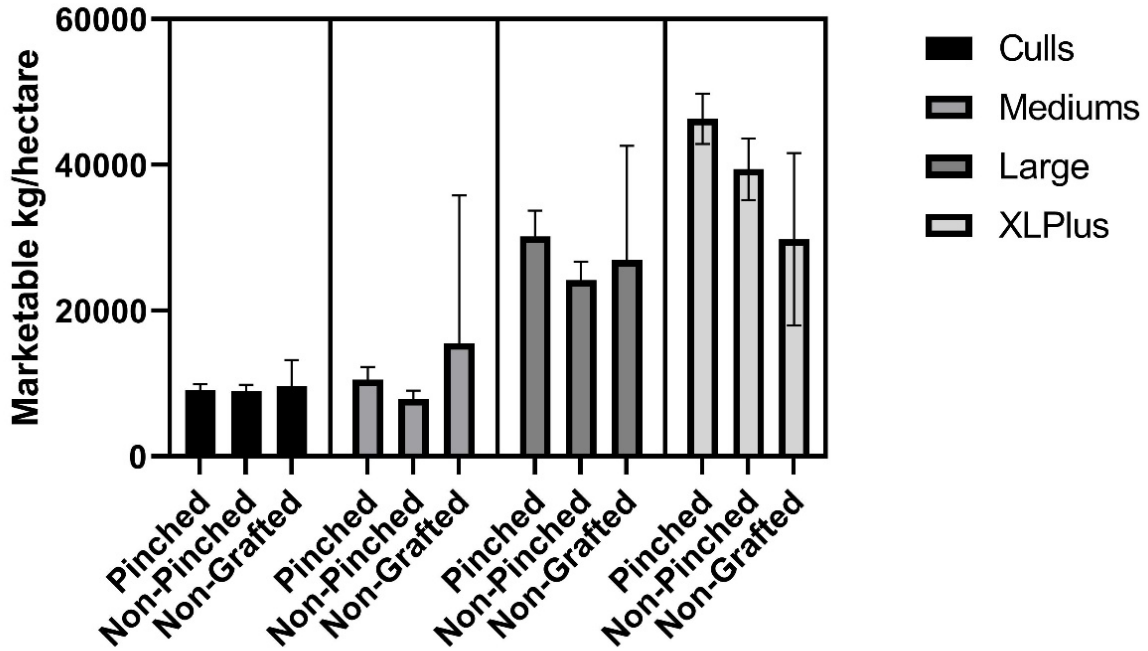

Figure 3.6. Mean fruit category distribution of all pinched and non-pinched TL tomatoes in 2018. Error bars represent the 95% confidence interval.

Supplemental Table S3.1: List of rootstock, spacing, pinching, and pruning treatment combinations with TL or MFP.

Scion/Variety	Rootstock	Spacing	Heads	Pruned
Tasti-lee or MFP	Arnold	56 cm	2	yes
Tasti-lee or MFP	Arnold	61 cm	2	yes
Tasti-lee or MFP	Arnold	56 cm	1	yes
Tasti-lee or MFP	Arnold	61 cm	1	yes
Tasti-lee or MFP	Shield	56 cm	2	yes
Tasti-lee or MFP	Shield	61 cm	2	yes
Tasti-lee or MFP	Shield	56 cm	1	yes
Tasti-lee or MFP	Shield	61 cm	1	yes
Tasti-lee or MFP	Beaufort	56 cm	2	yes
Tasti-lee or MFP	Beaufort	61 cm	2	yes
Tasti-lee or MFP	Beaufort	56 cm	1	yes
Tasti-lee or MFP	Beaufort	61 cm	1	yes
Tasti-lee or MFP	Beaufort	56 cm	2	no
Tasti-lee or MFP	Beaufort	61 cm	2	no
Tasti-lee or MFP	Beaufort	56 cm	1	no
Tasti-lee or MFP	Beaufort	61 cm	1	no
Tasti-lee or MFP	Fortamino*	61 cm	1	yes
Tasti-lee or MFP	none	46 cm	1	yes

*Fortamino was only planted in 2018.

Supplemental Table S3.2: Yields (kg/ha) of Tasti-lee grafted on to different rootstocks and submitted to different horticultural practices. Rootstock, spacing, number of heads (pinched = 2 head, non-pinched = 1 head), pruning combinations reported.

				Tasti-lee (kg/ha)			
				2017		2018	
Rootstock	Spacing	Heads	Pruning	Total	Marketable	Total	Marketable
Arnold	56 cm	2	Pruned	97307	79371	92879	83514
Arnold	61 cm	2	Pruned	94146	78936	102584	92320
Arnold	56 cm	1	Pruned	108358	90329	90698	79919
Arnold	61 cm	1	Pruned	108369	91261	84133	75777
Shield	56 cm	2	Pruned	99829	82484	96880	87076
Shield	61 cm	2	Pruned	90422	73422	86707	77650
Shield	56 cm	1	Pruned	84573	66553	71585	61224
Shield	61 cm	1	Pruned	80500	55092	73639	64692
Beaufort	56 cm	2	Pruned	111953	92775	99585	92090
Beaufort	61 cm	2	Pruned	107729	89363	97788	89023
Beaufort	56 cm	1	Pruned	116561	98647	80665	72276
Beaufort	61 cm	1	Pruned	108684	91338	81537	74493
Beaufort	56 cm	2	Non-Pruned	112917	95042	99528	90559
Beaufort	61 cm	2	Non-Pruned	102747	84678	103199	94508
Beaufort	56 cm	1	Non-Pruned	114495	97291	102324	94481
Beaufort	61 cm	1	Non-Pruned	128121	100247	120359	108748
Fortamino	61 cm	1	Pruned			95549	85058
none	46 cm	1	Pruned	83300	55216	81869	72216

Supplemental Table S3.3: Yields (kg/ha) of 'Mountain Fresh Plus' grafted on to different rootstocks and submitted to different horticultural practices. Rootstock, spacing, number of heads (pinched = 2 heads, non-pinched = 1 head), pruning combinations reported.

				Mountain Fresh Plus (kg/ha)			
				2017		2018	
Rootstock	Spacing	Heads	Pruning	Total	Marketable	Total	Marketable
Arnold	56 cm	2	Pruned	112175	77031	96352	82410
Arnold	61 cm	2	Pruned	105912	73241	105132	87702
Arnold	56 cm	1	Pruned	114208	77062	95020	80756
Arnold	61 cm	1	Pruned	108223	78096	91242	78026
Shield	56 cm	2	Pruned	104140	66283	98934	86785
Shield	61 cm	2	Pruned	103706	69030	93224	78932
Shield	56 cm	1	Pruned	96016	63311	79869	63019
Shield	61 cm	1	Pruned	94349	56203	81124	64623
Beaufort	56 cm	2	Pruned	105805	84311	100160	87558
Beaufort	61 cm	2	Pruned	104315	82785	109054	97900
Beaufort	56 cm	1	Pruned	98171	85717	103542	89596
Beaufort	61 cm	1	Pruned	89466	75729	100135	85260
Beaufort	56 cm	2	Non-Pruned	118627	97130	107382	95348
Beaufort	61 cm	2	Non-Pruned	106828	88209	108721	98251
Beaufort	56 cm	1	Non-Pruned	105840	93080	97945	87563
Beaufort	61 cm	1	Non-Pruned	108870	94783	102735	90431
Fortamino	61 cm	1	Pruned			93448	80413
none	46 cm	1	Pruned	102805	60918	88499	63547

CHAPTER 4

VERTICILLIUM WILT AND BACTERIAL SPOT RESISTANT TOMATO SCION WITH VIGOROUS ROOTSTOCKS INCREASES YIELDS IN GRAFTED TOMATOES IN NON-FUMIGATED FIELDS

ABSTRACT

In Western North Carolina non-race 1 *Verticillium dahliae* is a devastating soil-borne fungus that infects many economically important vegetable crops, including tomato. Grafting tomatoes for disease resistance has been a successful strategy in combating other soil-borne pathogens such as *Fusarium* wilt, root-knot nematodes, bacterial wilt, and *V. dahliae* race 1 (Vd1). While there are rootstocks resistant against *V. dahliae* race 2 (Vd2), the majority of fields in North Carolina are contaminated with non-race 1 or 2 strains (VdN). A field survey of 18 rootstocks was conducted in 2017, 2018, and 2019 in a highly *V. dahliae* infested field in Mills River, NC. Foliar necrosis and chlorosis associated with *V. dahliae* infections were rated and scores developed from those ratings. While several highly resistant tomato lines were identified, when used as rootstocks the susceptible scion 'Red Defender' (RD) was as diseased as the non-grafted and self-grafted controls. The most resistant tomato line was NC-7GEM which had the lowest severity of all lines tested. Additionally, NC-7GEM had the lowest incidence of symptoms? 33% compared to the RD susceptible controls with 100%. However, when used as a rootstock NC-7GEM was unable to protect the susceptible RD scion. In contrast, when NC-7GEM was used as a resistant scion with a susceptible RD rootstock, the scion showed little symptoms?. This indicated field resistance to VdN based on foliar necrosis and chlorosis symptoms in the scion is most likely controlled by the scion, not the rootstock. Additionally, the only rootstock to provide increased fruit size and overall yield in any year was 'Maxifort'.

‘Maxifort’ is resistant to Vd1 and susceptible to VdN and did not prevent foliar symptoms from forming in susceptible RD scion. This rootstock is highly vigorous and even under disease pressure was able to increase yields in susceptible plants. In 2019 a final field trial was conducted to demonstrate the optimal combination of rootstocks and scions in *V. dahliae* infested fields. NC-7GEM/Maxifort had the highest overall yield at 76,504 kg/ha, and the .area under the growth progress curve (AUGPC) score of 179.6. While this combination had significantly higher overall yields than the non-grafted RD (46,798 kg/ha) and RD/NC-7GEM (47,169 kg/ha), it was not significantly higher than the RD/Maxifort combination with an overall yield of 61,785 kg/ha. Red Defender plants also had significantly larger fruit, with more jumbo and extra-large fruit. These results indicate that while the resistant ‘NC-7GEM’ is excellent at reducing symptoms of VdN and bacterial spot (BS), it must be bred with elite breeding lines with better fruit qualities before release. Grafting tomatoes in VdN infested fields appears to greatly increase yield when vigorous disease tolerant rootstocks such as ‘Maxifort’ are used. Optimal conditions for growing in VdN infested fields appears to be a combination of VdN and BS resistant scions and vigorous/VdN tolerant rootstocks.

INTRODUCTION

Verticillium dahliae Kleb. is an insidious soil-borne pathogenic fungus that can infect many economically important plant species, including tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.), cotton (*Gossypium* spp. L.), and mint (*Mentha* spp. L.) (Dung et al. 2013; Klosterman et al. 2009). *V. dahliae* can survive for over a decade in soil, even with no viable host present (Green 1980; Xiao et al. 1998). Host resistance against *V. dahliae* is one of the few management practices that is truly effective for controlling *V. dahliae*. Unfortunately resistance against *V. dahliae* race 1 (Vd1) is currently the only well described and highly deployed resistance available in commercial cultivars (Diwan et al. 1999, 1; Klosterman et al. 2009). A newly described race 2 (Vd2) resistance phenotype is available in three commercial hybrids from Japan, 'Aibou', 'Ganbarune-Karis', and 'Bowman' (Usami et al. 2017; Kano and Usami 2019). Unfortunately, several unpublished reports indicate this resistance will be inconsistently effective in both North Carolina and California as many isolates in these states are not race 2 (Iott 2013) (unpublished data). The majority of fields sampled in North Carolina contain non-race 1 and race 2 isolates (VdN), only 1 field in Haywood county NC, and 1 isolate in San Benito county CA contained race 2 isolates (unpublished data).

Grafting tomatoes is a successful management strategy against a multitude of soil-borne pathogens. Allowing growers to grow their variety of choice is important. Resistance against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) races 1-3, and *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) is highly effective at controlling these soil-borne fungal diseases (Hibar et al. 2006; Ozbay and Newman 2004; Louws et al. 2010; Rivard and Louws 2008). Bacterial wilt of tomatoes (*Ralstonia solanacearum*) can also be controlled by grafting susceptible tomatoes onto resistant cultivars (Rivard et al. 2012; Suchoff et al. 2014). Tomato lines with the Mi gene is also

effective against root-knot nematodes (Lopez-Perez et al. 2006; Diwan et al. 1999; Verdejo-Lucas et al. 2013). In the case of FOL and bacterial wilt, the diseases will often completely wilt susceptible tomatoes.

In non-disease infested fields grafting can also provide a significant benefit (Masterson et al. 2016; Khah et al. 2006). Interspecific hybrids such as 'Maxifort' and 'Beaufort' (De Ruiter/Monsanto, Bergschenhoek, Netherlands) are the results of a cross between *S. lycopersicum* and *S. habrochaites*. These kinds of interspecific hybrids provide a high level of vigor and disease resistance and tolerance (Rivard et al. 2010; Djidonou et al. 2017). Interspecific hybrids may also provide some level of VdN tolerance/resistance (Bryan 1925; Iott 2013; Pegg and Brady 2002). 'Beaufort' has been used as a rootstock for eggplant (*Solanum melongena* L.) and provides some level of resistance against VdN (Johnson et al. 2014). Wild tomato lines are a rich source of genetic material, especially for grafting where breeders do not need to focus on fruit traits and can focus solely on vigor and disease resistance. Overall grafting with vigorous rootstocks provides a significant benefit over non-grafted plants, however, there are many accounts of grafting providing no benefit, or a negative benefit (Grieneisen et al. 2018). The maximum benefit for grafting in tomatoes may be grafting low-yielding low resistance varieties onto highly vigorous rootstocks. It is vital for growers and extension professionals to perform extensive field testing before deploying new rootstock scion combinations.

Reports of tomato varieties resistant to VdN are mixed in the literature. One of the main issues with breeding for resistance against any plant pathogen is a lack of understanding of pathogen populations. A study of multiple lines with reported resistance showed a specific race interaction between 'UC 82', where 1 (an isolate from Spain) out of 4 VdN isolates tested was unable to cause symptoms (Baergen et al. 1993). One of three pathogenic isolates on 'UC 82'

was from North Carolina, and all 4 genotypes were pathogenic on ‘IRAT-L3’ (Baergen et al. 1993). ‘IRAT-L3’ is a tomato variety developed from ‘Hawaii 7998’. Some initial reports indicated this line may be resistant to VdN (Gold et al. 1996). Another confounding factor is that some fields may be contaminated with multiple races or genotypes of *V. dahliae*. A study of 70 Japanese tomato fields showed that 25 fields contained only Vd2, 40 fields had only VdN (aka race 3), and 5 fields had both races (Usami et al. 2017).

The purpose of this study was to test a multitude of tomato rootstocks to discover the optimal conditions for growing in highly contaminated tomato fields in Western North Carolina. Susceptible ‘Red Defender’ (RD) tomatoes were grafted onto resistant rootstocks. A final test was conducted in 2019 to test the heterograft combinations of RD, ‘NC-7GEM’, and ‘Maxifort’ to test the most important traits for rootstock scion combinations. Our findings demonstrate that scions must have high levels of resistance to VdN and bacterial spot (*Xanthomonas perforans* and *Xanthamonas* spp.). Successful rootstocks must either be able to prevent disease from forming in the scion, increase yield, or both.

MATERIALS AND METHODS

Field conditions. In 2017, 2018, and 2019 grafted and non-grafted tomatoes were planted in a VdN infested field in Mills River, NC at locations 35°23'29.0"N 82°33'46.4"W, 35°23'38.1"N 82°33'41.8"W, and 35°23'32.6"N 82°33'26.6"W respectively. The planting dates were June 15th 2017, June 15th 2018, and June 20th 2019. Grafted and non-grafted plants were planted 5 weeks post-germination. All tomatoes were planted at 56 cm in-row spacing. Rows were 30 cm high and 100 cm wide with 1.5 meters between row centers. A 1 meter fallow section was left between rows. White 1.5ml polyethylene plastic was used to cover rows. In 2017 and 2018 the grafted and non-grafted tomatoes were planted in non-fumigated rows. In 2017 self-grafted and

non-grafted race 1 resistant RD (Red Defender, HM.CLAUSE, Inc Davis California) were also planted in an adjacent fumigated row. In 2018 only non-grafted plants were planted on the fumigated row. Fumigation was accomplished using Pic-Clor 60 (1,3-dichloropropene and chloropicrin) 90 kilograms of active ingredients per hectare. Drip tape was used to irrigate and fertilize the plants throughout the season, and plants were staked and strung at the standard rates (Ivors 2010).

Rootstock scion combinations. In 2017 Vd1 resistant RD plants were grafted onto 10 candidate rootstocks, non-grafted and self-grafted RD controls were grown simultaneously. In 2018 RD plants were grafted onto 8 candidate rootstocks, along with self-grafted and non-grafted controls. In 2019 only RD, NC-7GEM, and Maxifort heterographs were planted, along with experimental varieties (EV). Self-grafted and non-grafted plants were germinated at the same time. Plants were grafted 3 weeks post-germination. All grafted plants were placed in a healing cage with 2 layers of black shade cloth, 90% density per layer. Misters sprayed water for 10 seconds every 5 minutes. After 2 days post-grafting 1 layer of shade cloth was removed. At 3 days misters were set to 10 seconds every 10 minutes. At 5 days post-grafting all shade cloth was removed from the sides, and only the top shade cloth remained. At 8 days post grafting the plants were removed from the misting chamber and transported to a greenhouse. 3 days pre-planting plants were placed in a concrete platform to harden the plants off. Both grafted and non-grafted tomatoes were planted by hand with the graft union at least 4 cm above the soil line. In 2017, 2018, and 2019 the plants were planted in a randomized complete block design. In 2017 and 2018 there were four reps across four rows, with each plot containing 5-12 plants. In 2019 there were 8 rows, with each row being a rep containing up to 5 plants per plot.

Disease ratings. In all three years disease ratings for foliar necrosis/chlorosis were taken every 14 days on every single plant until symptoms emerged, then disease ratings were taken every 7 days until the second harvest. *V. dahliae* associated chlorosis and necrosis symptoms were rated at a percent of diseased tissue on the overall foliage. In 2019 bacterial spot ratings for *X. perferens* were taken as well. Disease ratings were used to calculate the area under the disease pressure curve (AUDPC) (Jeger and Viljanen-Rollinson 2001). The sum of the areas between two disease ratings Y_i is the disease score taken at a specific timepoint T_i.

$$AUDPC = \sum_{i=1}^{n_{i-1}} \left(\frac{Y_i + Y_{i+1}}{2} \right) (T_{i+1} - T_i)$$

Statistical analysis was conducted in SAS (Cary, NC, USA) using PROC GLIMMIX, and significant differences between treatments was determined by Tukey's honestly significant difference (HSD).

Yield and fruit ratings. Fruits were harvested when tomatoes reached the breaker stage. There were 5, 4, and 7 harvests taken in 2017, 2018, and 2019 respectively. In 2017 and 2018 only grafted and non-grafted RD and NC-1GEM plants/scions were harvested. No non-grafted resistant rootstock candidates were harvested in 2017 or 2018. In 2019 RD and NC-7GEM plants/scions were harvested. Harvests were taken every week except in 2018 where there was a 2-week gap between harvest 3 and 4. Each plot was harvested, fruit was sorted into culls (fruit 2.5 cm in diameter), smalls (2.5-5.71 cm), medium (5.71-6.43 cm), large (6.35-7.06 cm), extra-large (7.06-8.8 cm), and jumbo (>8.8 cm). Significant differences between treatments was determined using SAS (Cary, NC, USA) PROC GLM and students t-test.

Vigor ratings. In 2019 vigor ratings were taken from all tomato plants. Vigor ratings were taken at the time points as disease ratings, every two weeks until disease emerged, then every

week until the second harvest. Vigor ratings were taken on a 1-10, 10 being the largest plant in the field by height and foliage thickness, and 1 being a plant 10% the size of the largest plant. Area under the growth progress curve (AUGPC) scores were calculated from the vigor scores taken over the whole season. AUGPC is the sum of the averages of the vigor scores between time points where Y_i is the vigor score taken at a specific timepoint T_i (Shanner and Finney 1977)

$$AUGPC = \sum_{i=1}^{n_{i-1}} \left(\frac{Y_i + Y_{i+1}}{2} \right) (T_{i+1} - T_i)$$

Nutrient analysis. Macro and micro-nutrient analysis of tomatoes planted in 2019 was taken at the second harvest time. Five most recent mature leaves (MRML) were taken from plants within plots from reps 1, 3, 5, and 7. Samples were submitted to the North Carolina Department of Agriculture & Consumer Services (NCDA&CS) for processing. MRMLs from each plot were dried for 12-24 hours at 80 °C. Samples were ground in a stainless steel grinder (Wiley Mini-Mill; Thomas Scientific; Swedesboro, NJ) and filtered through a 20-mesh (1-mm) screen. Nitrogen concentration is established using an elemental analyzer (NA1500; CE Elantech Instruments; Lakewood, NJ) (Campbell and Plank 1992). Nutrient content of other nutrients is found using an inductively-couple-plasma (ICP) spectrophotometer (Optima 3300 DV ICP emission spectrophotometer; Perkin Elmer Corporation; Shelton, CT). Further information regarding methods used can be found at the NCDA&CS website http://www.ncagr.gov/agronomi/pdffiles/plantguide.pdf. Statistical analysis of each nutrient was determined using SAS (Cary, NC, USA) PROC GLM, significant differences was determined with students t-test.

Confirmation of VdN in field. In 2017 isolates were recovered from 144 susceptible RD guard plants. Stem cuttings from above the cotyledon scar were taken 90 days post planting. These stem cuttings were stored for 24-72 hours in cold storage. The cuttings were disinfested in 75% ethanol for 1 minute, washed in autoclaved deionized water, then placed in 1% sodium hypochlorite for 1minute, washed in autoclaved deionized water, placed on sterile filter paper for 1 minute, then placed onto Sorensons NP-10 (Kabir et al. 2004). Plates with stem cuttings were placed in a 26 °C incubator for 3 weeks. Plates with fungal colonies with microsclerotia were rated as positive for *V. dahliae*. ITS sequences of 5 isolates was used as secondary confirmation of *V. dahliae* species. Ave1 primers were used to determine isolates were non-race 1 (Diwan et al. 1999, 1).

RESULTS

Effects of self-grafting and fumigation on VW disease development and yield. In 2017 self-grafted non-fumigated RD plants had significantly lower AUDPC scores than non-grafted and self-grafted RD plants in the fumigated row. However, self-grafted plants did not have significantly lower AUDPC scores than non-grafted non-fumigated plants (Fig. 1). In 2018 there were no significant differences between self-grafted, non-grafted, fumigated, or non-fumigated disease scores (Fig. 2). In both 2017 and 2018 there were no significant differences between self-grafted, non-grafted, fumigated, or non-fumigated marketable or overall yield scores (Fig. 3-6).

Impact of grafting on VdN disease progression. No rootstock was able to show a consistent ability to prevent the susceptible RD scion from becoming diseased by VdN. In 2017 RD-‘IRAT-L3’ was the only scion-rootstock combination to have significantly less disease than the RD non-grafted and self-grafted controls (Table 1). However, in 2018 RD-‘IRAT-L3’ had the highest disease of all scion-rootstock combinations. ‘NC-7GEM’ and ‘SG06-220’ were the

only rootstocks to have significantly less disease than the RD controls in both 2017 and 2018 (Table 2). In 2018 and 2019 both non-grafted 'NC-7GEM' and 'NC-7GEM'-RD scion-rootstock combination had significantly less disease than RD controls with AUDPC scores (Tables 2&3).

Impact of grafting on bacterial spot disease progression. Bacterial spot (BS) incidence was 100% in 2019. All five of the EV lines, NC-7GEM, 'NC-7GEM'-RD, and 'NC-7GEM'-'Maxifort' had the lowest BS AUDPC scores. RD, 'RD-7GEM', and RD-'Maxifort', had the highest BS AUDPC scores. 'Maxifort' had intermediate BS scores, but did group significantly with the RD plants (Table 3).

Vigor comparisons. In 2019 non-grafted 'Maxifort' had the highest AUGPC rating of 212. 'NC-7GEM', 'NC-7GEM'-RD, and 'NC-7GEM'-'Maxifort' had the second highest AUGPC scores, but were significantly lower than the non-grafted 'Maxifort'. RD, RD-'NC-7GEM', and RD-'Maxifort' were all lower than any of the 'NC-7GEM' grafted or non-grafted plants at 95.3, 95.8, and 130.1 respectively. However, RD-'Maxifort' had significantly higher AUGPC scores than non-grafted RD and RD-'NC-7GEM' (Table 3).

Yield and fruit sizing. In 2017 and 2018 RD-'Maxifort' had the highest marketable and total yields. RD-Maxifort was the only grafted rootstock scion to have significantly higher average total or marketable yield than any of the RD controls for all three years (Fig. 7-8 & 10-11). Maxifort was also able to increase the number of jumbo and extra-large fruits in its RD scion in both 2017 and 2019 (Fig. 9)(Table 4). In 2019 'NC-7GEM'-'Maxifort' had the highest yields of all the treatments, however, the total yields and marketable yields were not significantly different from 'NC-7GEM' non-grafted, 'NC-7GEM'-RD. 'NC-7GEM' had significantly more medium, smalls, and culled fruits than any of the RD rootstock scion or non-grafted combinations.

Nutrient analysis. 'NC-7GEM' plants had significantly higher nitrogen, phosphorous, and potassium than RD grafted and non-grafted plants, or non-grafted 'Maxifort' plants, with the exception of the RD-'Maxifort' tomatoes. Calcium was significantly higher in RD plants than 'NC-7GEM' or 'Maxifort' plants. Maxifort had significantly lower sulfur than all other plants. There were no significant differences in zinc content between all plants. Micronutrients iron, manganese, copper, boron, molybdenum, sodium, chlorine, aluminum had highly variable results, and no consistent discernable pattern could be detected (Tables 5&6).

DISCUSSION

'Red Defender' (RD) plants are resistant to *V. dahliae* race 1 (Vd1), but susceptible to race 2 (Vd2) and VdN (race 3) isolates. Across 2017, 2018, and 2019 field trials, no potential source of Vd2 or VdN resistant rootstocks were able to consistently prevent RD plants from becoming heavily infected with VdN (Tables 1-3). Even 'NC-7GEM', which had lower incidence and severity than any variety, was unable to prevent RD from becoming infected. More importantly, the only rootstock to consistently increase yields in RD was 'Maxifort'. 'Maxifort' is resistant to race 1, but appears to be susceptible to VdN, at least the foliar necrosis effects of VdN. 'Maxifort' has a high level of vigor in fields infested with *V. dahliae*. It is more likely that the vigor component of 'Maxifort' simply prevents stunting and wilting that less vigorous varieties are afflicted with. 'Maxifort' was the best performing rootstock in this VdN infested field.

'NC-7GEM' is clearly a variety with a high degree of BS and VdN resistance or at least tolerance under field conditions. While 'NC-7GEM' did not produce as many of the extra-large and jumbo sizes, its overall marketable yield was still quite competitive. In 2017 'NC-1GEM' was highly susceptible to VdN, however, this line had the highest number of jumbo fruit. 3BS is

a cross between ‘NC-1GEM’ and ‘NC-7GEM’, and showed a high level of resistance to VdN in 2017. These data support the idea that the resistance in ‘NC-7GEM’ may be dominant, and it may be used as a parent to create highly lines that are highly resistant to BS and VdN, and with good fruit characteristics, in the future.

Race 1 resistant rootstocks slow but do not completely prevent susceptible scions (Paplomatas et al. 2000). With race 2 resistant lines the pathogen can often be found above the cotyledon (Usami et al. 2017, 2). It is entirely possible that scion resistance is just as important as rootstock resistance. When the highly resistant ‘NC-7GEM’ was used as a rootstock for RD, it failed to prevent RD from becoming infected. When RD was used as a rootstock for ‘NC-7GEM’, ‘NC-7GEM’-RD had statistically similar severity AUDPC scores for foliar necrosis-chlorosis, indicating the scion was able to fend off the disease, despite having a susceptible rootstock. More research is needed to understand the rootstock scion dynamics for Vd1, Vd2, and VdN resistance. One mechanism by which *V. dahliae* could move through a resistant plant and into susceptible scion is by conidia which could migrate through the xylem acropetally of infected roots into the healthy scion (Sewell and Wilson 1964; Garas et al. 1986; Wright 1968). More research is also needed in understanding the intervascular movement of *V. dahliae* in tomato and other important plant species.

Overall the optimal conditions for growing tomatoes in non-fumigated VdN infested fields appears to be a combination of vigorous/VdN tolerant rootstocks, and BS and VdN resistant scions. While more work needs to be done to merge VdN resistance with desirable fruit characteristics, it is clear ‘NC-7GEM’ will be a valuable resource in tomato breeding. Highly vigorous rootstocks such as ‘Maxifort’ can be used to boost yields in fields, despite their inability to prevent VdN or Vd2 from infecting the scion. More research needs to be done to understand

the nature of VdN resistance. VdN infested fields are only increasing and given this funguses ability to survive for over a decade without a host present, we will certainly need solutions to this ever-increasing issue.

REFERENCES

1. Baergen, K., Hewitt, J., and Clair, D. S. 1993. Resistance of tomato genotypes to four isolates of Verticillium dahliae race 2. HortScience. 28:833–836.
2. Bryan, M. K. 1925. Phytopathological notes-Verticillium wilt of tomato. Phytopathology. 15:187–188.
3. Campbell, C., and Plank, C. O. 1992. Determination of total nitrogen in plant tissue by combustion. Plant Anal. Ref. Proced. South. US South. Coop Ser Bull. 368:20–22.
4. Diwan, N., Fluhr, R., Eshed, Y., Zamir, D., and Tanksley, S. D. 1999. Mapping of Ve in tomato: a gene conferring resistance to the broad-spectrum pathogen, Verticillium dahliae race 1. Theor. Appl. Genet. 98:315–319.
5. Djidonou, D., Zhao, X., Brecht, J. K., and Cordasco, K. M. 2017. Influence of interspecific hybrid rootstocks on tomato growth, nutrient accumulation, yield, and fruit composition under greenhouse conditions. HortTechnology. 27:868–877.
6. Dung, J. K., Peever, T. L., and Johnson, D. A. 2013. Verticillium dahliae populations from mint and potato are genetically divergent with predominant haplotypes. Phytopathology. 103:445–459.
7. Garas, N., Wilhem, S., and Sagen, J. 1986. Relationship of cultivar resistance to distribution of Verticillium dahliae in inoculated cotton plants and to growth of single conidia on excised stem segments. Phytopathology. 76:1005–1010.
8. Gold, J., Lee, B., and Robb, J. 1996. Colonization of tomatoes by Verticillium dahliae: determinative phase II. Can. J. Bot. 74:1279–1288.
9. Green, R. J. 1980. Soil factors affecting survival of microsclerotia of Verticillium dahliae. Phytopathology. 70:353–355.

10. Grieneisen, M. L., Aegerter, B. J., Stoddard, C. S., and Zhang, M. 2018. Yield and fruit quality of grafted tomatoes, and their potential for soil fumigant use reduction. A meta-analysis. Agron. Sustain. Dev. 38:29.
11. Hibar, K., Daami-Remadi, M., Jabnoun-Khiareddine, H., and El Mahjoub, M. 2006. Control of Fusarium crown and root rot of tomato, caused by Fusarium oxysporum f. sp. radicis-lycopersici by grafting onto resistant rootstocks. Plant Pathol. J. 5:161–165.
12. Iott, M. C. 2013. Utility of Grafting and Evaluation of Rootstocks for the Management of Verticillium Wilt in Tomato Production in Western North Carolina.
13. Ivors, K. 2010. Commercial production of staked tomatoes in the Southeast. N. C. Coop Ext Serv Bul AG-405.
14. Jeger, M., and Viljanen-Rollinson, S. 2001. The use of the area under the disease-progress curve (AUDPC) to assess quantitative disease resistance in crop cultivars. Theor. Appl. Genet. 102:32–40.
15. Johnson, S., Inglis, D., and Miles, C. 2014. Grafting effects on eggplant growth, yield, and verticillium wilt incidence. Int. J. Veg. Sci. 20:3–20.
16. Kabir, Z., Bhat, R., and Subbarao, K. 2004. Comparison of media for recovery of Verticillium dahliae from soil. Plant Dis. 88:49–55.
17. Kano, K., and Usami, T. 2019. Race 3 tomato Verticillium wilt pathogen ptoentially derives from race 1 isolate. Soil Microorg. 73:71–78.
18. Khah, E., Kakava, E., Mavromatis, A., Chachalis, D., and Goulas, C. 2006. Effect of grafting on growth and yield of tomato (Lycopersicon esculentum Mill.) in greenhouse and open-field. J. Appl. Hortic. 8:3–7.

19. Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. 2009. Diversity, Pathogenicity, and Management of Verticillium Species. Annu. Rev. Phytopathol. 47:39–62.

20. Lopez-Perez, J.-A., Le Strange, M., Kaloshian, I., and Ploeg, A. T. 2006. Differential response of Mi gene-resistant tomato rootstocks to root-knot nematodes (Meloidogyne incognita). Crop Prot. 25:382–388.

21. Louws, F. J., Rivard, C. L., and Kubota, C. 2010. Grafting fruiting vegetables to manage soilborne pathogens, foliar pathogens, arthropods and weeds. Sci. Hortic. 127:127–146.

22. Masterson, S. A., Kennelly, M. M., Janke, R. R., and Rivard, C. L. 2016. Scion shoot removal and rootstock cultivar affect vigor and early yield of grafted tomatoes grown in high tunnels in the central United States. HortTechnology. 26:399–408.

23. Ozbay, N., and Newman, S. E. 2004. Fusarium crown and root rot of tomato and control methods. Plant Pathol J. 3:9–18.

24. Paplomatas, E., Elena, K., Tsagkarakou, A., and Perdikaris, A. 2000. Control of Verticillium wilt of tomato and cucurbits through grafting of commercial varieties on resistant rootstocks. In , p. 445–449.

25. Pegg, G. F., and Brady, B. L. 2002. *Verticillium Wilts*. CABI.

26. Rivard, C. L., and Louws, F. J. 2008. Grafting to manage soilborne diseases in heirloom tomato production. HortScience. 43:2104–2111.

27. Rivard, C., O'Connell, S., Peet, M., and Louws, F. 2010. Grafting tomato with interspecific rootstock to manage diseases caused by Sclerotium rolfsii and southern root-knot nematode. Plant Dis. 94:1015–1021.

28. Rivard, C., O'connell, S., Peet, M., Welker, R., and Louws, F. 2012. Grafting tomato to manage bacterial wilt caused by Ralstonia solanacearum in the southeastern United States. Plant Dis. 96:973–978.

29. Sewell, G., and Wilson, J. 1964. Occurrence and dispersal of Verticillium conidia in xylem sap of the hop (Humulus lupulus L.). Nature. 204:901–901.

30. Shanner, G., and Finney, R. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology. 67:1051–1056.

31. Suchoff, D., Gunter, C., Schultheis, J., and Louws, F. 2014. On-farm grafted tomato trial to manage bacterial wilt. In , p. 119–127.

32. Usami, T., Momma, N., Kikuchi, S., Watanabe, H., Hayashi, A., Mizukawa, M., et al. 2017. Race 2 of Verticillium dahliae infecting tomato in Japan can be split into two races with differential pathogenicity on resistant rootstocks. Plant Pathol. 66:230–238.

33. Verdejo-Lucas, S., Blanco, M., Cortada, L., and Sorribas, F. J. 2013. Resistance of tomato rootstocks to Meloidogyne arenaria and Meloidogyne javanica under intermittent elevated soil temperatures above 28 C. Crop Prot. 46:57–62.

34. Wright, D. 1968. Verticillium wilt of tobacco: VIII. Movement of conidia of Verticillium dahliae Kleb. in tobacco plants. N. Z. J. Agric. Res. 11:803–811.

35. Xiao, C. L., Subbarao, K. V., Schulbach, K. F., and Koike, S. T. 1998. Effects of Crop Rotation and Irrigation on *Verticillium dahliae* Microsclerotia in Soil and Wilt in Cauliflower. Phytopathology. 88:1046–1055.

Table 4.1: AUDPC scores developed from *V. dahliae* chlorosis/necrosis symptom ratings of grafted and non-grafted tomato plants. Tomatoes were grown in *V. dahliae* infested soil in Mills River, NC in 2017.

Scion/Cultivar	Rootstock	n	Severity AUDPC[y]	Incidence%[z]
Red Defender	Beefsteak	45	47.6 a	99.81 a
Red Defender Guard		145	41 ab	97.44 abc
Red Defender	FG06-301	47	38.3 bc	91.51 abc
Red Defender	EarlyPak7	35	37.6 bcd	96.95 abc
FG06-302		48	37.2 bcd	100 a
Red Defender Fumigated	Red Defender	48	35.7 bcd	100 a
Red Defender Fumigated		47	34.9 bcd	100 a
Red Defender		48	31.9 cde	100 a
Red Defender	OH-316	40	29.9 cdef	97.5 abc
EarlyPak7		47	28.6 def	89.38 abcde
Red Defender	Red Defender	48	25.9 ef	89.58 abcd
Red Defender	NC7GEM	48	24.3 efg	89.58 abcd
Red Defender	NC6GEM	47	24 efgh	95.81 abc
IRAT-L3		48	21.7 fghi	87.5 cde
Red Defender	TD-17B	33	21.4 fghij	90.88 abcd
NC1GEM		46	21.3 fghij	95.78 abc
FG06-304		47	21.1 fghij	91.47 abc
Red Defender	Maxifort	48	21 fghij	93.75 abc
H7998		48	20.7 fghij	89.58 abcd
SG06-219		48	20.7 fghij	97.92 ab
SG06-210		48	20.3 fghij	93.75 abc
Red Defender	H7998	19	19.5 fghijk	94.9 abc
NC6GEM		48	17.2 ghijk	75 f
FG06-301		48	15.8 hijk	87.5 bcde
Red Defender	IRAT-L3	45	15 ijk	73.53 f
Maxifort		25	11.4 jkl	75.88 ef
BeefSteak		44	11 kl	79.55 def
3BS		48	10.7 kl	87.5 bcde
TD-17B		32	10.4 kl	72 f
SG06-220		46	10.2 kl	89.01 abcde
NC7GEM		42	1.8 l	33.35 g

[y]Severity AUPDC mean determined from ratings of foliar chlorosis and necrosis. Letters indicate significant differences based on Tukey's HSD (P>0.05)

[z]Percent plant incidence of chlorosis and necrosis symptoms. Letters indicate significant differences based on Tukey's HSD (P>0.05)

Table 4.2: AUDPC scores developed from *V. dahliae* chlorosis/necrosis symptom ratings of grafted and non-grafted tomato plants. Tomatoes were grown in *V. dahliae* infested soil in Mills River, NC in 2018.

Scion/Cultivar	Rootstock	n	Severity AUDPC[y]	Incidence%[z]
Red Defender	IRAT-L3	11	136.32 a	99.97 ab
Red Defender		40	96.1 bc	100 ab
Red Defender Fumigated		40	94.9 bc	100 ab
Red Defender	FG06-301	11	78.7 cdef	99.97 ab
Red Defender Guard		97	75.3 cdef	95.94 ab
Red Defender	Maxifort	33	74 cdef	99.97 ab
Red Defender	SG06-220	29	72.1 cdef	100 ab
Maxifort		40	71.2 cdef	100 ab
Red Defender	Red Defender	22	70.9 cdef	100 ab
Red Defender	Happy Root	33	64.4 def	100 ab
BHN line		40	61.2 defg	100 ab
Bowman		13	60.5 defgh	92.33 ab
Red Defender	EarlyPak7	26	58.8 defghi	100 ab
Mtn. Majesty	Bowman	17	56.2 defghi	100 ab
Mtn. Majesty		20	54.1 defghi	100 ab
Red Defender	OH-316	26	53.9 defghi	86.28 ab
Red Defender	NC-7GEM	29	51.5 efghi	93.19 ab
Aibou		24	48.6 fghi	95.83 ab
OH-316		40	45 fghi	80 b
FG06-301		40	42.5 ghi	99.97 ab
IRAT-L3		39	38.2 hi	87.21 ab
EarlyPak7		23	36.6 hi	91.29 ab
HappyRoot		38	27 i	68.41 c
SG06-220		40	25.4 i	76.91 bc
NC-7GEM		40	10.8 i	50 d
NC-7GEM	Red Defender	13	9.6 i	69.79 bc

[y]Severity AUPDC mean determined from ratings of chlorosis and necrosis. Letters indicate significant differences based on Tukey's HSD (P>0.05)

[z]Percent incidence of chlorosis and necrosis symptoms. Letters indicate significant differences based on Tukey's HSD (P>0.05)

Table 4.3: AUDPC and AUGPC scores from grafted and non-grafted tomatoes grown in a *V. dahliae* (*Vd*) and bacterial spot (BS) infested field in Mills River NC in 2019. *Vd* and BS AUDPC scores based on multiple foliar necrosis and chlorosis symptom ratings from the start of symptoms up to the second harvest.

	Vd AUDPC[z]		*Vd* Inc[z]		BS AUDPC[z]		AUGPC[z]	
EV-1	102.54	c	100	a	132.9	d	136.9	e
EV-2	82.24	c	100	a	191.6	d	166.2	c
EV-3	62.41	c	100	a	124.1	d	146.9	d
EV-4	95.16	c	94.8	a	120.2	d	131.1	e
EV-5	87.56	c	100	a	102.1	d	147.8	d
RD	192.57	b	100	a	320.5	ab	95.3	f
NC7GEM	31.84	c	76.1	b	113.2	d	171.9	bc
Maxifort	244.46	a	100	a	283	bc	212.5	a
RD-NC7GEM	172.86	b	100	a	365.5	a	95.8	f
RD-Maxifort	171.57	b	100	a	341.7	a	130.1	e
NC7GEM-RD	99.68	c	100	a	172.01	d	174.5	bc
NC7GEM-Maxifort	54.16	c	78.8	b	139.95	d	179.6	b

[z]AUDPC and AUGPC scores with the same letter are not significantly different from each other based on Tukey's HSD (P=0.05).

Table 4.4: Specific fruit sizes, marketable, and total yields from 2019 field trial conducted in Mills River NC in a *V. dahliae* infested field. Measurements are in kilograms per hectare squared.

Scion-RST	Cull[z]	Small[z]	Medium[z]	Large[z]	Extra Large[z]	Jumbo[z]	Marketable[z]	Total[z]
NC-7GEM	11099 bc	9183 a	12087 ab	11974 a	10897 c	209 b	44317 ab	55415 ab
NC-7GEM-Maxifort	15008 ab	9067 a	15288 a	19480 a	16732 bc	791 b	61496 a	76504 a
NC-7GEM-RD	18095 a	7119 ab	12002 ab	8945 a	7354 c	7 b	35491 b	53587 ab
RD-Maxifort	7102 cd	2300 b	7074 bc	11152 a	29140 a	4960 a	54682 ab	61785 ab
RD-NC7GEM	5951 d	1692 b	5146 c	18949 a	15094 bc	297 b	41217 ab	47169 b
RD	6763 cd	1917 b	5154 c	10944 a	21514 ab	504 b	40035 ab	46798 b

[z]Yield measurements kg*ha^{-1} with the same letter are not significantly different from each other based on students t-test (P=0.05).

Table 4.5: Nutrient analysis of foliar tissue from five non-meristem newly formed leaves of grafted and non-grafted tomatoes.

	N[z]		P[z]		K[z]		Ca[z]		Mg[z]		S[z]		Fe[z]		Mn[z]	
Maxifort-NG	3.02	d	0.32	abc	2.68	bc	2.13	b	0.54	c	0.62	b	414	b	66	d
NC7GEM-Maxifort	4.43	ab	0.35	a	3.51	a	2.55	b	0.47	c	1.04	a	395	b	67.6	d
NC7GEM-NG	3.98	bc	0.29	abc	3.29	ab	2.71	b	0.7	b	1.07	a	579	ab	86.8	cd
NC7GEM-RD	4.77	a	0.33	ab	3.32	ab	2.42	b	0.75	b	1.01	a	445	ab	87.3	cd
RD-Maxifort	3.99	bc	0.33	ab	2.63	c	4.47	a	0.73	b	1.25	a	925	a	126	a
RD-NC7GEM	3.48	cd	0.26	bc	2.42	c	4.28	a	0.8	ab	1.21	a	555	ab	120	ab
RD-NG	3.54	cd	0.27	bc	2.38	c	4.77	a	0.9	a	1.24	a	576	ab	103	bc

[z]Nutrient scores with the same letter are not significantly different from each other based on students t-test (P=0.05).

Table 4.6: Nutrient analysis of foliar tissue from five non-meristem newly formed leaves of grafted and non-grafted tomatoes.

	Zn[z]		Cu[z]		B[z]		Mo[z]		Na[z]		Cl[z]		Al[z]	
Maxifort-NG	15.8	a	16.7	d	48.5	b	0.31	ab	0	c	1.1	b	505	b
NC7GEM-Maxifort	18.8	a	25.3	ab	51.5	b	0.5	a	0.01	bc	1.48	ab	493	b
NC7GEM-NG	15.9	a	20.1	dc	57.1	ab	0.33	ab	0.02	a	1.45	ab	771	ab
NC7GEM-RedDefender	17.3	a	23.3	abc	56.7	ab	0.47	ab	0.02	ab	1.72	a	571	b
RedDefender-Maxifort	20.3	a	26.2	a	58	ab	0.34	ab	0	c	1.77	a	1279	a
RedDefender-NC7GEM	20.8	a	21.5	bc	65.9	a	0.45	ab	0.01	abc	1.29	ab	661	ab
RedDefender-NG	19.5	a	22.9	abc	64.5	a	0.31	b	0.01	abc	1.76	a	719	ab

[z]Nutrient scores with the same letter are not significantly different from each other based on students t-test (*P*=0.05).

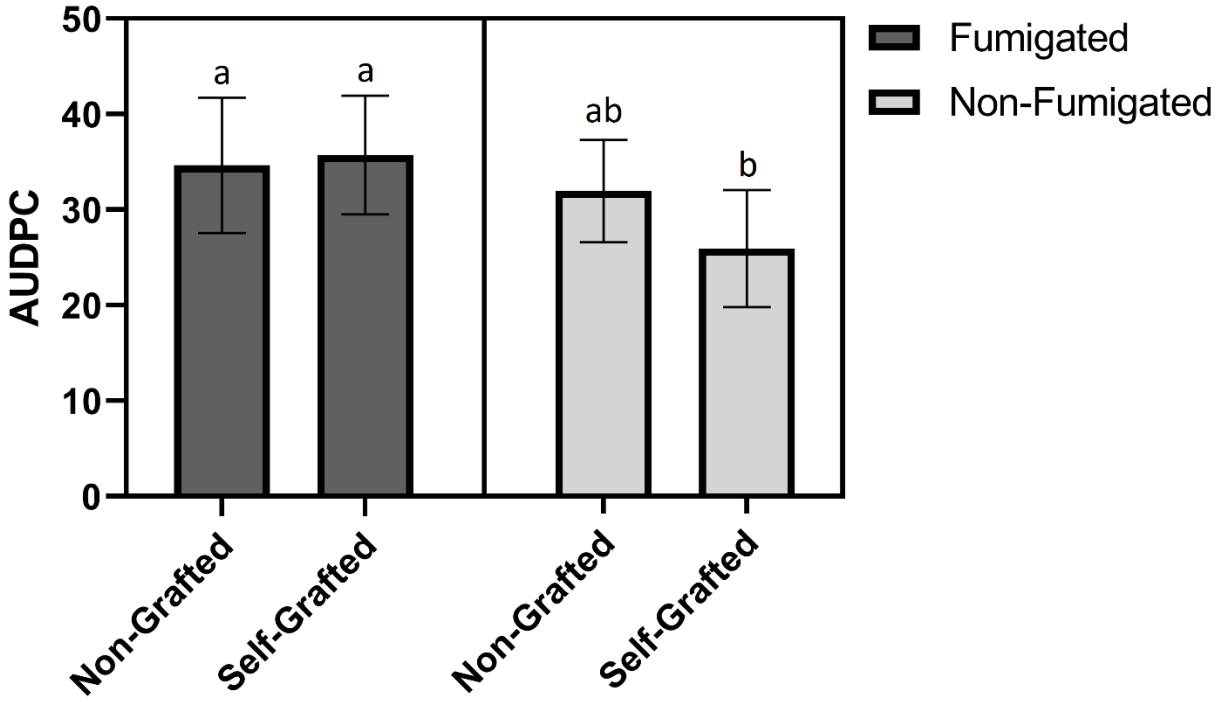

Figure 4.1: AUDPC scores from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2017. Treatments with the same letter are not significantly different from each other (P=0.05).

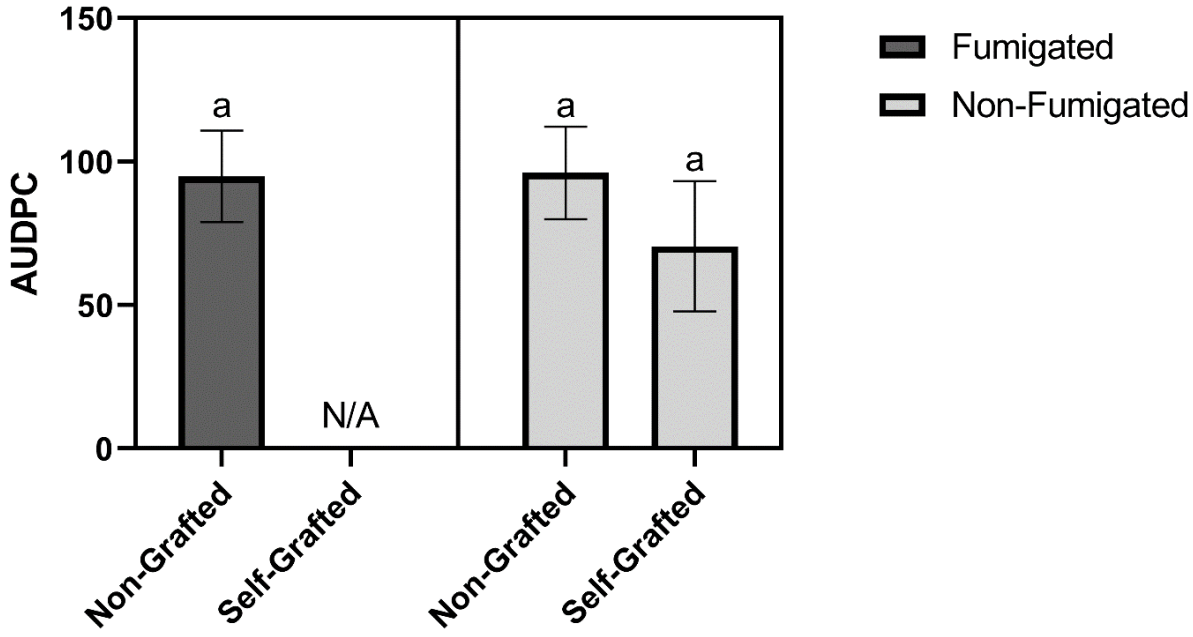

Figure 4.2: AUDPC scores from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2018. Treatments with the same letter are not significantly different from each other (P=0.05).

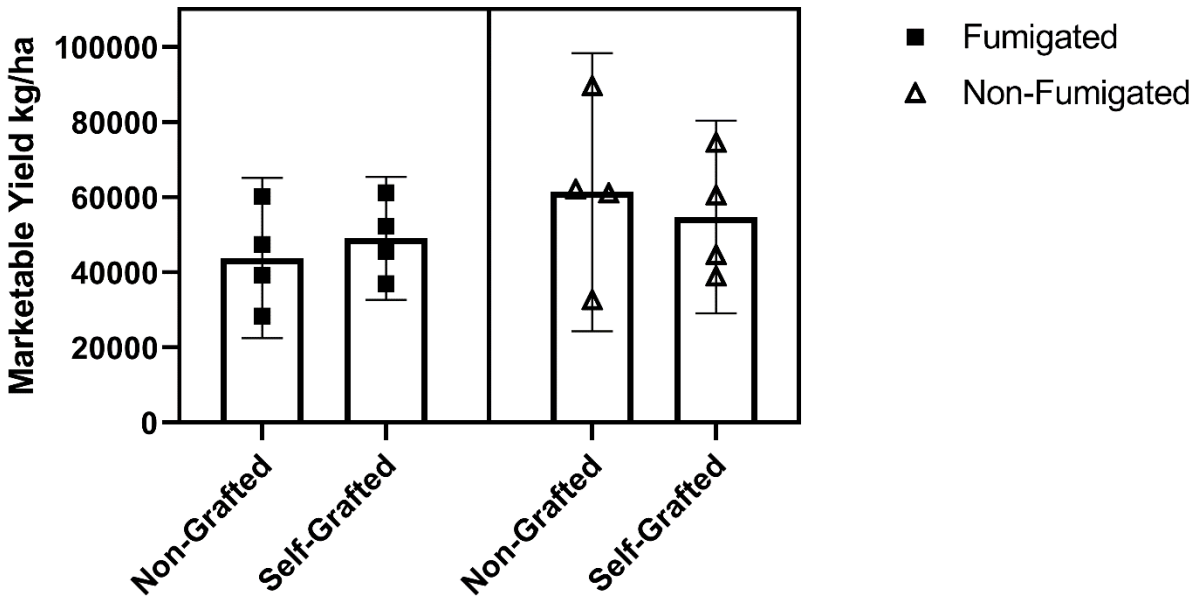

Figure 4.3: Marketable (no culls) yields from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2017. No significant differences were detected between these treatments.

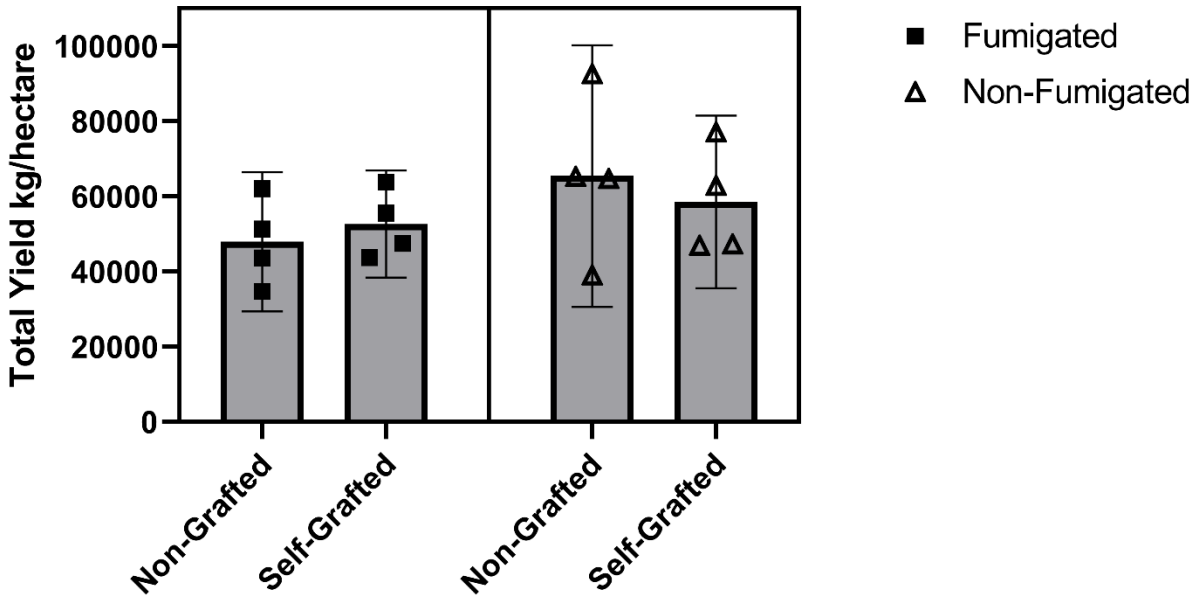

Figure 4.4: Total yields from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2017. No significant differences were detected between these treatments.

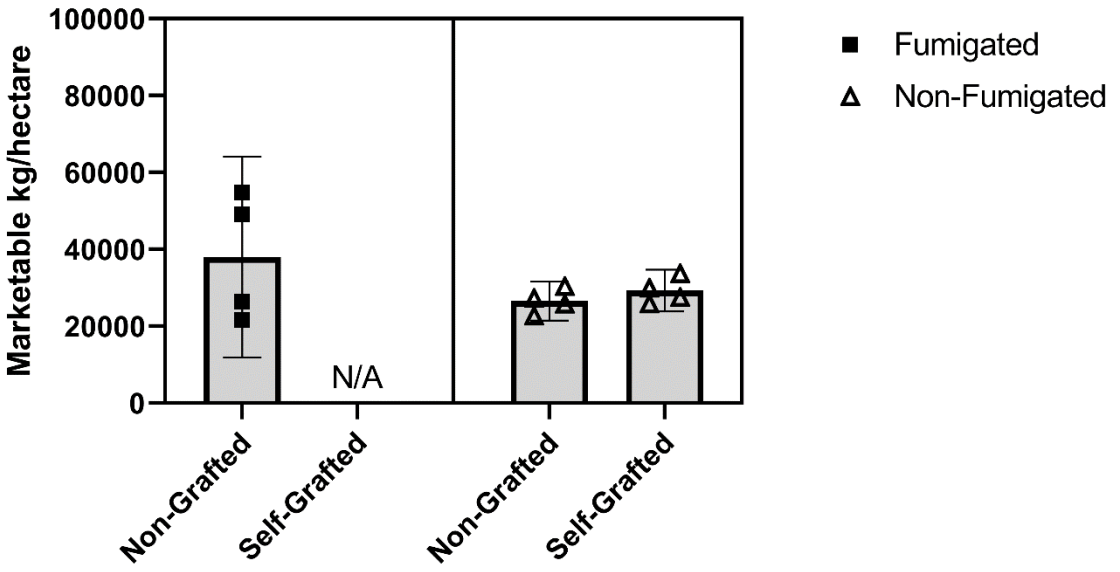

Figure 4.5: Marketable (no culls) yields from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2018. No significant differences were detected between these treatments.

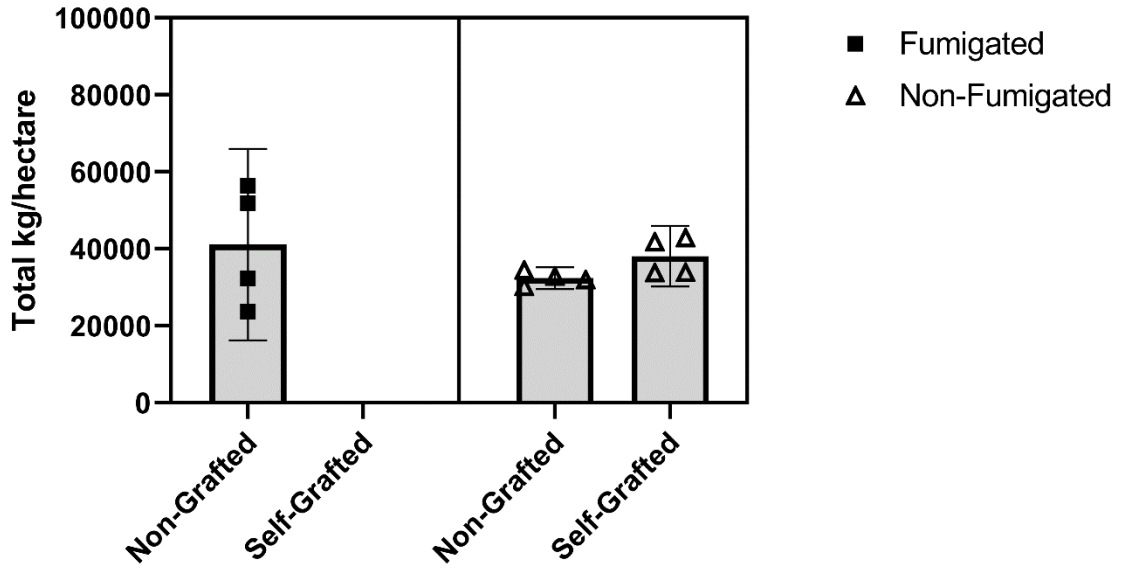

Figure 4.6: Total yields from fumigated, non-fumigated, non-grafted, and self-grafted 'Red Defender' combinations planted in *V. dahliae* infested field in Mills River NC in 2018. No significant differences were detected between these treatments.

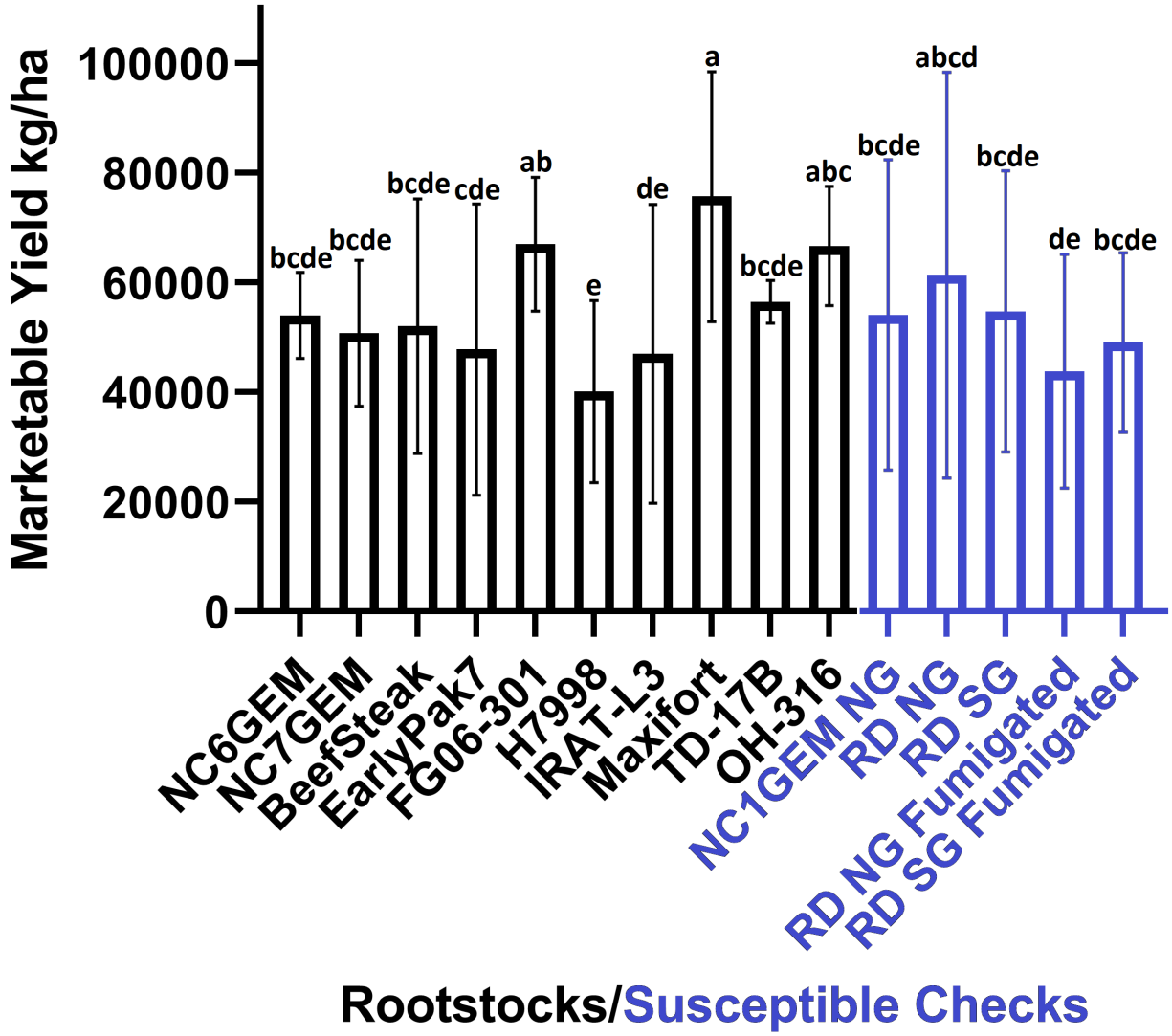

Figure 4.7: 2017 marketable yields (no culls) from grafted 'Red Defender' plants compared to non-grafted (NG), self-grafted (SG), fumigated checks, and non-grafted NC1GEM planted in a *V. dahliae* infested field in Mills River NC.

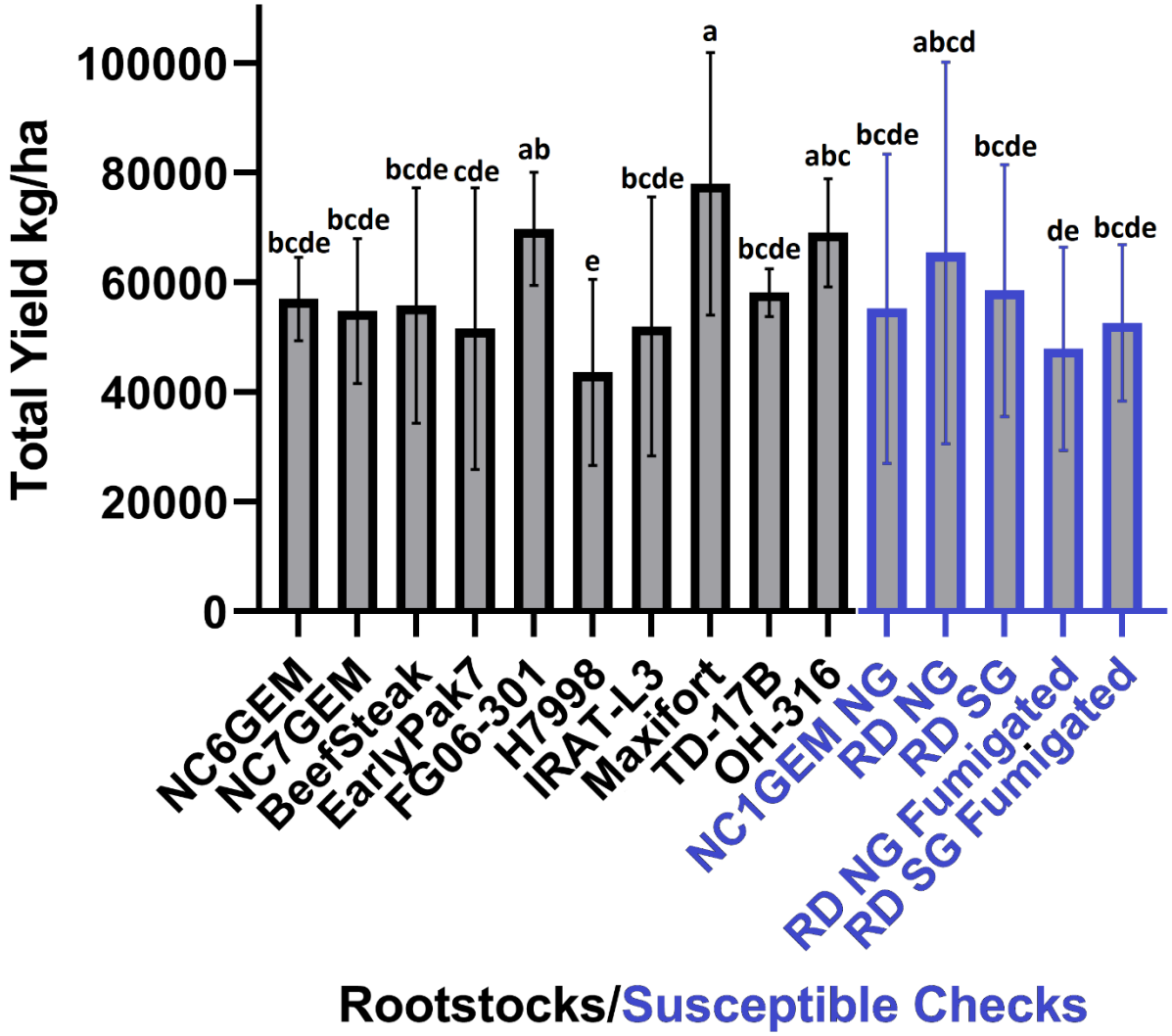

Figure 4.8: 2017 total yields from grafted 'Red Defender' plants compared to non-grafted (NG), self-grafted (SG), fumigated checks, and non-grafted NC1GEM planted in a *V. dahliae* infested field in Mills River NC.

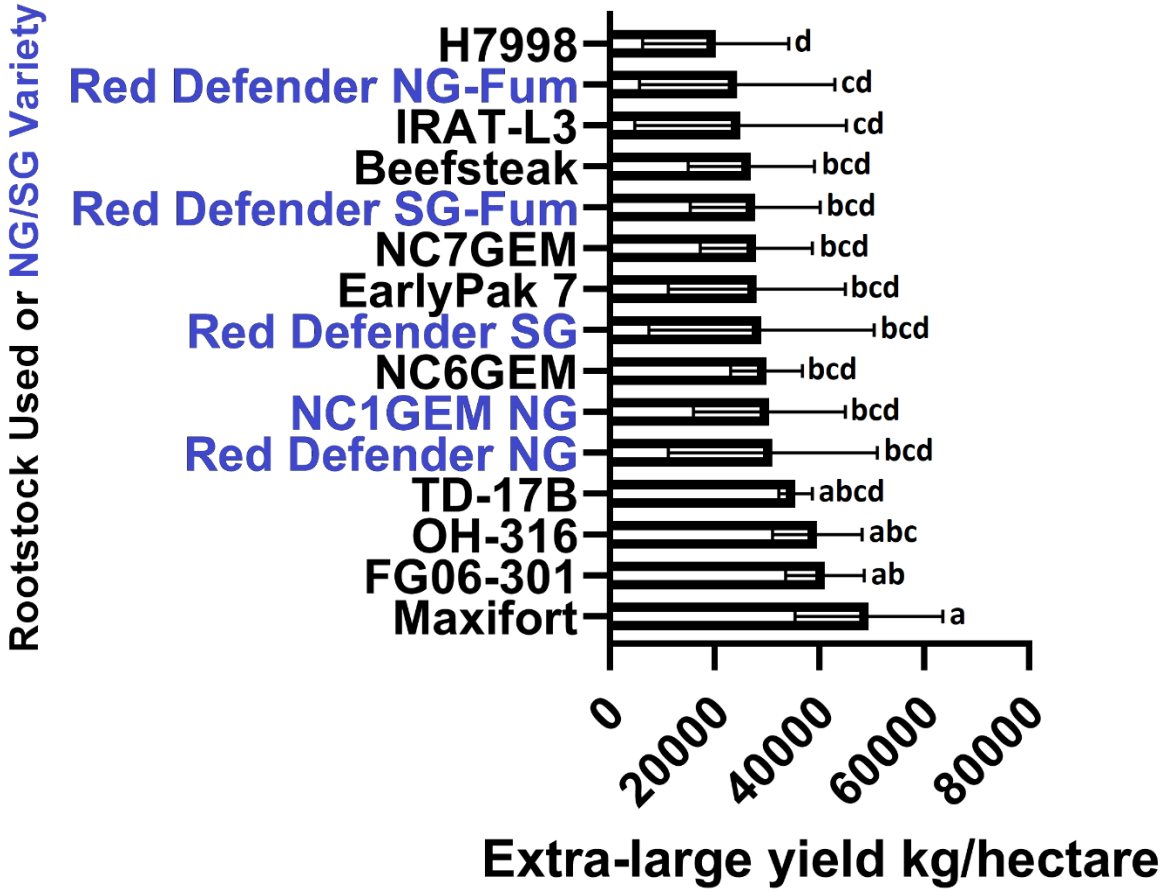

Figure 4.9: Extra-large fruit yields harvested from 2017 RD grafted plants. Error bars represent the 90% confidence intervals around the mean. Treatments with the same letter are not significantly different from each other according to Tukey's HSD.

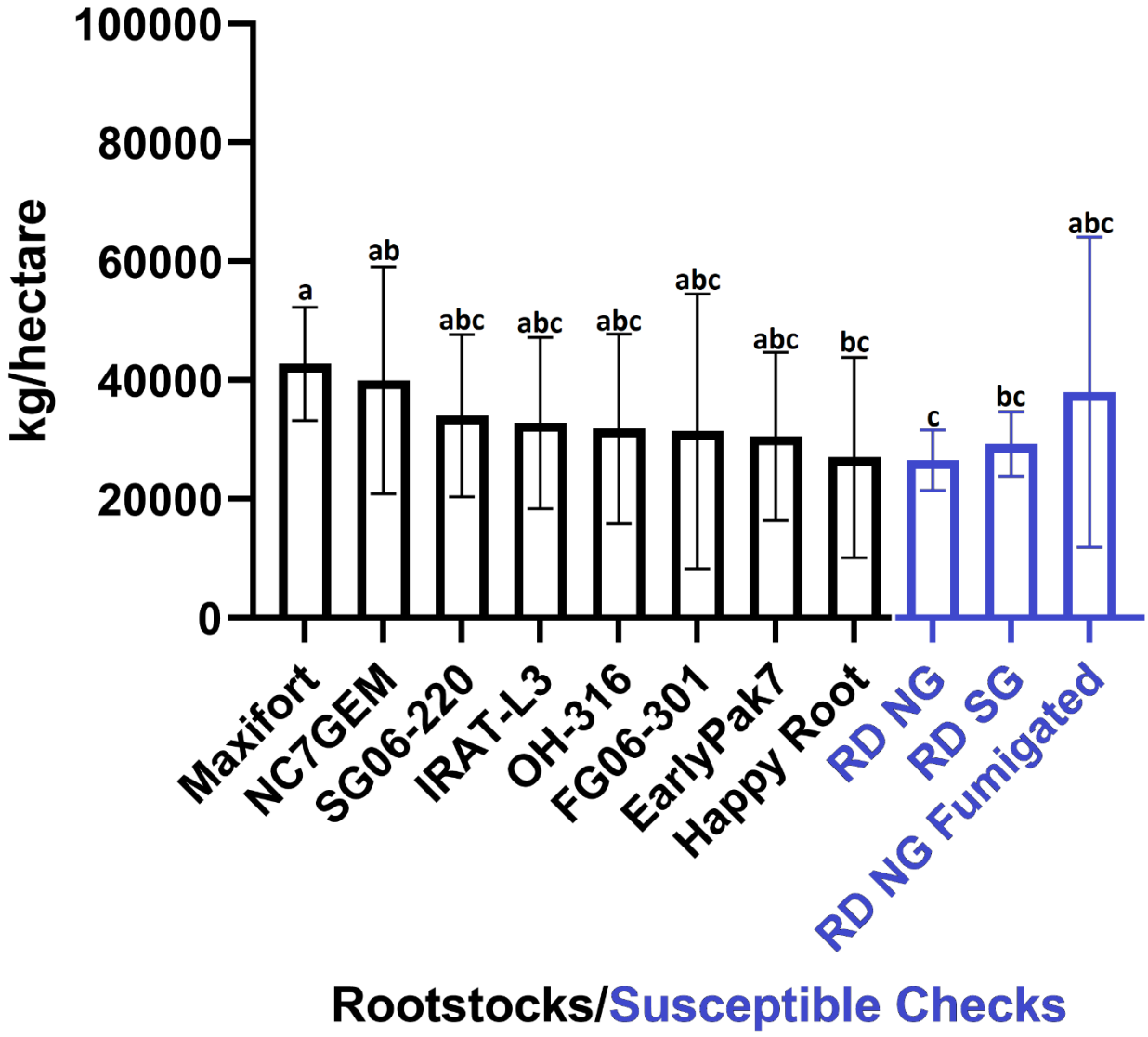

Figure 4.10: 2018 marketable yields (no culls) from grafted 'Red Defender' plants compared to non-grafted (NG), self-grafted (SG), and fumigated checks planted in a *V. dahliae* infested field in Mills River NC.

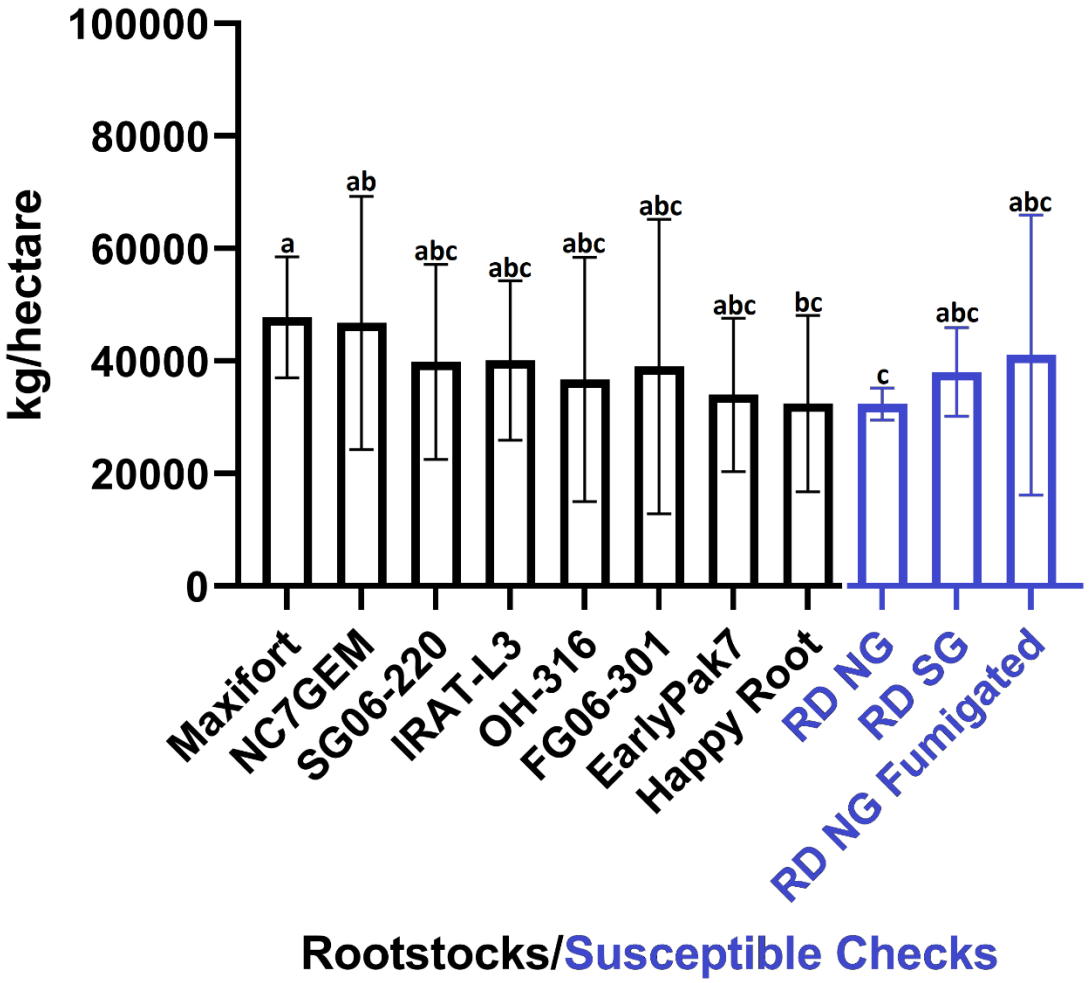

Figure 4.11: 2018 total yields from grafted 'Red Defender' plants compared to non-grafted (NG), self-grafted (SG), and fumigated checks planted in a *V. dahliae* infested field in Mills River NC.

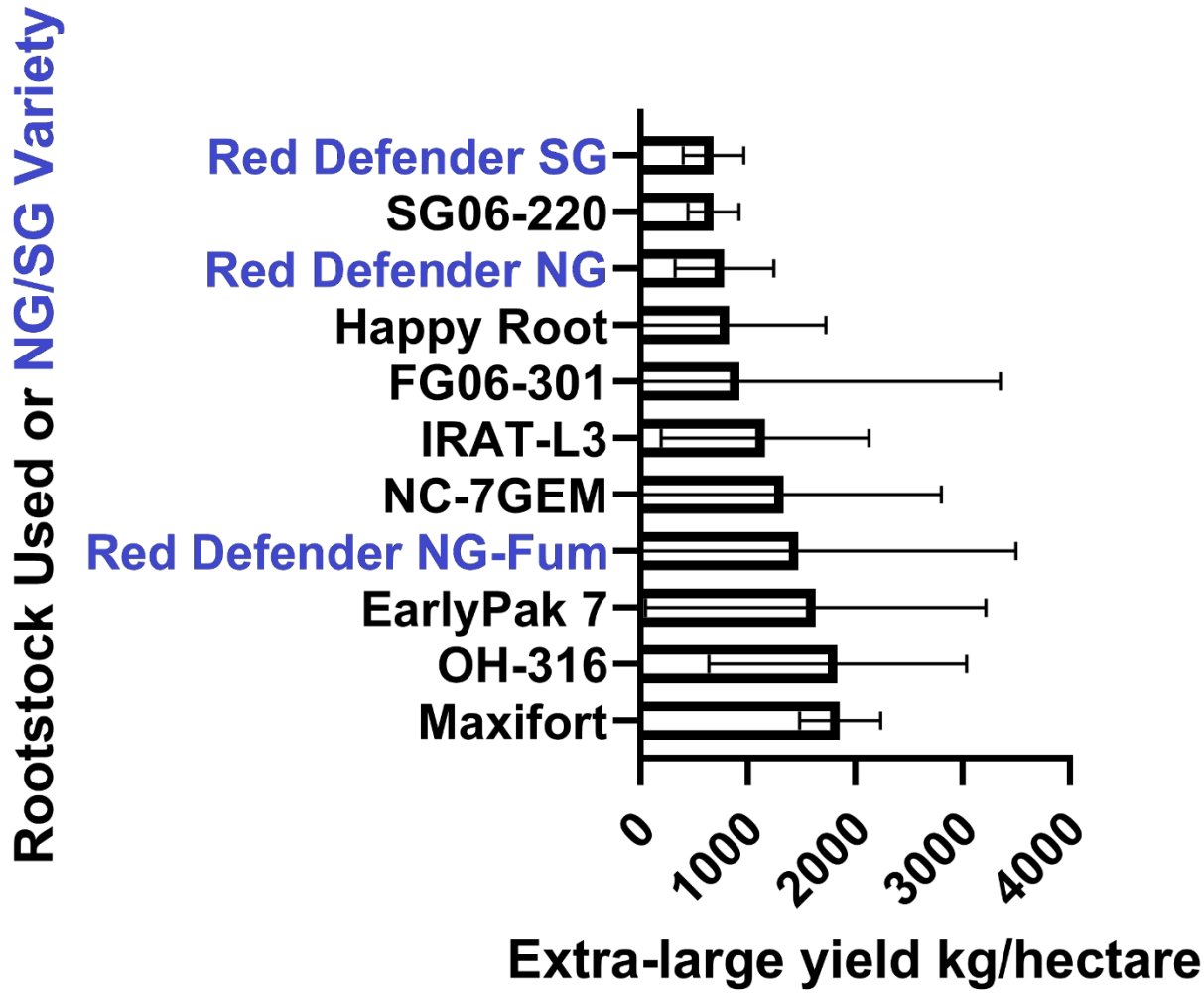

Figure 4.12: Extra-large fruit yields harvested from 2017 RD grafted plants. Error bars represent the 90% confidence intervals around the mean. There were no significant differences between treatments according to a Tukey's HSD analysis.

Table S4.1: Cultivars used in 2017, 2018, and 2019 field trials.

Cultivar	Source	Resistance
Red Defender	HM.CLAUSE Inc., Davis, CA, USA	Vd1
IRAT-L3	CGN Wageningen, Netherlands	Unknown
FG06-301	Dr. David Francis, OSU, Wooster, Ohio, USA	Unknown
SG06-220	Dr. David Francis, OSU, Wooster, Ohio, USA	Unknown
OH-316	Dr. David Francis, OSU, Wooster, Ohio, USA	Unknown
SG06-220	Dr. David Francis, OSU, Wooster, Ohio, USA	Unknown
Maxifort	De Reuter, Cambridge, United Kingdom	Vd1
Happy Root	Asahi Industries, Tokyo, Japan	Vd1
Aibou	Asahi Industries, Tokyo, Japan	Vd1, Vd2
Bowman	Sakata Seed America, Morgan Hill, CA, USA	Vd1, Vd2
Mtn. Majesty	North Carolina State University, Raleigh, NC, USA	Vd1
Beefsteak	Unknown	Vd1
EarlyPak7	Asgrow Seed Co. Kalamazoo, Michigan, USA	Unknown
NC-1GEM	North Carolina State University, Raleigh, NC, USA	Vd1
NC-6GEM	North Carolina State University, Raleigh, NC, USA	Vd1
NC-7GEM	North Carolina State University, Raleigh, NC, USA	Vd1
EV-1	North Carolina State University, Raleigh, NC, USA	Unknown
EV-2	North Carolina State University, Raleigh, NC, USA	Unknown
EV-3	North Carolina State University, Raleigh, NC, USA	Unknown
EV-4	North Carolina State University, Raleigh, NC, USA	Unknown
EV-5	North Carolina State University, Raleigh, NC, USA	Unknown

Figure S4.1: Red Defender plants on September 19th 2019 (91 days post-planting).

Figure S4.2: ‘NC-7GEM’ plants on September 19th 2019 (91 days post-planting).

Figure S4.3: RD-'Maxifort' plants on September 19th 2019 (91 days post-planting).

Figure S4.4: ‘NC-7GEM’-‘Maxifort’ plants on September 19th 2019 (91 days post-planting).

Figure S4.5: RD-'NC-7GEM' plants on September 19th 2019 (91 days post-planting).

Figure S4.6: ‘NC-7GEM’-RD plants on September 19th 2019 (91 days post-planting).

CHAPTER 5

WHOLE GENOME RESEQUENCING AND MACHINE LEARNING REVEAL CANDIDATE GENE FOR VERTICILLIUM DAHLIAE RACE 2

ABSTRACT

Host resistance is one of few strategies available to combat the soil borne pathogenic fungus *Verticillium dahliae.* Resistance in tomato against *V. dahliae* race 1 is well described. Until recently there were no other race-resistance interactions described in tomato. *V. dahliae* (race 2, which is unable to infect the tomato cultivar 'Aibou', was discovered in Japan in 2017. However, isolates that can infect 'Aibou' were described as race 3. The genomes of 18 *V. dahliae* isolates of races 1 (n = 2), 2 (n = 4) and 3 *(*n = 12) from Japan, California, and North Carolina were sequenced and compared with the reference genomes of JR2 (from tomato) and VdLs.17 (from lettuce) to identify putative effectors specific to race 2. Phylogenomic analysis of the largest assembled contig (2.51 Mb) showed that all 18 isolates and the reference genomes belonged to one of four distinct groups. Race 2 was only found in group 4, while race 3 was present in all four groups. Coding sequences were extracted, and filtered through two machine learning algorithms, SignalP and EffectorP, to determine candidate secreted effectors. A total of 201 and 222 secreted effector candidate genes were discovered by mapping to the VdLs17 and JR2 reference genomes, respectively. From the genomes mapped to the VdLs17 reference genome, one candidate effector was observed to contain a single amino acid change that distinguished race 2 isolates from race 3. Two other hypothetical effectors unique to race 2 isolates were also discovered in the isolates mapped to the JR2 reference genome. Analysis of the unassembled reads revealed 9 hypothetical effectors unique to race 2. Together, 12 candidate genes were identified that may be the race 2 effector and are worthy of further research.

INTRODUCTION

Verticillium dahliae is a destructive soil-borne pathogen that infects hundreds of plant species (Pegg and Brady 2002; Bhat and Subbarao 1999; Klosterman et al. 2009). The pathogen can survive in soil for over a decade without a viable host present (Green 1980; Xiao et al. 1998). Selected soil fumigants are widely used in vegetable fields as one of the few chemical-based strategies to suppress this pathogen (Gullino et al. 2002). Host resistance is a key management strategy for tomato growers (Klosterman et al. 2009) where race 1 predominates. Race 1 *V. dahliae* is characterized by the presence of a functional copy of the *Ave1* effector which is recognized by the host resistance gene *Ve1* (Diwan et al. 1999; de Jonge et al. 2012). Non-race 1 isolates have been discovered in large quantities in North Carolina since the 80s (Bender and Shoemaker 1984). Until recently the population of *V. dahliae* isolates infecting tomato were separated into race 1 and non-race 1 strains. In 2017, Usami et al. revealed that new races could be distinguished amongst non-race 1 strains. Isolates that were pathogenic on tomato lines harboring the *Ve1* gene only, but found non-pathogenic on the tomato rootstock 'Aibou' (aka 'Aiboh' or V2) and 'Ganbarune-Karis', which contain an additional gene, were designated as race 2. Isolates pathogenic on 'Aibou' and 'Ganbarune-Karis' were described as race 3. It was also shown that selfed 'Aibou' resulted in progeny with a resistant to susceptible ratio of 3:1, indicating the race 2 resistance is conferred by a single dominant locus present in 'Aibou' (Usami et al. 2017). In a more recent study, it was shown that race 3 could be derived from the race 1 strain Vdp4 by knocking out the race 1 effector *Ave1* (Kano and Usami 2019). These findings suggest that races 2 and 3 isolates may have emerged from different phylogenetic origins.

The first layer of biochemical plant defense is achieved through the detection of pathogen associated molecular patterns (PAMPs) and microbe-associated molecular patterns (MAMPs) such as flagella and chitin (Jones and Dangl 2006; Newman et al. 2013: Akamatsu et al. 2013; Hayafune et al. 2014). Upon detection of PAMPs and MAMPs plants activate their immune systems through PAMP triggered immunity (PTI). salicylic acid, reactive oxygen species, MAP kinase pathways, phytoalexin production, and other biochemical processes become activated (Howe et al. 1992; Raskin 1992; Ebel 1986; Peumans and Van Damme 1995; Kombrink and Somssich 1997; Sharma et al. 2011). In *V. dahliae,* chitin-binding lysin motif (LysM) effectors sequester chitin and thus increase host susceptibility by preventing the activation of PTI (Kombrink et al. 2017a). The LysM effector *VdPDA1* (polysaccharide deacetylase) is one of several effectors responsible for scavenging chitin pre-resistance gene recognition, which hides chitin from plants PAMP recognition genes in *Fusarium* spp. and *Verticillium* spp. (Gao et al. 2019).

Effector triggered immunity (ETI) is typically a much more aggressive defense response than PTI (Tsuda and Katagiri 2010). Host resistance genes typically encode proteins that contain nucleotide binding site leucine rich repeats (NBS-LRR), which can directly or indirectly recognize the presence of pathogen effectors (Belkhadir et al. 2004). In foliar tissue, ETI often results in the hypersensitive response (HR), which leads to localized cell death and eventually systemic acquired resistance (SAR) (Kombrink and Schmelzer 2001). While ETI clearly results in HR in plant foliage, very little is known about the ETI in roots. The *Ve1* locus in tomatoes results in nearly complete immunity to strains containing the *Ave1* effector (de Jonge et al. 2012). Co-expression of the *Ave1* gene and *Ve1* gene in foliar tissue of *Nicotiana glutinosa* results in the hypersensitive response (HR)(Song et al. 2017). However, there is only

fragmentary evidence that HR occurs in tomato roots (Sutherland 1991). A comparison of *Ve1*+ and *Ve1*- plants infected with *V. dahliae* strains Le1087 (race 1) and Le1811 (non-race 1) indicates that many defense reactions such as phenylalanine ammonia-lyase (PAL) and other enzymes are differentially expressed in incompatible interactions in roots (Hu et al. 2019). It is unclear whether race 1 resistant plants are eliciting a HR response or a more complex resistance response more akin to PTI or the post-HR SAR (Hu et al. 2019).

Currently, there are two publicly available annotated *V. dahliae* genomes, JR2 (GCA_000400815.2) and VdLs17 (GCF_000150675.1). JR2 was isolated from a tomato plant in Canada, and sequenced using PacBio at 250x coverage, and the scaffolds have been assembled into 8 distinct chromosomes. The JR2 genome was annotated using a combination of *in silico* gene prediction and 35 fungal proteomes, which resulted in the prediction of 11,426 genes (de Jonge et al. 2012). VdLs17 was isolated from lettuce in California and was sequenced using Illumina next generation sequencing at 7x coverage and assembled to 55 scaffolds. The VdLs17 genome was annotated using a combination of manual curation, BLAST prediction, and *ab initio* gene prediction uncovering 10,535 genes (Klosterman et al. 2011). Genome annotation typically involves ab initio gene discovery through the in silico recognition of open reading frames (ORFs), and the input of messenger RNA data from RNA-sequencing projects (Campbell, Holt, et al. 2014; Campbell, Law, et al. 2014). Both methods have their pitfalls as ab initio annotation has difficulties accounting for introns within coding sequences, and mRNA data relies on specific genes being expressed in large quantities at the time of sampling (Yandell and Ence 2012). In contrast, whole genome re-sequencing relies on mapping reads from next generation sequencing (NGS) of different isolates to reference genomes and subsequent extraction of gene information, observing indels and nucleotide polymorphisms. However, mapping to a reference

genome relies on the reference genome and the re-sequenced isolate genome being closely related. Machine learning algorithms such as EffectorP and SignalP can be used to filter annotated genes. SignalP detects signal peptide motifs on the N-terminus of protein sequences that is required for secretion through the canonical secretion pathway to outside the cell. As virtually all effectors must be secreted from fungal cells to interact with their hosts, this is a powerful tool for recognizing candidate effectors (Armenteros et al. 2019; Melhem et al. 2013). However, many other non-effector proteins are secreted. To refine predictions of candidate effectors other machine learning algorithms such as EffectorP have been developed. EffectorP was trained on 94 experimentally confirmed effector genes from a diverse set of fungal pathogens (Sperschneider et al. 2016). In 2011, 127 effectors were discovered (using SignalP 3.0 and EffectorP 1.0) on the *V. dahliae* VdLs17 genome and 112 on the *V. alfalfae* VaMs.102 genome (Klosterman et al. 2011). *V. dahliae* is an asexually reproducing ascomycete, with no known sexual stage (Short et al. 2014; Usami et al. 2009). Chromosomal rearrangement via transposable elements (TEs), random mutation, and horizontal gene transfer all contribute to *V. dahliae* diversity (Chen et al. 2018; Shi-Kunne et al. 2018). VdLs17 (non-race 1) and JR2 (race 1) have vastly different chromosomal arrangements despite having few nucleotide differences (de Jonge et al. 2013). More research is needed into the evolution of *V. dahliae* to understand how these processes are affecting pathogenesis.

The main objectives of this study were to: (i) illuminate the phylogenetic structure through whole genome re-resequencing and race patterns using select differential tomato varieties within *V. dahliae* isolates from Japan, California, and North Carolina, and (ii) identify genes (specifically effectors) associated with the race. By combining whole genome

resequencing and mining for candidate effector genes with macroscopic phenotypic data, we provide new insights into this pathogen's diversity and pathogenicity factors.

MATERIALS AND METHODS

Fungal isolation. North Carolina isolates were obtained from V. dahliae infested tomato fields from Henderson, Jackson, Haywood, and Buncombe counties, all in the temperate, high elevation, growing region of western North Carolina. V. dahliae was isolated from infected tomato by surface sterilizing stem segments and placing them on Sorenson's NP-10 media for 2 weeks at 26° C (Kabir et al. 2004). Spore suspensions were diluted to 1×10^2 conidia per mL and streaked on potato dextrose agar (PDA, Difco Lab., Detroit). Single spore isolates were obtained by hyphal tip isolation from 3-day old single spore colonies. California isolates Le1087 (race 1) and Le1811 (race 2) were supplied by Dr. Krishna V. Subbarao at UC Davis. California isolates CA70 and CA36 were supplied by Suraj Gurung from Sakata Seed America, Inc., Salinas, CA. DNA from all Japanese isolates (GFCa2, To22, Vdp4, Vd141, GFCB5, and HoMCF) was kindly provided by Dr. Toshiyuki Usami at Chiba University in Japan (Usami et al. 2017).

DNA extraction. Single spore isolates were grown on PDA for 10 days. Plugs from those plates were used to inoculate autoclaved 150mL conical flasks filled with 50mL of potato dextrose broth (PDB, Difco Lab., Detroit). After 4 days on PDB, the resulting mycelia was decanted into a 50 mL Falcon centrifuge tube and spun at 6000 rpm for 10 minutes. The supernatant was discarded, and the pelleted mycelia was dried under a laminar flow hood on autoclaved filter paper for 5-10 minutes. Pellets were frozen with liquid nitrogen and ground with a mortar and pestle to a fine powder. DNA was extracted using phenol-chloroform extraction (Usami et al. 2007). High molecular weight and overall DNA quality was confirmed

by North Carolina State University (NC State) Genome Sciences Laboratory (GSL) in Raleigh NC using an Agilent 2200 TapeStation and the Agilent 2100 Bioanalyzer (Santa Clara, CA).

Whole genome sequencing. Library preparation and sequencing took place at the NC State GSL. Genomic DNA libraries of Vdp4 and GFCa2 were prepared using Nextera DNA Flex Library Prep Kit from Illumina (San Diego, CA, USA). Vdp4 and GFCa2 genomes were sequenced at 1600x and 700x coverage using MiSeq v.2 150 bp PE flow cell. Genome libraries from all other isolates was prepared using TruSeq Nano LT DNA kit by Illumina (San Diego, CA, USA) and were sequenced at 20-30x coverage using MiSeq v3 300 bp PE flow cell.

Coding sequence extraction. Adapters were removed, paired end reads were merged, and QC scores <10 were trimmed. The paired reads were mapped to the 55 contigs of the VdLs17 reference genome bioproject PRJNA28529 (NCBI) using the Geneious mapper (Biomatters, Ltd., Auckland, NZ) with the only non-default option turned on being "Find structural variants, short insertions, and deletions of any size". Annotations were transferred to the consensus sequence at 75% percent similarity and a cost matrix of 65% similarity (5.0/-4.0). Coding sequences were extracted from the consensus sequence for each individual isolate sequence with a minimum coverage of 2x. Each coding sequence was translated in Geneious (Biomatters, Ltd., Auckland, NZ). This process was repeated for the 18 isolates mapped to the 8 chromosomes of the JR2 reference genome (Bioproject Accession PRJNA175765).

Secreted effector discovery. Coding sequences from the consensus sequence of each isolate were filtered through SignalP 5.0 to determine if a signal peptide was present on the N-terminus of the gene coding sequence (Armenteros et al. 2019). During SignalP filtering the signal peptide, a ~25 amino acid long sequence, was removed. Secreted sequences were then filtered through EffectorP (Sperschneider et al. 2016).

Phylogenetic analysis. Paired reads from each sequence were mapped to the VdLs17 contig NW_009276921.1. This contig was chosen because it was the largest continuous contig present in both reference genomes. The consensus sequences of all 18 isolates, as well as the identical region from JR2 chromosome 4 (Bioproject Accession PRJNA175765), and VdLs17 NW_009276921.1 were aligned using MAFFT v.1.4.0 (Biomatters Ltd, Auckland NZ). Gaps and ambiguous sequences were removed. Genetic distances were calculated using the Jukes-Cantor model (Jukes and Cantor 1969). Resampling was completed using the bootstrap method with 1000 replicates.

Amino acid sequences from 64 core effectors present in all isolates were concatenated from each isolate. Concatenated sequences were aligned using MAFFT 1.4.0 (Biomatters Ltd., Auckland NZ), and gaps and ambiguous sequences were removed using Geneious (Biomatters Ltd., Auckland NZ) sequence masking. A phylogenetic tree was developed using the aligned sequences and the Jukes-Cantor model was applied.

Unassembled reads effector discovery. The unassembled reads that did not map to the VdLs17 reference genome were assembled using the Geneious de novo assembler at the default settings in version 2020.1.0. Open reading frames (ORFs) were annotated to the assembled contigs. ORF settings were set to 'Alternative Yeast' with the start codons ATG and CTG, and a minimum nucleotide length of 100. All ORFs annotations were extracted and translated to amino acids in Geneious. Amino acid sequences were processed by SignalP v. 5.0, then EffectorP v. 2.0. Sequences were analyzed for secreted effectors specific to race 2 isolates.

Pathogenicity assay. Pathogenic races of 13 isolates were confirmed by inoculating 3-week-old differential tomato cultivars in the greenhouse. Three tomato cultivars tested were 'Bonny Best' (universal susceptible), 'Red Defender' (Ve1+/race 1 resistant, race 2 susceptible),

and ‘Aibou’ (Ve1+/race 1 resistant, race 2 resistant). Inoculum was prepared by growing each isolate on PDA for 1-2 weeks. Distilled de-ionized autoclaved water at ~22° C was used to wash spores from PDA plates. Spores were filtered through a double layer of autoclaved cheese cloth and diluted to 1 $\times 10^7$ conidia mL-1. Ten mL of this spore suspension were injected into the soil ~1 cm from the base of the plant stem using a 10 mL sterile pipette. Disease ratings from 0-5 (0 = 0%; 1 = 1-20%; 2 = 21-40%; 3 = 41-60%; 4 = 61-80%; 5 = 81-100%) for V-shaped foliar chlorosis/necrosis symptoms were recorded every 7 days for 45 days post inoculation. Each experiment contained 4 reps. Plants were planted in 18 oz plastic cups with 2-ply Sungro (70-80% Canadian sphagnum peat moss; 5-10% vermiculite plus dolomite limestone) potting soil. Plants were top watered for 1-week post inoculation. Holes were cut in the bottom of the cups, which were placed in trays 12 plants per tray with 2-5 cm of tap water. A 100mL aliquot of fertilizer “Miracle-Gro Water-Soluble All-Purpose Plant Food” at a concentration of 1g/1000 mL was added to each tray once a week. Photosynthetic photon flux density was 251 µmol photon m^{-2} s^{-1} with a blue, green, and red percentages being 37, 37.1, and 25.9% respectively and were on for 12 hours each day. Temperatures ranged between 23 and27C, and relative humidity ranged between 30 and 55%. Susceptibility herein was defined as having levels of chlorosis and necrosis on leaflets with an average AUDPC score > 100. Additional pathogenicity assays were conducted with ‘Aibou’ for six key isolates (Le1087, Ca36, NC86, JL5c, KJ14a, VdLs17) and a water control. To determine if ‘Bowman’ and ‘Ganbarune-Karis’ contained the race 2 resistance gene, they were inoculated with water, Ca36, NC86, and KJ14a using identical parameters.

RESULTS

Comparison of coding sequences of the 18 *V. dahliae* isolates assembled with the VdLs17 and JR2 reference genomes. Vdp4 had a coverage of 813x (based on an estimated

genome size of 32 Mbp) and 95.8% of the total 86,689,970 paired reads (150 bp paired end MiSeq) assembled to the VdLs17 genome. GFCa2 had a coverage of 245x and 95.5% of the total 26,130,444 paired reads (150 bp paired end MiSeq) assembled to the VdLs17 genome. The other 16 genomes had coverages ranging from 20x to 25x and 94.3-95.9% of the totals of 1,046,827 and 1,329,386 paired reads (300 bp paired end MiSeq) assembled to the VdLs17 reference genome (Table S1). Between 10,082-10,535 coding sequences were extracted from the 18 genomes and the VdLS17 reference genome (Supplementary Table S2).

Reads assembled to the JR2 reference genome had similar coverages to those assembled to the VdLs17 reference genome (Supplementary Table S3). The unassembled reads varied more in *V. dahliae* isolates assembled to the JR2 genome, ranging from 1.41%-9%. The number of coding sequences from the 18 sequenced genomes ranged from 10,896 - 11,233 (Supplementary Table S4).

Race Designation of *V. dahliae* Isolates. All isolates tested were pathogenic on 'Bonny Best', and only Le1087 was non-pathogenic on 'Red Defender' (race 1 resistant) (data not shown). Le1087, CA36, and TC18a were non-pathogenic on 'Aibou' (race 1 and 2 resistant). NC86, FL10b, NC85, KJ14a, FF5a, FL7a, JL5c, Le1811, FL9b, and VdLs17 were all pathogenic on 'Aibou' (Figure 1). The water control showed no symptoms in all cultivars. In a second trial race typing on 'Aibou' confirmed that Le1087 and Ca36 were non-pathogenic on 'Aibou', while NC86, JL5c, KJ14a, and VdLs17 were pathogenic on 'Aibou' (Supplementary Figure S3). A parallel third trial conducted on 'Bowman' showed that Ca36 was non-pathogenic on 'Bowman', while NC86 and KJ14a were pathogenic on 'Bowman' (Supplementary Figure S4). A fourth trial on Ganbarune-Karis showed that Ca36 was non-pathogenic on Ganbarune-Karis, but KJ14a was pathogenic (Supplementary Figure S5). In sum, the data confirm Le1087 is race 1, that CA36,

and TC18a are race 2 and NC86, FL10b, NC85, KJ14a, FF5a, FL7a, JL5c, Le1811, FL9b, and VdLs17 are race 3. The race typing of Japanese isolates included in this study were Vdp4 (race 1, Vdp4Δ*Ave1* = race 3), GFCa2 (race 2), Vd141 (race 2), TO22 (Race 2), GFCB5 (Race 3), HoMCF (Race 3) and these data were documented in two separate publications (Usami et al. 2017; Kano and Usami 2019).

Genetic Diversity in *V. dahliae*. To compare with the reference genome of VdLs17, the 18 isolates of *V. dahliae* from tomatoes in Japan, California, and North Carolina were aligned to a 2.51 million nucleotide region NW_009276921., which isthe largest contig from the reference genome of VdLs17. Based on this analysis, 18 isolates classified into 4 major groups (Figures 2 and 3). After gaps and ambiguous sequences were removed, the single nucleotide polymorphism (SNP) differences within isolates in a group were less than 0.05%. Between-group SNP differences ranged from 0.11-0.45%. Group 1 comprised isolates least similar to one another with the highest number of SNP differences ranging between 0.4-0.45% differences (Fig. 3). Group 1 contained VdLs17, Vdp4, and HoMCF; Group 2: FL9b, JL5c, CA70, Le1811, and GFCB5; Group 3: KJ14a, FF5a, FL7a, and NC85; and Group 4: FL10b, NC86, CA36, Le1087, GFCa2, and Vd141. Overall, the groups revealed no evidence for geographic clustering. Groups 1-3 contained primarily race 3 isolates, whereas Group 4 contained isolates of all 3 races.

Effector Analysis of 18 *V. dahliae* Isolates and the Reference Genome VdLs17. The phylogenetic tree developed from the concatenated alignment of the 64 core effectors (effectors present in all isolates) (Figure 4) indicated the non-synonymous mutations (NSMs) within the secreted effectors mirrored the phylogenomic groups from whole genome sequences (Figure 1). Very few nucleic acid changes (<0.1%) were evident within group 4 (Figures 2 and 3), despite this group containing 3 different races. Within group 3 NC85 and FF5a had a large >3 NSMs

differences between each other and the other group 3 isolates KJ14a and FL7a with a bootstrap value of 92. Similarly, CA70 differed from other isolates in group 2 with a bootstrap value of 95. The 3 isolates in group 1 which included the VdLs17 reference strain were very distinct from each other (Figure 4).

Across the 18 re-sequenced *V. dahliae* genomes and the VdLs17 reference genome 201 unique secreted effector regions were discovered using SignalP and EffectorP (Table 1). Eighty completely conserved core effector genes that contained no NSMs were present across the 19 genomes. These core effectors were present on all the VdLs17 chromosomes (Supplementary Table S5). An additional 64 effector genes, present in all strains with NSMs, were identified. Of these, 40 effectors with specific NSMs could be assigned to each of the 4 groups. Group 1 contained 33 secreted effector genes with NSMs unique to that group, whereas Group 2, 3 and 4 contained 4, 1 and 3 secreted effector genes with NSMs unique to each group. Of note, a single effector gene *VdPDA1* (accession number XP_009654342) on the NW_009276934.11 contig within Group 4 contained NSM for isoleucine in isolates corresponding to races 1 and 2. In contrast all race 3 isolates in Group 4 and present in other groups contained Phenylalanine at that location. No other haplotype profile matched the race structure. There were 22 core secreted effector regions that had either 5 or more haplotypes, or large truncated regions within their sequences between isolates (Table 1). There were 57 secreted effector genes that were differentially absent/present in one or multiple isolates (Table 1).

Effector Analysis of 18 *V. dahliae* Isolates and the Reference Genome JR2. There were 222 unique effector loci identified from across all 18 re-sequenced genomes and the JR2 reference genome. Eighty-one core effectors were identified in all isolates and contained no amino acid changes in their coding sequences. There were 85 core effectors present in all

genomes but with at least 1 NSM, or large indels, in their sequences. Fifty-six effectors were differentially present in one or more of the isolate's genomes, but exhibited no clear pattern as related to the 4 groups and were considered as non-core (Table 2). Candidate effectors were distributed across all 8 chromosomes. Chromosome 1, the largest chromosome, had the greatest number of effectors at 48 (1 per 193 Kb), whereas chromosome 3 had the least number of effectors at 20 (1 per 208 Kb) (Supplementary Table S6). Of the 18 re-sequenced isolates, Vdp4 had the highest number of candidate effectors at 191, while KJ14a had the fewest at 182 (Supplementary Table S4). Notably, *VdPDA1* was not recognized as a secreted effector in the JR2 reference genome because the provided annotation starts with a TTG start codon that adds 18 amino acids to the beginning of the gene sequence. JR2 contains the race 3 haplotype of *VdPDA1*, although its phenotype is unknown.

Two candidate effectors were discovered that were only present in the race 2 isolates, Le1087 (race 1), and JR2 (race 1), but not in any of the race 3 isolates or VdP4 (race 1 and 3). Both genes are present on the same region of JR2 chromosome 4: VDAG_JR2_Chr4g03650a-00001 and VDAG_JR2_Chr4g03680a-00001. Race 3 genomes do not contain the regions where these genes are found. This region is absent in the VdLs17 reference genome. Both candidate effectors yielded no nucleotide or protein matches (default search parameters) in the nr/nt database.

De novo Assembly of Unmapped Reads (not mapped to the VdLs17 genome), Open Reading Frame (ORF), and Effector Analysis. Three contigs unique to race 2 isolates were assembled from paired reads not assembled to the VdLs17 reference genome. These were 29.4 (contig 1), 14.6, and 16.0 Kb in length. While these contigs were are similar to regions on the JR2 chromosome 4, they contained ORFs which were not annotated on the JR2 reference

genome. Nine candidate effectors were found only in race 2 isolates; 3 from each of the 3 contigs. Five of the secreted effectors had CTG as the start codon, while 4 had ATG as the start codon. The JR2 cDNA library (VDAG_JR2v.4.0) was searched for sequences similar to the 9 ORFs, however no similarities were found. The RNA transcript of each ORF was blasted against the reference RNA sequences 'refseq-rna' database, and but no similar genes were found. Nucleotide and protein BLAST results yielded no genes similar (default search parameters) to these sequences in the nr/nt database. Only 1 of 9 secreted effectors found in the ORF analysis of unused reads had a TATA box promoter site that was less than 150 bp from the start codon. There are some large insertions and deletions which cause contigs 1-3 to not match identically with JR2 chromosome 4, however the regions that do match are 99% similar. Unassembled contig 1 aligns to several regions of JR2 chromosome 4, and is ~29.4 Kb in length. Unassembled contig 2 is at the 1.165-1.180 Mb region of JR Chr. 4 and is 14.6 Kb in length. Unassembled contig 3 starts at the 1.09-1.0 Mbp region and is 22.6 Kb in length. The locations and names of the candidate effectors from the ORF and other analysis are located in table 3 and 4.

DISCUSSION

We sequenced and compared the genome sequences of different races of *Verticillium dahlia* and candidate effector profiles, and identified candidates that were unique, or had unique NSMs, that may explain the race 2 phenotype. Twelve candidate effectors were found in race 2 isolates, or race 1 isolates that lack race 2 phenotype data, such as Le1087 and JR2 (Table 3). Interestingly, one candidate effector, matching *VdPDA1* (R2C1) contained an NSM that converted isoleucine in race 2 to phenylalanine in race 3. Of all the race 2 candidate genes identified, *VdPDA1* is the only gene that has been experimentally shown to be have role in pathogenesis that is vital for successful infection of tomatoes (Gao et al 2019). None of the 9

secreted effectors found in the ORF analysis of unused reads, or 2 effectors found in race 2 isolates and the race 1 isolates JR2 and Le1087, have been shown experimentally to be functional genes. However, R2C2 and R2C3 have both been found the cDNA library of JR2, indicating they may play a role in pathogenicity. Further research is needed to elucidate which candidate gene is the correct race 2 gene.

Effector cassettes were primarily lineage specific, rather than specific to any geographic region. Group 1 isolates were found in California, Japan, and Canada. Group 3 was the only group unique to North Carolina, although the sample size of this study was rather small. Group 4 is the most informative of all the groups because it contains all 3 races, despite being closely related. In this study *V. dahliae* isolates pathogenic on tomato were shown to have between 169-193 secreted effectors. A study of the VdLs17 genome in 2011 showed only 127 effectors; however, the method by which they were classified as effectors was by sequence length (< 400 bp) and being rich in cysteine (Klosterman et al 2011). A more recent study of the VdLs17 (using SignalP v4.1 and EffectorP v1.0) reported 179 (Gibriel et al 2019). In this study, additional secreted effectors were found. It is unclear if this increase in secreted effector is due to the discovery of more true genes or more false positives. Phenotyping secreted effectors should be a priority to increase our understanding of these crucial genes. Both core and lineage specific effectors may play vital functions that could be exploited by researchers to influence pathogenicity.

With the discovery of a new source of host resistance in tomato against *V. dahliae* race 2, new questions emerge about the nomenclature surrounding this pathosystem. Except for Vdp4 (race 1 and 3) and JR2 (race 1), all isolates in groups 1-3, as circumscribed in this study, are race 3 (i.e. can infect ‘Aibou’). *Ave1* is the effector responsible for the race 1 phenotype (Jonge et al.

2012). Interestingly, in 2019 it was shown that deletion of Ave1 from Vdp4 resulted in the ability to be pathogenic on 'Aibou', i.e. become race 3 (Kano and Usami 2019). In contrast the race 1 isolate Le1087 is in the phylogenetically distinct group 4. Le1087 also contains all the race 2 candidate secreted effectors. Group 4 is the only group with race 2 isolates, which suggests that within this group race 2 evolved from race 1 by loss of the *Ave1* effector. Indeed, with the exception of Le1087, all isolates in group 4 lack Ave1. Further evidence for this hypothesis could be provided by deleting Ave1 from Le1087 and evaluating pathogenicity on Aibou. Our results also call into the question the race nomenclature. Race 3 may simply represent isolates that lack race 1 and 2 effectors, which could mean there are currently many races within the race 3 designation. Race 3 and further race designations will require the identification of additional differential germplasm. Before the Usami 2017 race 2 publication, many scientific publications referred to non-race 1 isolates as race 2.

The largest number of nucleotide differences were between group 1 and all other groups (0.4-0.45% differences) (Figures 3, S1, S2). Group 1 also contained the largest number of group specific NSMs at 33, more than all the other groups combined. Group 1 also contains the reference genomes VdLs17 and JR2. VdLs17 and JR2 also have radically different chromosome structures, despite being quite similar phylogenetically to each other, having undergone chromosomal rearrangement in the not so distant past (de Jonge et al. 2013; Shi-Kunne et al. 2018). These data raise productive avenues of further research concerning the comparative chromosomal structure of groups 2, 3, and 4. Further research should be conducted into the chromosome structures of groups 2, 3, and 4. Interestingly group 3 was only found in North Carolina, USA. An expanded analysis of a world-wide collection could ascertain the distribution of groups 1-4, potentially uncover additional groups, and provide a broader phylogenetic

framework to offer additional insights regarding *V. dahliae* biology, race and host range attributes. More research into the distribution of groups 1-4 from a broader world-wide collection would be productive to undercover additional groups, if any.

The only candidate with identified function is *VdPDA1*, which contains a chitin binding domain. Effector triggered immunity against LysM effectors is unknown in tomatoes. The non-LysM effector Avr4 in *Cladosporium fulvum* protects chitin from hydrolysis by plant chitinases and can be recognized by host defenses in tomato (van der Burg et al 2006). The *VdPDA1* may have chitin binding properties found in other virulence factors for *V. dahliae* (Kombrink et al. 2017b; Miya et al. 2007; Gao et al. 2019). A homologous *VdPDA1* (and functional) locus also exists in *Fusarium oxysporum* f. sp. *vasinfectum* which may function similarly to the *V. dahliae* gene (ref or explain). It is unclear whether the race 2 resistance would also confer resistance against *Fusarium* species.

Conclusions and Perspectives. We identified novel candidate effectors which showed specific variations in *V. dahliae* race 2, and may potentially be associated with virulence. Further phenotypic and functional studies are needed to fully characterize these effectors to evaluate their roles of in the virulence of *V. dahliae.* This may simplify future studies using the candidate effectors identified here by molecular genetic manipulation (silencing, knock out and knock in experiments). Determining the role of *VdPDA1* presents a significant challenge. Knocking out the gene completely has the effect of greatly inhibiting pathogenicity (Gao et al 2019). A more effective strategy would be to replace the race 2 haplotype of *VdPDA1* with the race 3 haplotype and testing pathogenicity on differential germplasm. R2C2 and R2C3 also present excellent candidates for further research, as there is evidence they are being expressed, and they contain both signal peptides and similarities to other effectors.

Within this study both the phylogenetic relationships and the race 2 phenotype of *V. dahliae* have been illuminated via whole genome resequencing. More experiments are necessary to confirm that *VdPDA1*, or any of the race 2 specific hypothetical effectors, are the effector responsible for the race 2 phenotype. Combining whole genome sequencing and machine learning algorithms provided new insights into specific host-pathogen interactions.

To determine the nature of the race 2 resistance in tomato, 'Aibou' and 'Ganbarune-Karis' were crossed and selfed, and their progeny were evaluated for resistance to race 2. We found the ratio of resistant to susceptible plants was 3:1. This indicates that the resistance to race 2 is controlled by a single dominant locus. To maximize the genetic gain in this population, focus must be given to elucidate the plant-pathogen interactions at molecular level. It appears that race 2 is widely prevalent in the region. Thus, rapid and precision breeding is necessary by strategically integrating modern genomics approaches with advanced breeding techniques. Cost-effective third-generation sequencing technologies are now available to capture the SNPs via high-throughput genotyping. The identification and functional characterization of genes associated disease resistance for multiple races would be useful to ensure for broad-spectrum and durable resistance to *V. dahliae.*

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Toshiyuki Usami at Graduate School of Horticulture, Chiba University Japan for his contribution of DNA from *V. dahliae* isolates from Japan. We would also like to thank Dr. Krishna Subbarao at University of California, Davis for his contribution of *V. dahliae* isolates from California. Sakata Seed America, Inc. for providing USA isolates and seed for this project.

Data Availability

Raw data for this project is available through sequence read archive (SRA) bioproject PRJNA640002 https://www.ncbi.nlm.nih.gov/bioproject/640002. Biosamples SAMN15297318-SAMN15297335.

REFERENCES

1. Akamatsu, A., Wong, H. L., Fujiwara, M., Okuda, J., Nishide, K., Uno, K., et al. 2013. An OsCEBiP/OsCERK1-OsRacGEF1-OsRac1 module is an essential early component of chitin-induced rice immunity. Cell Host Microbe. 13:465–476.
2. Almagro Armenteros, J. J., Sønderby, C. K., Sønderby, S. K., Nielsen, H., and Winther, O. 2017. DeepLoc: prediction of protein subcellular localization using deep learning. Bioinformatics. 33:3387–3395.
3. Armenteros, J. J. A., Tsirigos, K. D., Sønderby, C. K., Petersen, T. N., Winther, O., Brunak, S., et al. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 37:420–423.
4. Belkhadir, Y., Subramaniam, R., and Dangl, J. L. 2004. Plant disease resistance protein signaling: NBS–LRR proteins and their partners. Curr. Opin. Plant Biol. 7:391–399.
5. Bender, C. G., and Shoemaker, P. B. 1984. Prevalence of Verticillium wilt of tomato and virulence of Verticillium dahliae race 1 and race 2 isolates in western North Carolina. Plant Dis. 68:305–309.
6. Bhat, R. G., and Subbarao, K. V. 1999. Host Range Specificity in *Verticillium dahliae*. Phytopathology. 89:1218–1225.
7. Campbell, M. S., Holt, C., Moore, B., and Yandell, M. 2014. Genome annotation and curation using MAKER and MAKER-P. Curr. Protoc. Bioinforma. 48:4–11.
8. Campbell, M. S., Law, M., Holt, C., Stein, J. C., Moghe, G. D., Hufnagel, D. E., et al. 2014. MAKER-P: a tool kit for the rapid creation, management, and quality control of plant genome annotations. Plant Physiol. 164:513–524.

9. Diwan, N., Fluhr, R., Eshed, Y., Zamir, D., and Tanksley, S. D. 1999. Mapping of Ve in tomato: a gene conferring resistance to the broad-spectrum pathogen, Verticillium dahliae race 1. Theor. Appl. Genet. 98:315–319.
10. Ebel, J. 1986. Phytoalexin synthesis: the biochemical analysis of the induction process. Annu. Rev. Phytopathol. 24:235–264.
11. Gao, F., Zhang, B.-S., Zhao, J.-H., Huang, J.-F., Jia, P.-S., Wang, S., et al. 2019. Deacetylation of chitin oligomers increases virulence in soil-borne fungal pathogens. Nat. Plants. 5:1167-+.
12. Green, R. J. 1980. Soil factors affecting survival of microsclerotia of Verticillium dahliae. Phytopathology. 70:353–355.
13. Gullino, M. L., Minuto, A., and Garibaldi, A. 2002. Soil fumigation with chloropicrin in Italy: experimental results on melon, eggplant and tomato. Meded. Rijksuniv. Te Gent Fak. Van Landbouwkd. En Toegepaste Biol. Wet. 67:171–180.
14. Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., et al. 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. Proc. Natl. Acad. Sci. 111:E404–E413.
15. Howe, L. R., Leevers, S. J., Gómez, N., Nakielny, S., Cohen, P., and Marshall, C. J. 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell. 71:335–342.
16. Hu, X., Puri, K. D., Gurung, S., Klosterman, S. J., Wallis, C. M., Britton, M., et al. 2019. Proteome and metabolome analyses reveal differential responses in tomato -Verticillium dahliae-interactions. J. Proteomics. 207:103449.
17. Jones, J. D., and Dangl, J. L. 2006. The plant immune system. nature. 444:323–329.

18. de Jonge, R., Bolton, M. D., Kombrink, A., van den Berg, G. C., Yadeta, K. A., and Thomma, B. P. 2013. Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen. Genome Res. 23:1271–1282.

19. Jonge, R. de, Esse, H. P. van, Maruthachalam, K., Bolton, M. D., Santhanam, P., Saber, M. K., et al. 2012. Tomato immune receptor Ve1 recognizes effector of multiple fungal pathogens uncovered by genome and RNA sequencing. Proc. Natl. Acad. Sci. 109:5110–5115.

20. Kabir, Z., Bhat, R., and Subbarao, K. 2004. Comparison of media for recovery of Verticillium dahliae from soil. Plant Dis. 88:49–55.

21. Kano, K., and Usami, T. 2019. Race 3 tomato Verticillium wilt pathogen ptoentially derives from race 1 isolate. Soil Microorg. 73:71–78.

22. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nat. Protoc. 10:845.

23. Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. 2009. Diversity, Pathogenicity, and Management of Verticillium Species. Annu. Rev. Phytopathol. 47:39–62.

24. Klosterman, S. J., Subbarao, K. V., Kang, S., Veronese, P., Gold, S. E., Thomma, B. P., et al. 2011. Comparative genomics yields insights into niche adaptation of plant vascular wilt pathogens. PLoS Pathog. 7.

25. Kombrink, A., Rovenich, H., Shi-Kunne, X., Rojas-Padilla, E., van den Berg, G. C., Domazakis, E., et al. 2017a. Verticillium dahliae LysM effectors differentially contribute to virulence on plant hosts. Mol. Plant Pathol. 18:596–608.

26. Kombrink, A., Rovenich, H., Shi-Kunne, X., Rojas-Padilla, E., van den Berg, G. C., Domazakis, E., et al. 2017b. Verticillium dahliae LysM effectors differentially contribute to virulence on plant hosts. Mol. Plant Pathol. 18:596–608.

27. Kombrink, E., and Schmelzer, E. 2001. The hypersensitive response and its role in local and systemic disease resistance. Eur. J. Plant Pathol. 107:69–78.

28. Kombrink, E., and Somssich, I. 1997. Pathogenesis-related proteins and plant defense. In *Plant relationships*, Springer, p. 107–128.

29. Melhem, H., Min, X. J., and Butler, G. 2013. The impact of SignalP 4.0 on the prediction of secreted proteins. In IEEE, p. 16–22.

30. Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. Proc. Natl. Acad. Sci. 104:19613–19618.

31. Newman, M.-A., Sundelin, T., Nielsen, J. T., and Erbs, G. 2013. MAMP (microbe-associated molecular pattern) triggered immunity in plants. Front. Plant Sci. 4:139–139.

32. Pegg, G. F., and Brady, B. L. 2002. *Verticillium Wilts*. CABI.

33. Peumans, W. J., and Van Damme, E. 1995. Lectins as plant defense proteins. Plant Physiol. 109:347.

34. Raskin, I. 1992. Role of salicylic acid in plants. Annu. Rev. Plant Biol. 43:439–463.

35. Sharma, N., Sharma, K., Gaur, R., and Gupta, V. 2011. Role of chitinase in plant defense. Asian J Biochem. 6:29–37.

36. Shi-Kunne, X., Faino, L., van den Berg, G. C., Thomma, B. P., and Seidl, M. F. 2018. Evolution within the fungal genus Verticillium is characterized by chromosomal rearrangement and gene loss. Environ. Microbiol. 20:1362–1373.

37. Short, D. P., Gurung, S., Hu, X., Inderbitzin, P., and Subbarao, K. V. 2014. Maintenance of sex-related genes and the co-occurrence of both mating types in Verticillium dahliae. PLoS One. 9.

38. Song, Y., Zhang, Z., Seidl, M. F., Majer, A., Jakse, J., Javornik, B., et al. 2017. Broad taxonomic characterization of Verticillium wilt resistance genes reveals an ancient origin of the tomato Ve1 immune receptor. Mol. Plant Pathol. 18:195–209.

39. Sperschneider, J., Gardiner, D. M., Dodds, P. N., Tini, F., Covarelli, L., Singh, K. B., et al. 2016. EffectorP: predicting fungal effector proteins from secretomes using machine learning. New Phytol. 210:743–761.

40. Sutherland, M. W. 1991. The generation of oxygen radicals during host plant responses to infection. Physiol. Mol. Plant Pathol. 39:71–93.

41. Tsuda, K., and Katagiri, F. 2010. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Curr. Opin. Plant Biol. 13:459–465.

42. Usami, T., Ishigaki, S., Takashina, H., Matsubara, Y., and Amemiya, Y. 2007. Cloning of DNA fragments specific to the pathotype and race of Verticillium dahliae. J. Gen. Plant Pathol. 73:89–95.

43. Usami, T., Itoh, M., and Amemiya, Y. 2009. Asexual fungus Verticillium dahliae is potentially heterothallic. J. Gen. Plant Pathol. 75:422.

44. Usami, T., Momma, N., Kikuchi, S., Watanabe, H., Hayashi, A., Mizukawa, M., et al. 2017. Race 2 of Verticillium dahliae infecting tomato in Japan can be split into two races with differential pathogenicity on resistant rootstocks. Plant Pathol. 66:230–238.

45. Xiao, C. L., Subbarao, K. V., Schulbach, K. F., and Koike, S. T. 1998. Effects of Crop Rotation and Irrigation on *Verticillium dahliae* Microsclerotia in Soil and Wilt in Cauliflower. Phytopathology. 88:1046–1055.

46. Yandell, M., and Ence, D. 2012. A beginner's guide to eukaryotic genome annotation. Nat. Rev. Genet. 13:329–342.

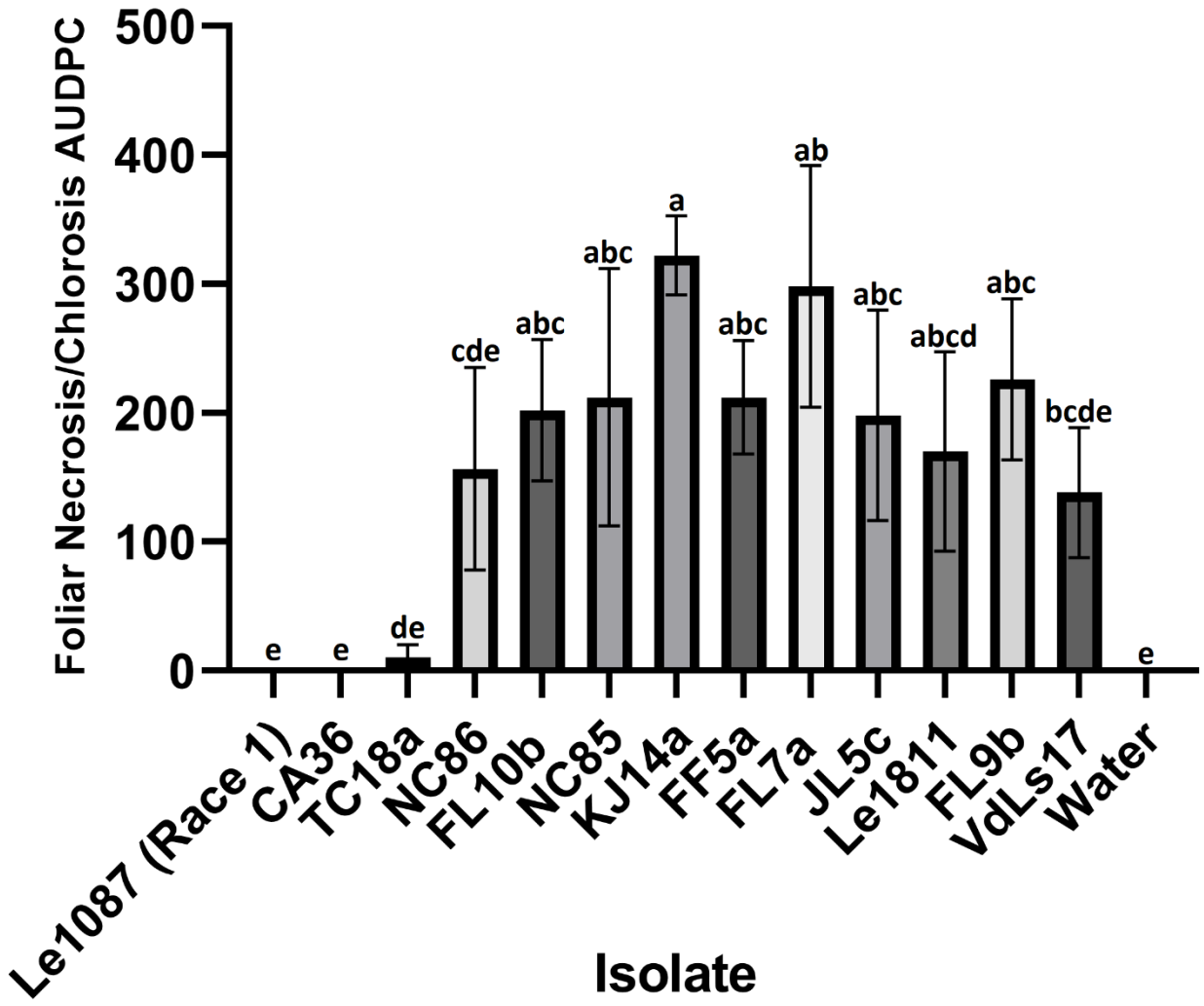

Figure 5.1: Area under the disease pressure curve scores (AUDPC) scores developed from chlorosis/necrosis foliar symptoms present on Aibou (resistant to race 1 and 2) plants inoculated with conidia from *V. dahliae* strains. Letters indicate significance groupings determined with students t-test (P=0.05). Error bars represent the standard error of the mean.

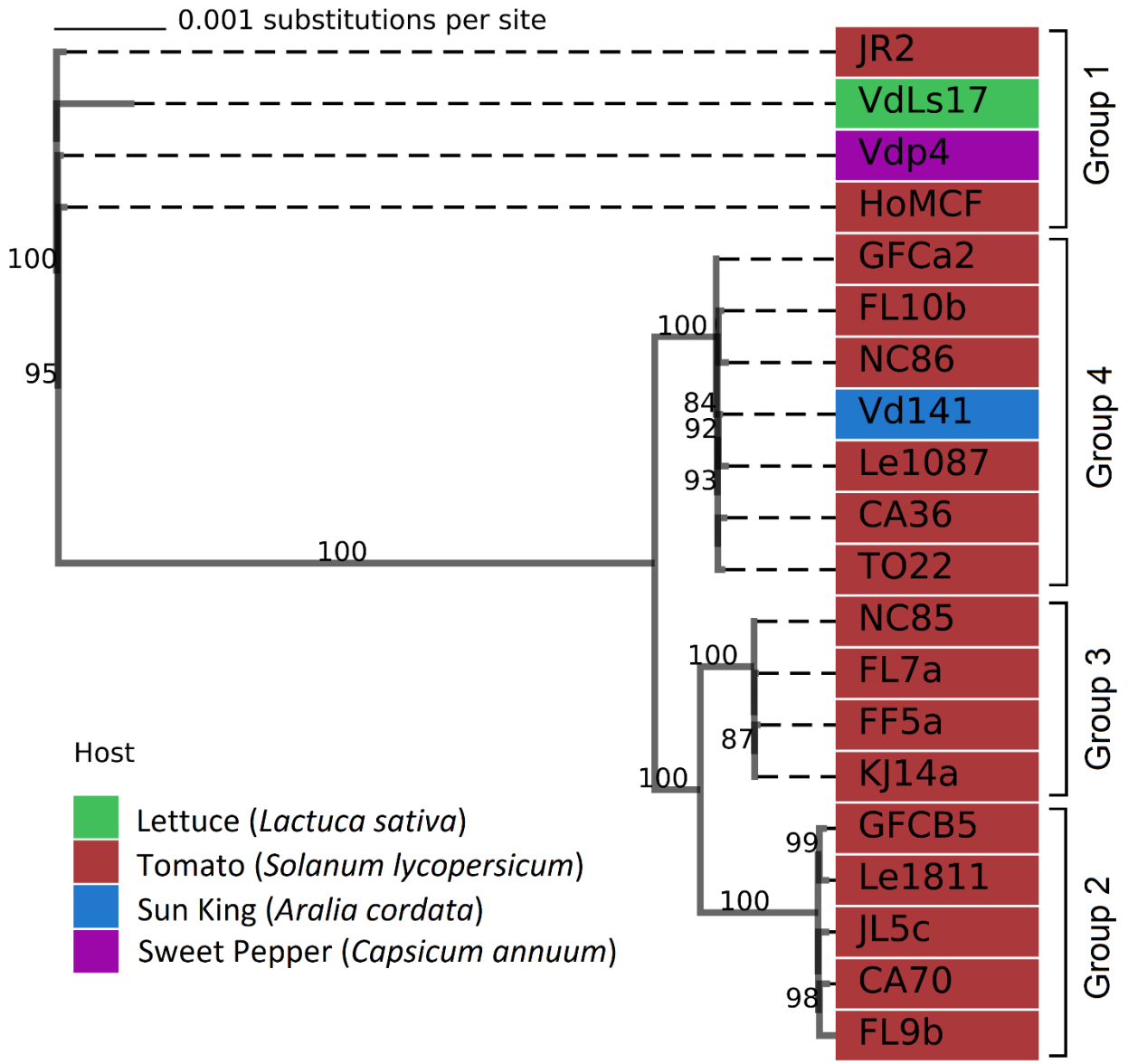

Figure 5.2: Neighbor-joining consensus tree of reads mapped to the VdLs17 contig NW_009276921.1. Consensus sequences were compared to the identical region found on the JR2 chromosome 1. After gaps and ambiguous sequences were removed the alignment length was 2.51 million nucleotides in length. Alignment was completed by MAFFT 1.4.0 (Biomatters Ltd, Auckland NZ). Genetic distances were calculated using the Jukes-Cantor model. Resampling was completed using the bootstrap method with 1000 replicates with bootstrap values assigned to each branching nodule.

	VdLs17	JL5c	KJ14a	NC86	Ca36	Le1087
VdLs17 (Race 3, Group 1)		11283 (99.55)	10608 (99.57)	10230 (99.59)	10150 (99.59)	10126 (99.6)
JL5c (Race 3, Group 2)	11283 (99.55)		2807 (99.88)	3634 (99.85)	3557 (99.85)	3565 (99.86)
KJ14a (Race 3, Group 3)	10608 (99.57)	2807 (99.88)		2754 (99.89)	2700 (99.89)	2683 (99.89)
NC86 (Race 3, Group 4)	10230 (99.59)	3634 (99.85)	2754 (99.89)		868 (99.96)	863 (99.97)
Ca36 (Race 2, Group 4)	10150 (99.59)	3557 (99.85)	2700 (99.89)	868 (99.96)		779 (99.97)
Le1087 (Race 1, Group 4)	10126 (99.59)	3565 (99.85)	2683 (99.89)	863 (99.96)	779 (99.97)	

Figure 5.3: Single nucleotide differences matrix with representative isolates from all four groups and between races within group 4. Reads from isolates were mapped to the VdLs17 contig (NW_009276921.1). The consensus sequences were extracted and aligned using MAFFT 1.4.0 (Biomatters Ltd, Auckland, NZ). Gaps and ambiguous sequences were removed to create a 2.51 million nucleotide long alignment. Genetic distances calculated using the Jukes-Cantor model. Within group differences were less than 0.05%.

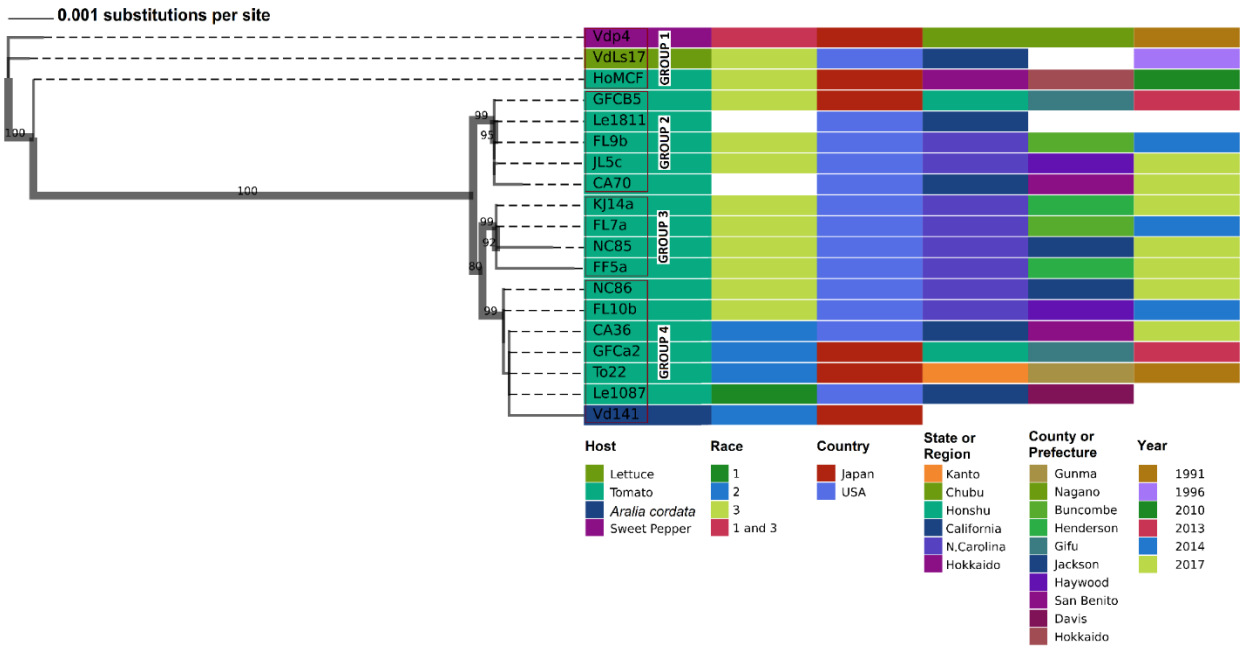

Figure 5.4: Phylogenetic tree developed from 64 concatenated protein sequences of secreted effector regions present in all isolates but containing non-synonymous mutations. The concatenated sequences were aligned using MAFFT 1.4.0 (Biomatters Ltd., Auckland NZ), and gaps and ambiguous sequences were removed. The tree was developed using the Jukes-Cantor model with 1000 bootstrap replications. Host indicates the plant species the fungus was originally isolated from. Race designations confirmed experimentally in this study or in Usami et al. 2017.

Table 5.1: Unique effector-like regions discovered through filtering coding sequences through SignalP and EffectorP. Coding sequences were extracted from mapping reads from 18 *V. dahliae* genomes compared to the VdLs17 reference genome (ASM15067v2).

Effector Groupings[x]	Number[z]
Core effectors with no NSMs	80
Core effectors with group 1 NSMs	33
Core effectors with group 2 NSMs	4
Core effectors with group 3 NSMs	1
Core effectors with group 4 NSMs	3
Effectors with NSMs only found in Race 2 Isolates	1
Core effectors with cross group NSMs or truncation	22
Non-Core effectors	57
Total individual effector regions discovered	201

[x]Groupings of effectors present or absent in 19 sequenced genomes containing Non-Synonymous Mutations (NSMs) or truncated regions

[z]Individual effectors derived from mapping to VdLs17 reference genome and filtered by SignalP and EffectorP

Table 5.2. Unique effector-like regions discovered through filtering coding sequences through SignalP and EffectorP. Coding sequences were extracted from mapping reads from 18 *V. dahliae* genomes to the JR2 reference genome (ASM15067v2)

Effector Groupings	Number[z]
Core effectors with no NSMs	81
Core effectors with group 1 NSMs	35
Core effectors with group 2 NSMs	6
Core effectors with group 3 NSMs	4
Core effectors with group 4 NSMs	5
Effectors with NSMs only found in Race 2 Isolates, JR2, and Le1087	2
Core effectors with cross group NSMs or truncation	33
Non-Core effectors	56
Total	222

[x]Core effectors present in all 18 sequenced genomes and JR2

[z]Individual effectors derived from mapping to JR2 reference genome

Table 5.3. Summary of candidate resistance genes found from either the isolates assembled to the VdLs17 (VdLs17 RG) or JR2 reference genomes, or from open reading frame (ORF) analysis of the reads not assembled to the VdLs17 genome (UR ORF). Presence of the race 2 haplotype indicated in JR2 genomic DNA, JR2 cDNA, or Le1087 genomic DNA.

Gene ID	Gene name	Method	Type[w]	JR2[x]	JR2 cDNA[y]	Le1087[z]
R2C1	VdPDA1	VdLs17 RG	NSM	-	-	+
R2C2	VDAG_ Chr4g03650.1-1	JR2 RG	Present	+	+	+
R2C3	VDAG_ Chr4g03680.1-3	JR2 RG	Present	+	+	+
R2C4	Contig 1 ORF 1	UR ORF	Present	+	-	+
R2C5	Contig 1 ORF 2	UR ORF	Present	+	-	+
R2C6	Contig 1 ORF 3	UR ORF	Present	+	-	+
R2C7	Contig 2 ORF 1	UR ORF	Present	+	-	+
R2C8	Contig 2 ORF 2	UR ORF	Present	+	-	+
R2C9	Contig 2 ORF 3	UR ORF	Present	+	-	+
R2C10	Contig 3 ORF 1	UR ORF	Present	+	-	+
R2C11	Contig 3 ORF 2	UR ORF	Present	+	-	+
R2C12	Contig 3 ORF 3	UR ORF	Present	+	-	+

[w]Indicates how this gene is different between race 2 and race 3 isolates. NSM = gene present in both races but contains an NSM present only in race 2 but not in race 3. Present = gene and surrrounding contig are only present in race 2 isolates, or race 1 isolates with no known race 2 phenotype (Le1087 or JR2), and are not present in race 3 isolates.

[x]Race 2 haplotype present in the JR2 genomic DNA.

[y]Race 2 haplotype present in the JR2 cDNA

[z]Race 2 haplotype present in the Le1087 genomic DNA

Table 5.4. Start Codon and location of each race 2 candidate gene on the JR2 chromosome.

Gene ID	Start Codon	JR2 Chr4 Location
R2C1	ATG	1523795 to 1524695
R2C2	ATG	1170784 to 1170406
R2C3	ATG	1179336 to 1179785
R2C4	ATG	1075295 to 1075066
R2C5	CTG	1065892 to 1066032
R2C6	CTG	1073775 to 1074068
R2C7	ATG	1172205 to 1172606
R2C8	ATG	1167261 to 1167106
R2C9	CTG	1168770 to 1169135
R2C10	CTG	1104678 to 1104157
R2C11	CTG	1105738 to 1105932
R2C12	CTG	1104567 to 1104157

	CA70	GFCB5	Le1811	FL9b	JL5c	FL10b	NC86	GFCa2	Ca36	Vd141	TO22	Le1087	FF5a	KJ14a	NC85	FL7a	HoMCF	JR2	Vdp4	VdLs17
CA70		241	277	311	263	2851	2902	2821	2901	2848	2876	2913	2217	2181	2173	2192	9021	9055	8993	9840
GFCB5	241		206	305	245	2817	2868	2787	2867	2816	2846	2882	2181	2149	2141	2160	8979	9013	8951	9803
Le1811	277	206		326	270	2847	2898	2817	2897	2844	2858	2911	2203	2175	2168	2186	9018	9052	8990	9841
FL9b	311	305	326		332	2919	2967	2887	2965	2912	2944	2980	2282	2248	2240	2259	9082	9116	9054	9905
JL5c	263	245	270	332		2842	2893	2814	2890	2839	2869	2903	2210	2172	2168	2183	9008	9042	8980	9830
FL10b	2851	2817	2847	2919	2842		187	142	198	153	165	214	2015	1985	1983	2000	7847	7881	7819	8669
NC86	2902	2868	2898	2967	2893	187		189	257	204	233	269	2072	2038	2034	2051	7900	7936	7872	8723
GFCa2	2821	2787	2817	2887	2814	142	189		162	107	139	180	1997	1961	1950	1970	7781	7815	7749	8601
Ca36	2901	2867	2897	2965	2890	198	257	162		173	199	248	2069	2033	2031	2048	7893	7929	7865	8714
Vd141	2848	2816	2844	2912	2839	153	204	107	173		154	178	2020	1986	1974	1995	7850	7886	7822	8673
TO22	2876	2846	2858	2944	2869	165	233	139	199	154		215	2039	2010	2008	2025	7869	7905	7841	8693
Le1087	2913	2882	2911	2980	2903	214	269	180	248	178	215		2086	2053	2046	2062	7919	7955	7891	8742
FF5a	2217	2181	2203	2282	2210	2015	2072	1997	2069	2020	2039	2086		106	112	131	8342	8378	8314	9167
KJ14a	2181	2149	2175	2248	2172	1985	2038	1961	2033	1986	2010	2053	106		80	91	8308	8344	8280	9134
NC85	2173	2141	2168	2240	2168	1983	2034	1950	2031	1974	2008	2046	112	80		91	8302	8338	8274	9127
FL7a	2192	2160	2186	2259	2183	2000	2051	1970	2048	1995	2025	2062	131	91	91		8323	8359	8295	9145
HoMCF	9021	8979	9018	9082	9008	7847	7900	7781	7893	7850	7869	7919	8342	8308	8302	8323		245	185	1031
JR2	9055	9013	9052	9116	9042	7881	7936	7815	7929	7886	7905	7955	8378	8344	8338	8359	245		202	1026
Vdp4	8993	8951	8990	9054	8980	7819	7872	7749	7865	7822	7841	7891	8314	8280	8274	8295	185	202		990
VdLs17	9840	9803	9841	9905	9830	8669	8723	8601	8714	8673	8693	8742	9167	9134	9127	9145	1031	1026	990	

Figure S5.1. Comparative analysis of nucleic acid differences between isolates from an alignment of consensus sequences of reads mapped to the VdLs17 contig NW_009276921.1 (2.51 Mb). Gaps and ambiguous sequences were removed.

	CA70	GFCB5	Le1811	FL9b	JL5c	FL10b	NC86	GFCa2	Ca36	Vd141	TO22	Le1087	FF5a	KJ14a	NC85	FL7a	HoMCF	JR2	Vdp4	VdLs17
CA70		99.99	99.989	99.987	99.989	99.885	99.883	99.886	99.883	99.885	99.884	99.882	99.91	99.912	99.912	99.911	99.635	99.633	99.636	99.602
GFCB5	99.99		99.992	99.988	99.99	99.886	99.884	99.887	99.884	99.886	99.885	99.883	99.912	99.913	99.913	99.913	99.636	99.635	99.638	99.603
Le1811	99.989	99.992		99.987	99.989	99.885	99.883	99.886	99.883	99.885	99.884	99.882	99.911	99.912	99.912	99.911	99.635	99.634	99.636	99.602
FL9b	99.987	99.988	99.987		99.987	99.882	99.88	99.883	99.88	99.882	99.881	99.879	99.908	99.909	99.909	99.909	99.632	99.631	99.633	99.599
JL5c	99.989	99.99	99.989	99.987		99.885	99.883	99.886	99.883	99.885	99.884	99.882	99.911	99.912	99.912	99.912	99.635	99.634	99.636	99.602
FL10b	99.885	99.886	99.885	99.882	99.885		99.992	99.994	99.992	99.994	99.993	99.991	99.918	99.92	99.92	99.919	99.682	99.681	99.683	99.649
NC86	99.883	99.884	99.883	99.88	99.883	99.992		99.992	99.99	99.992	99.991	99.989	99.916	99.917	99.918	99.917	99.68	99.679	99.681	99.647
GFCa2	99.886	99.887	99.886	99.883	99.886	99.994	99.992		99.993	99.996	99.994	99.993	99.919	99.921	99.921	99.92	99.685	99.684	99.686	99.652
Ca36	99.883	99.884	99.883	99.88	99.883	99.992	99.99	99.993		99.993	99.992	99.99	99.916	99.918	99.918	99.917	99.68	99.679	99.682	99.647
Vd141	99.885	99.886	99.885	99.882	99.885	99.994	99.992	99.996	99.993		99.994	99.993	99.918	99.92	99.92	99.919	99.682	99.681	99.683	99.649
TO22	99.884	99.885	99.884	99.881	99.884	99.993	99.991	99.994	99.992	99.994		99.991	99.917	99.919	99.919	99.918	99.681	99.68	99.683	99.648
Le1087	99.882	99.883	99.882	99.879	99.882	99.991	99.989	99.993	99.99	99.993	99.991		99.916	99.917	99.917	99.917	99.679	99.678	99.681	99.646
FF5a	99.91	99.912	99.911	99.908	99.911	99.918	99.916	99.919	99.916	99.918	99.917	99.916		99.996	99.995	99.995	99.662	99.661	99.663	99.629
KJ14a	99.912	99.913	99.912	99.909	99.912	99.92	99.917	99.921	99.918	99.92	99.919	99.917	99.996		99.997	99.996	99.664	99.662	99.665	99.63
NC85	99.912	99.913	99.912	99.909	99.912	99.92	99.918	99.921	99.918	99.92	99.919	99.917	99.995	99.997		99.996	99.664	99.662	99.665	99.63
FL7a	99.911	99.913	99.911	99.909	99.912	99.919	99.917	99.92	99.917	99.919	99.918	99.917	99.995	99.996	99.996		99.663	99.662	99.664	99.63
HoMCF	99.635	99.636	99.635	99.632	99.635	99.682	99.68	99.685	99.68	99.682	99.681	99.679	99.662	99.664	99.664	99.663		99.99	99.993	99.958
JR2	99.633	99.635	99.634	99.631	99.634	99.681	99.679	99.684	99.679	99.681	99.68	99.678	99.661	99.662	99.662	99.662	99.99		99.992	99.958
Vdp4	99.636	99.638	99.636	99.633	99.636	99.683	99.681	99.686	99.682	99.683	99.683	99.681	99.663	99.665	99.665	99.664	99.993	99.992		99.96
VdLs17	99.602	99.603	99.602	99.599	99.602	99.649	99.647	99.652	99.647	99.649	99.648	99.646	99.629	99.63	99.63	99.63	99.958	99.958	99.96	

Figure S5.2. Comparison of nucleic acid differences (%) between isolates from an alignment of consensus sequences of reads mapped to the VdLs17 contig NW_009276921.1 (2.51 Mb). Gaps and ambiguous sequences were removed.

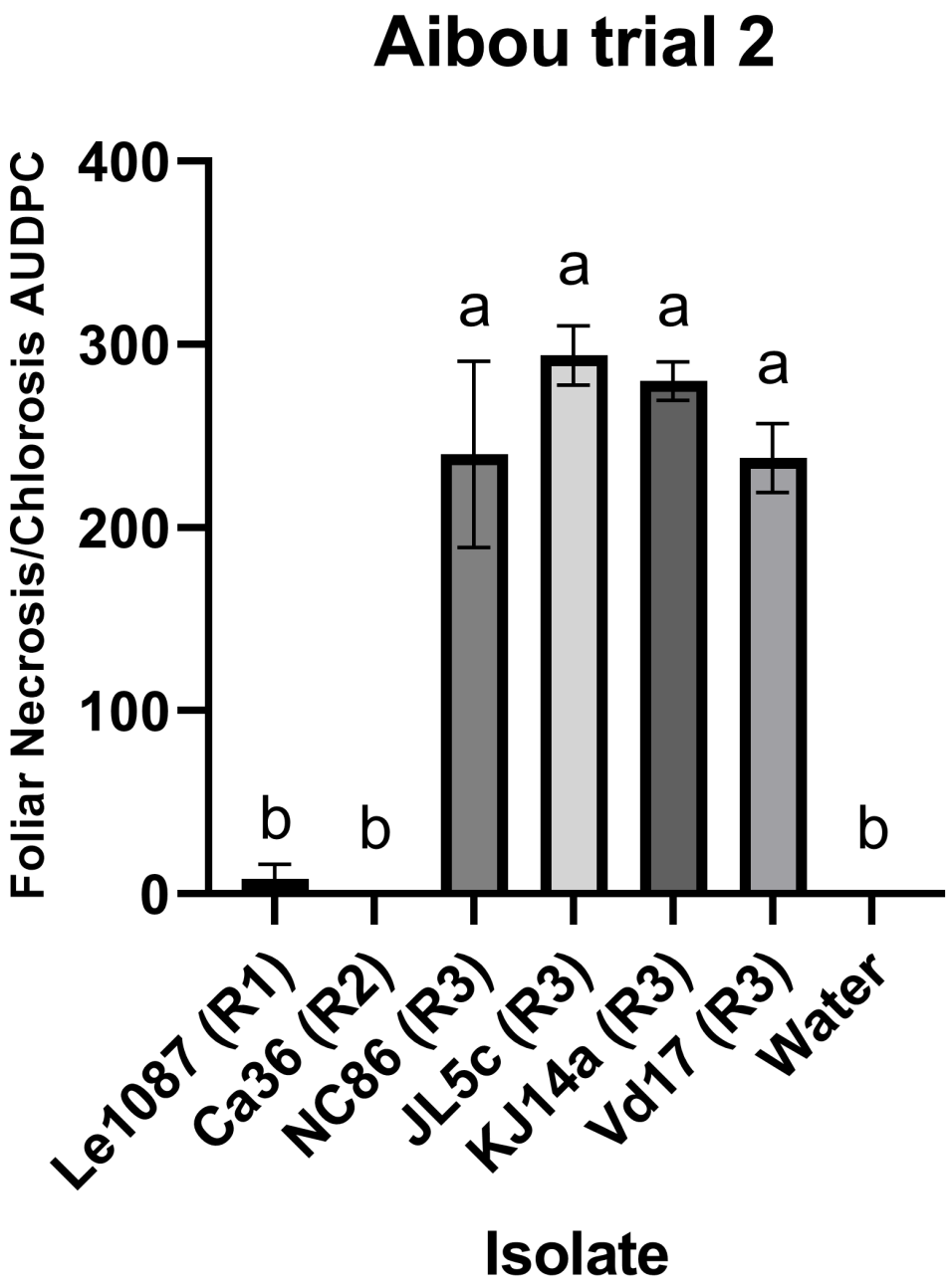

Figure S5.3: 'Aibou' seedlings inoculated with race 1, 2, and 3 and rated for *V. dahliae* related foliar chlorosis and necrosis symptoms for 45 days.

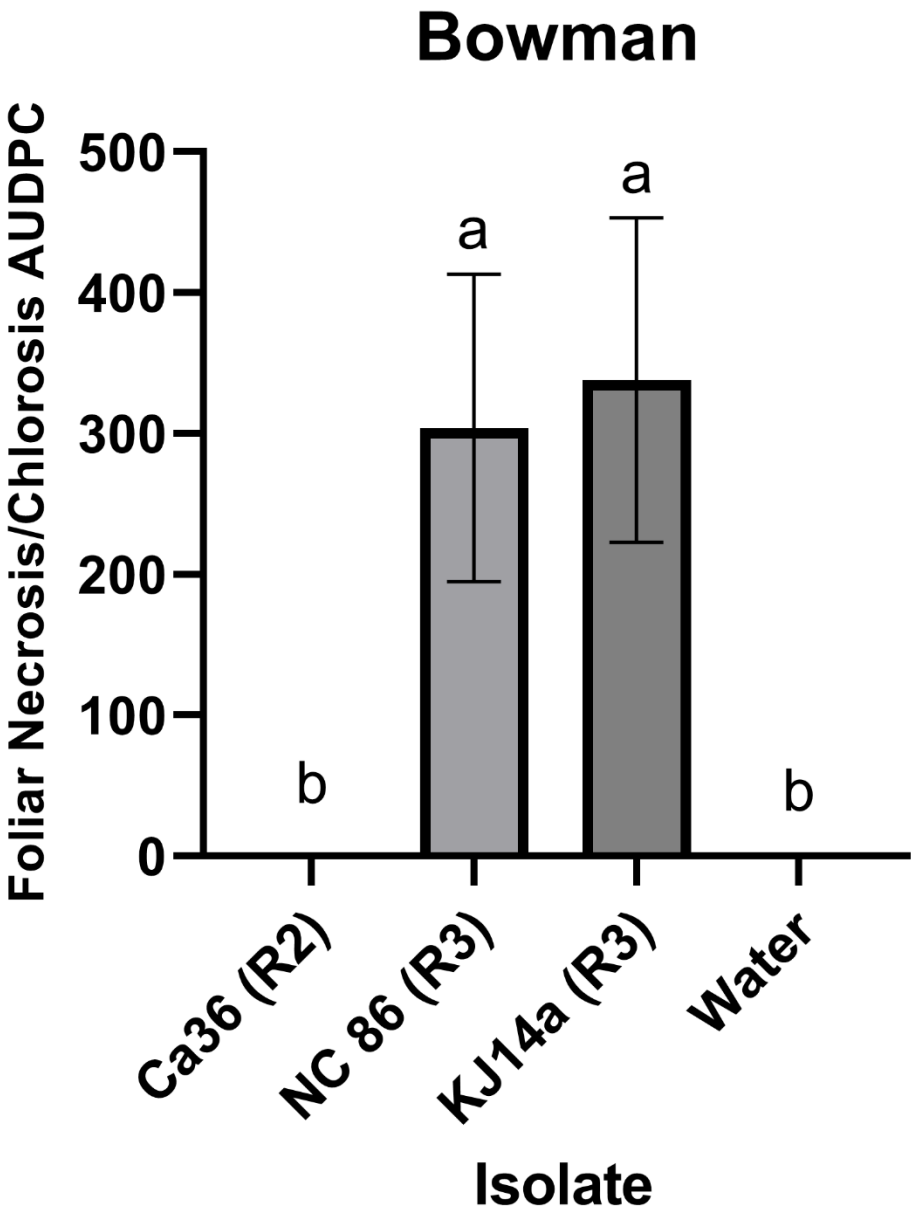

Figure S5.4: 'Bowman' seedlings inoculated with race 1, 2, and 3 and rated for *V. dahliae* related foliar chlorosis and necrosis symptoms for 45 days.

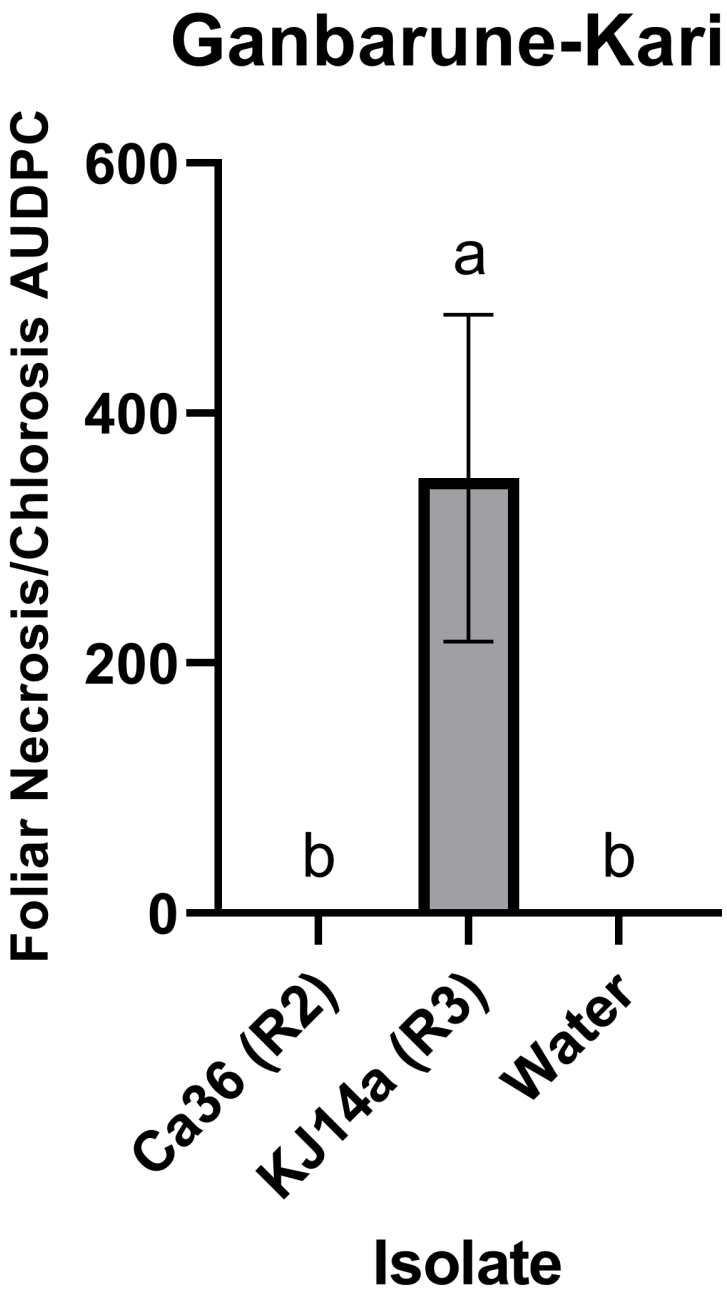

Figure S5.5: 'Ganbarune-Karis' seedlings inoculated with race 1, 2, and 3 and rated for *V. dahliae* related foliar chlorosis and necrosis symptoms for 45 days.

Figure S5.6: ‘Bowman’ seedling 45 days after having its roots dipped in water.

Figure S5.7: ‘Bowman’ seedling 45 days after having its roots dipped in a spore suspension prepared from the Ca36 (race 2) isolate.

Figure S5.8: ‘Bowman’ seedling 45 days after having its roots dipped in a spore suspension prepared from the KJ14a (race 3) isolate.

Figure S5.9: ‘Bowman’ seedling 45 days after having its roots dipped in a spore suspension prepared from the NC86 (race 3) isolate.

Table S5.1: Total, assembled, and unassembled paired reads to the VdLs17 reference genome.

				Assembled Reads		Unassembled Reads	
Isolate	Group[t]	Paired Reads[u]	Coverage[v]	ANo.[w]	A%[x]	UNo.[y]	U%[z]
HoMCF	1	1161501	22x	1108494	95.4%	53007	4.6%
Vdp4	1	86689970	813x	83022801	95.8%	3667169	4.2%
CA70	2	1046827	20x	996272	95.2%	50555	4.8%
FL9b	2	1171506	22x	1104617	94.3%	66889	5.7%
GFCB5	2	1155626	22x	1098622	95.1%	57004	4.9%
Le1811	2	1203789	23x	1146068	95.2%	57721	4.8%
JL5c	2	1198065	22x	1144093	95.5%	53972	4.5%
FL7a	3	1132651	21x	1076825	95.1%	55826	4.9%
NC85	3	1272050	24x	1219284	95.9%	52766	4.1%
FF5a	3	1238753	23x	1185698	95.7%	53055	4.3%
KJ14a	3	1329386	25x	1262600	95.0%	66786	5.0%
NC86	4	1068019	20x	1012741	94.8%	55278	5.2%
FL10b	4	1137452	21x	1077263	94.7%	60189	5.3%
CA36	4	1081891	20x	1031070	95.3%	50821	4.7%
GFCa2	4	26130444	245x	24942431	95.5%	1188013	4.5%
TO22	4	1056938	20x	1004199	95.0%	52739	5.0%
Vd141	4	1128839	21x	1073598	95.1%	55241	4.9%
Le1087	4	1130435	21x	1075008	95.1%	55427	4.9%

[t]Phylogenetic group

[u]Total paired reads from sequenced genome DNA of specified isolate

[v]Mean coverage of reads assembled to the VdLs17 reference genome

[w]Number of paired reads assembled to the VdLs17 reference genome

[x]Percent of paired reads assembled to the VdLs17 reference genome

[y]Assembled reads and percent of reads that were not assembled to the VdLs17

[z]Percent of paired read that were not assembled to the VdLs17 reference genome

Table S5.2: Total number of secreted coding sequence regions and secreted effectors present on the consensus sequence of reads mapped to the VdLs17 reference genome.

Isolate	Group[w]	Coding Sequences[x]	Secreted[y]	Effectors[z]
VdLs17	1	10535	1086	181
HoMCF	1	10138	1062	169
Vdp4	1	10288	1068	170
CA70	2	10149	1054	169
FL9b	2	10146	1059	170
GFCB5	2	10148	1061	176
Le1811	2	10153	1057	173
JL5c	2	10108	1056	174
FL7a	3	10082	1059	174
NC85	3	10089	1058	172
FF5a	3	10094	1057	170
KJ14a	3	10088	1055	171
NC86	4	10134	1054	171
FL10b	4	10142	1051	172
CA36	4	10118	1053	172
GFCa2	4	10151	1058	173
TO22	4	10126	1052	173
Vd141	4	10109	1048	175
Le1087	4	10137	1054	171

[w]Phylogenetic grouping
[x]Coding sequences extracted from consensus sequences of reads mapped to the VdLs17 reference genome
[y]Total secreted genes extracted from consensus sequences of reads mapped to the VdLs17 reference genome
[z]Total secreted effectors extracted from consensus sequences of reads mapped to the VdLs17 reference genome

Table S5.3: Total, assembled, and unassembled paired reads to the JR2 reference genome.

				Assembled Reads		Unassembled Reads	
Isolate	Group[t]	Paired Reads[u]	Coverage[v]	ANo.[w]	A%[x]	UNo.[y]	U%[z]
HoMCF	1	1161501	22x	1123919	96.76%	37582	3.24%
Vdp4	1	8.7E+07	813x	85204180	98.29%	1485790	1.71%
CA70	2	1046827	20x	985540	94.15%	61287	5.85%
FL9b	2	1171506	22x	1110799	94.82%	60707	5.18%
GFCB5	2	1155626	22x	1135977	98.30%	19649	1.70%
Le1811	2	1203789	23x	1152209	95.72%	51580	4.28%
JL5c	2	1198065	22x	1129963	94.32%	68102	5.68%
FL7a	3	1132651	21x	1078251	95.20%	54400	4.80%
NC85	3	1272050	24x	1205702	94.78%	66348	5.22%
FF5a	3	1238753	23x	1157925	93.48%	80828	6.52%
KJ14a	3	1329386	25x	1255407	94.44%	73979	5.56%
NC86	4	1068019	20x	996071	93.26%	71948	6.74%
FL10b	4	1137452	21x	1056118	92.85%	81334	7.15%
CA36	4	1081891	20x	984557	91.00%	97334	9.00%
GFCa2	4	8627278	167x	8505728	98.59%	121550	1.41%
TO22	4	1056938	20x	1024208	96.90%	32730	3.10%
Vd141	4	1128839	21x	1099519	97.40%	29320	2.60%
Le1087	4	1130435	21x	1064004	94.12%	66431	5.88%

[t]Phylogenetic group

[u]Total paired reads from sequenced genome DNA of specified isolate

[v]Mean coverage of reads assembled to the JR2 reference genome

[w]Number of paired reads assembled to the JR2 reference genome

[x]Percent of paired reads assembled to the JR2 reference genome

[y]Assembled reads and percent of reads that were not assembled to the JR2

[z]Percent of paired read that were not assembled to the JR2 reference genome

Table S5.4: Total number of secreted coding sequence regions and secreted effectors present on the consensus sequence of reads mapped to the JR2 reference genome.

Isolate	Group[w]	Coding Sequences[x]	Secreted[y]	Effectors[z]
JR2	1	11404	1055	193
HoMCF	1	10945	1028	187
Vdp4	1	11233	1052	191
CA70	2	11035	1033	183
FL9b	2	11034	1036	184
GFCB5	2	11025	1036	184
Le1811	2	11033	1034	183
JL5c	2	10979	1030	184
FL7a	3	10948	1029	189
NC85	3	10965	1032	183
FF5a	3	10960	1029	184
KJ14a	3	10952	1029	182
NC86	4	10989	1026	187
FL10b	4	11008	1032	187
CA36	4	11005	1030	184
GFCa2	4	11034	1036	185
TO22	4	11014	1033	185
Vd141	4	10896	1025	184
Le1087	4	11049	1030	186

[w]Phylogenetic grouping

[x]Coding sequences extracted from consensus sequences of reads mapped to the JR2 reference genome

[y]Total secreted genes extracted from consensus sequences of reads mapped to the JR2 reference genome

[z]Total secreted effectors extracted from consensus sequences of reads mapped to the JR2 reference genome

Table S5.5: Numbers of secreted effectors from 19 genomes found on consensus sequences of reads mapped to VdLs17 contigs.

			Secreted Effectors			
Chromosome	Contig	Length (bp)	Total	Core no NSM[x]	Core w/ NSM[y]	Variable[z]
1	NW_009276916.1	2529034	13	8	2	3
1	NW_009276917.1	1482790	7	3	2	2
1	NW_009276918.1	624446	1	1	0	0
1	NW_009276919.1	371638	2	0	2	0
2	NW_009276921.1	2667998	8	3	3	2
2	NW_009276922.1	1129899	5	1	1	3
2	NW_009276923.1	824884	5	3	2	0
2	NW_009276924.1	641803	4	0	1	3
3	NW_009276925.1	2001273	15	6	4	5
3	NW_009276926.1	1747785	13	5	3	5
3	NW_009276927.1	1352351	10	4	6	0
3	NW_009276928.1	433381	2	0	2	0
4	NW_009276929.1	1273651	7	4	2	1
4	NW_009276930.1	1106702	8	4	2	2
4	NW_009276931.1	557237	2	0	0	2
4	NW_009276932.1	532607	6	1	4	1
5	NW_009276934.1	938973	6	2	4	0
5	NW_009276935.1	907127	8	0	4	4
5	NW_009276936.1	843700	5	3	1	1
5	NW_009276937.1	559547	1	0	0	1
6	NW_009276938.1	1801442	16	6	5	5
6	NW_009276939.1	736048	9	5	0	4
7	NW_009276940.1	2207176	14	4	4	6
7	NW_009276941.1	499255	5	5	0	0
7	NW_009276942.1	441290	2	1	1	0
8	NW_009276943.1	1018472	5	2	0	3
8	NW_009276944.1	1020271	13	6	5	2
8	NW_009276945.1	946171	2	0	2	0
Unknown	NW_009276953.1	15454	2	0	0	2
Unknown	NW_009276956.1	62837	1	1	0	0
Unknown	NW_009276958.1	90006	1	0	1	0
Unknown	NW_009276963.1	127187	1	1	0	0
Unknown	NW_009276967.1	643454	2	1	1	0
		Totals:	201	80	64	57

[x]Secreted effectors found in 1-18 genomes

[y]Secreted effectors found in all 19 genomes with at least 1 non-synonymous mutation

[z]Secreted effectors found in all 19 genomes with no non-synonymous mutations

Table S5.6: Numbers of secreted effectors from 18 genomes found on consensus sequences of reads mapped to JR2 reference genome and from the JR2 genome.

			Secreted Effectors			
Chr.	Length (bp)	Bp per effector	Total	Core no NSM[x]	Core w/ NSM[y]	Variable[z]
1	9275483	193239	48	17	21	10
2	4277765	178240	24	9	6	9
3	4168633	208432	20	6	5	9
4	4086908	151367	27	6	14	7
5	4171808	173825	24	6	12	6
6	3530890	130774	27	12	7	8
7	3277570	148980	22	11	9	2
8	3361230	112041	30	14	9	7
		Totals:	222	81	83	58

[x]Secreted effectors found in only 1-18 genomes

[y]Secreted effectors found in all 19 genomes with at least 1 non-synonymous mutation

[z]Secreted effectors found in all 19 genomes with no non-synonymous mutations