Optimizing the Effectiveness of Induced Resistance in Tomato for Bacterial Disease Management

by

Cheryl Trueman

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Doctor of Philosophy
in
Environmental Biology

Guelph, Ontario, Canada

© Cheryl Trueman, December, 2017
ABSTRACT

OPTIMIZING THE EFFECTIVENESS OF INDUCED RESISTANCE IN TOMATO FOR BACTERIAL DISEASE MANAGEMENT

Cheryl Lynn Trueman       Advisor:  
University of Guelph, 2017      Professor P. H. Goodwin

As alternatives for managing bacterial speck (Pseudomonas syringae pv. tomato (Pst)) and bacterial spot (Xanthomonas gardneri (Xg)) of tomato (Solanum lycopersicum), the synthetic chemical defense activator, acibenzolar-s-methyl (ASM) combined with the synthetic plant growth regulator (PGR) uniconazole (UNI), the natural chemical defense activator, para-aminobenzoic acid (PABA), and the putative biological defense activator, B. mycoides/weihenstephanensis R17, were examined. No consistent benefits for bacterial spot and speck control or tomato growth were observed in field experiments from 2011-2013 with the combination of ASM and UNI, whether applied to seedlings (<6 weeks old) or post-transplanting. However, greenhouse applied ASM or ASM+UNI reduced late season disease severity 18 to 24% compared to nontreated and CuOH -treated controls for cv. TSH4 in 2012, indicating that ASM applied to tomato seedlings can have long-term benefits under certain conditions. Fitness costs in terms of reduced growth are a concern with ASM, so UNI was added to ASM to determine if that could be ameliorated, but this approach was ineffective. Greenhouse ASM applications to cv. H9909 in 2012 reduced total yield by 20% compared to the nontreated control, indicating a fitness cost, and ASM+UNI treated plants showed a similar loss. Fitness costs may have occurred in 2012 due to stress from dry conditions after transplanting. For PABA, effectiveness was affected by application method, concentration and host genotype. Despite optimizing PABA efficacy under controlled conditions, PABA was ineffective in the field. A bacterial endophyte, R17, was isolated from Solanum arcanum and its ability to putatively induce resistance against Pst under controlled conditions was affected by its concentration, application method, and host genotype. PABA and R17 reduced bacterial speck lesion incidence up to 43 and 51%, respectively, but the lesions that developed were larger in treated than nontreated plants resulting in no reduction in Pst population or total symptomatic leaf area. This suggests that certain defense activators can reduce the ability of Pst to infect but then allow for greater Pst population growth post-infection. While defense activators have potential, they need to be more effective and consistent before they are integrated into bacterial disease management strategies of tomato.
DEDICATION

For Justin, you believed in me, and Alexis, you helped me believe in myself. Also for Dad, who told me to find a summer job in Ontario, and Mom for all those kilometres you drove with me and for me.
ACKNOWLEDGEMENTS

Thank you to my advisor, Dr. Paul Goodwin, and the members of my advisory committee, Dr. Kari Dunfield, Dr. Tom Hsiang, Dr. Annette Nassuth, and Dr. Istvan Rajcan for their guidance and advice during this journey. Your time and patience is truly appreciated.

This research was completed at the Ridgetown Campus, University of Guelph, and there are many people who contributed time and effort toward its completion. These people include the Field Vegetable Pest Management Technician, Phyllis May, former co-op students Sherri Tedford and Tina Simonton, and many summer students and research assistants. Thank you for your dedication to the program.

I would also like to thank the many colleagues at the Ridgetown Campus who provided advice and support during this journey including Janice LeBoeuf, Steve Loewen, Ken McEwan, Elaine Roddy, Dr. Darren Robinson, Dr. Art Schaafsma, and Dr. Laura Van Eerd. I would also like to thank Dr. Diane Cuppels, with whom I had a valuable conversation about bacterial disease in tomato after her retirement from Agriculture and Agri-Food Canada in London.

To my partner in life, Justin Kritikos, thank you for being my soft place to fall. Your patience and encouragement did not go unnoticed. Thank you for embracing what it means to be an equal partner in life.

Funding for this research was provided in part by the Ontario Tomato Research Institute, Syngenta Canada, Valent Canada, Ridgetown Campus – University of Guelph, and the Ontario Ministry and Food and Rural Affairs.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. ii

DEDICATION ............................................................................................................................................. iii

ACKNOWLEDGEMENTS ........................................................................................................................ iv

TABLE OF CONTENTS .............................................................................................................................. v

LIST OF TABLES ........................................................................................................................................ x

LIST OF FIGURES .................................................................................................................................... xv

LIST OF ABBREVIATIONS ..................................................................................................................... xxi

Chapter 1: Literature Review ........................................................................................................................ 1

1.1 Introduction ................................................................................................................................... 1

1.2 Processing tomatoes in Ontario ..................................................................................................... 1

1.3 Tomato genetics ............................................................................................................................ 3

1.4 Bacterial endophytes of tomato ..................................................................................................... 3

1.5 Bacterial speck pathogen of tomato .............................................................................................. 4

1.6 Bacterial spot pathogen of tomato ................................................................................................ 9

1.7 Current bacterial speck and spot management practices in tomato ............................................ 13

1.8 SIR and its use in bacterial disease management ........................................................................ 15

1.8.1 SAR ..................................................................................................................................... 15

1.8.1.1 Mechanisms of SAR ................................................................................................... 15

1.8.1.2 Biological activators of SAR ................................................................................... 17

1.8.1.3 PAMP activators of SAR ........................................................................................ 18

1.8.1.4 Plant activators of SAR ........................................................................................... 18

1.8.1.5 Synthetic activators of SAR .................................................................................... 19

1.8.1.6 Plant fitness costs associated with SAR ................................................................... 20

1.8.1.7 Variation in SAR response among cultivars ........................................................... 22

1.8.1.8 SAR in disease management ................................................................................. 23

1.8.1.9 SAR in management of bacterial speck and spot of tomato ................................... 24

1.8.2 ISR ...................................................................................................................................... 26

1.8.2.1 Mechanisms of ISR ................................................................................................. 26

1.8.2.2 Biological activators of ISR .................................................................................. 28

1.8.2.3 PAMP activators of ISR ...................................................................................... 30
1.8.2.4 Synthetic activators of ISR ................................................................. 31
1.8.2.5 Plant growth effects associated with ISR ........................................... 31
1.8.2.6 Factors affecting ISR ........................................................................... 33
1.8.2.7 ISR as a disease management tool .......................................................... 35
1.8.2.8 ISR as a disease management tool in tomato ........................................... 36
1.8.3 Interactions and relationships between SAR and ISR pathways ............... 38
1.9 GA-related PGRs ......................................................................................... 39
1.10 Hypothesis ............................................................................................... 40
1.11 Objectives ................................................................................................. 40

Chapter 2: Effects of para-aminobenzoic acid on the incidence of bacterial speck disease, *P. syringae* pv. *tomato* growth, and plant growth in processing tomato ......................................................... 41

2.1 Introduction .................................................................................................. 41
2.2 Materials & Methods ................................................................................. 42
2.2.1 Direct antimicrobial effects of PABA on *Pst* ........................................... 42
2.2.2 Growth room evaluation of foliar applications of PABA against bacterial speck .......... 43
2.2.2.1 Optimal concentration .......................................................................... 43
2.2.2.2 Host genotype effect, foliar PABA application number, foliar PABA response
duration and soil PABA application ..................................................................... 44
2.2.2.3 *Pst* populations in tomato leaves .......................................................... 44
2.2.2.4 *Pst* lesion sizes in tomato leaves ......................................................... 45
2.2.3 Field evaluation of foliar applications of PABA ........................................ 45
2.2.4 Statistical analysis .................................................................................... 46
2.3 Results ........................................................................................................ 46
2.3.1 PABA direct antimicrobial effect .............................................................. 46
2.3.2 PABA soil drench application ................................................................. 47
2.3.3 PABA foliar application ........................................................................... 47
2.3.4 Effect of plant genotype on PABA response ............................................. 48
2.3.5 PABA applications ................................................................................. 48
2.3.6 PABA response duration ........................................................................... 49
2.3.7 PABA effect on *Pst* populations and lesions ........................................ 49
2.3.8 Field evaluation ....................................................................................... 50
2.4 Discussion .................................................................................................. 71
2.4.1 Direct antimicrobial effects of PABA ....................................................... 71


<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.2</td>
<td>PABA concentrations and application methods</td>
<td>71</td>
</tr>
<tr>
<td>2.4.3</td>
<td>Host genotype effect</td>
<td>73</td>
</tr>
<tr>
<td>2.4.4</td>
<td>Parameters affecting protection and the duration of protection by PABA</td>
<td>74</td>
</tr>
<tr>
<td>2.4.5</td>
<td>Effect of PABA on bacterial speck lesion incidence versus <em>Pst</em> population</td>
<td>76</td>
</tr>
<tr>
<td>2.4.6</td>
<td>Effects of PABA on bacterial speck incidence in the field</td>
<td>78</td>
</tr>
<tr>
<td>2.4.7</td>
<td>Effects of PABA on plant growth and development</td>
<td>79</td>
</tr>
<tr>
<td>2.4.8</td>
<td>Conclusions</td>
<td>80</td>
</tr>
</tbody>
</table>

Chapter 3: Effects of the plant growth regulator uniconazole with the plant defense activator acibenzolar-S-methyl on incidence and severity of bacterial speck (*P. syringae* pv. *tomato*) and bacterial spot (*X. gardneri*) in tomato

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Introduction</td>
<td>81</td>
</tr>
<tr>
<td>3.2</td>
<td>Materials &amp; Methods</td>
<td>83</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Weather conditions</td>
<td>83</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Greenhouse treatment with UNI followed by field treatment with ASM</td>
<td>84</td>
</tr>
<tr>
<td>3.2.2.1</td>
<td>Experimental design and treatments</td>
<td>84</td>
</tr>
<tr>
<td>3.2.2.2</td>
<td>Pathogen inoculations</td>
<td>84</td>
</tr>
<tr>
<td>3.2.2.3</td>
<td>Disease and plant growth assessments</td>
<td>85</td>
</tr>
<tr>
<td>3.2.2.4</td>
<td>Statistical analysis</td>
<td>86</td>
</tr>
<tr>
<td>3.2.3</td>
<td>Greenhouse treatment with UNI followed by greenhouse treatment with ASM or CuOH</td>
<td>86</td>
</tr>
<tr>
<td>3.2.3.1</td>
<td>Experimental design and treatments</td>
<td>86</td>
</tr>
<tr>
<td>3.2.3.2</td>
<td>Pathogen inoculations</td>
<td>87</td>
</tr>
<tr>
<td>3.2.3.3</td>
<td>Disease and plant growth assessments</td>
<td>87</td>
</tr>
<tr>
<td>3.2.3.4</td>
<td>Statistical analysis</td>
<td>88</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>91</td>
</tr>
<tr>
<td>3.3.1</td>
<td>Weather Conditions</td>
<td>91</td>
</tr>
<tr>
<td>3.3.2</td>
<td>Greenhouse treatment with UNI followed by field treatment with ASM</td>
<td>91</td>
</tr>
<tr>
<td>3.3.2.1</td>
<td>Effect of greenhouse UNI followed by field ASM on bacterial speck and bacterial spot</td>
<td>91</td>
</tr>
<tr>
<td>3.3.2.2</td>
<td>Effect of greenhouse UNI followed by field ASM on tomato growth, yield, and quality</td>
<td>93</td>
</tr>
<tr>
<td>3.3.3</td>
<td>Greenhouse treatment with UNI followed by greenhouse treatment with ASM</td>
<td>93</td>
</tr>
<tr>
<td>3.3.3.1</td>
<td>Effect of greenhouse UNI followed by greenhouse ASM on bacterial speck and bacterial spot</td>
<td>93</td>
</tr>
</tbody>
</table>
3.3.3.2  Effect of greenhouse UNI followed by greenhouse ASM on tomato growth, yield and quality ............................................................................................................................... 95

3.4  Discussion ................................................................. 119

3.4.1  Greenhouse UNI with field ASM application ................................................................. 119
3.4.2  Greenhouse UNI and greenhouse ASM application .......................................................... 121
3.4.3  Greenhouse UNI with greenhouse CuOH application ...................................................... 122
3.4.4  Improvement of ASM effectiveness ................................................................................. 124
3.4.5  Environmental impacts on disease and control measures ................................................. 125
3.4.6  Duration of ASM effectiveness ......................................................................................... 126
3.4.7  Comparison of disease assessment methods ..................................................................... 127
3.4.8  Conclusions ....................................................................................................................... 128

Chapter 4: Use of bacterial endophytes to control the incidence of bacterial speck disease (*Pseudomonas syringae* pv. *tomato*) and alter plant growth in processing tomato seedlings ........................................... 129

4.1  Introduction ............................................................................................................................... 129

4.2  Materials & Methods ................................................................................................................ 131

4.2.1  Isolation of endophytes from tomato and wild tomato species ......................................... 131
4.2.2  Screening of endophytes for induced resistance and plant growth effects ...................... 132
4.2.3  Standard curve of CFU versus A600 ................................................................................ 134
4.2.4  Direct antimicrobial effects of R17 and R21 .................................................................... 134
4.2.5  Optimization of bacterial endophyte treatment ................................................................. 135
4.2.6  Effect of R17 inoculation on populations in planta .......................................................... 136
4.2.7  Effect of R17 inoculation on *Pst* populations and lesion size in planta ......................... 137
4.2.8  Identification of endophytes R17 and R21 ........................................................................ 138
4.2.9  Statistical analysis ............................................................................................................. 139

4.3  Results ....................................................................................................................................... 139

4.3.1  Bacterial endophytes isolated from tomato and wild tomato species ............................... 139
4.3.2  Screening of bacterial endophytes for induced resistance and plant growth effects ......... 139
4.3.3  Direct antimicrobial effects of R17 and R21 .................................................................... 140
4.3.4  Optimization of bacterial endophyte treatment ................................................................. 140
4.3.5  Effect of R17 inoculation on *Pst* populations and lesion size in planta ......................... 142
4.3.6  Effect of R17 inoculation on populations *in planta* ......................................................... 143
4.3.7  Identification of R17 and R21 ........................................................................................... 143

4.4  Discussion .................................................................................................................................. 171
LIST OF TABLES

Table 2.1 Effect of PABA concentration on growth of P. syringae pv. tomato (Pst) in a filter disc assay. Filter discs soaked in 0, 1, 9, 18, and 27 mM para-aminobenzoic acid (PABA) dissolved in water, and 0, 18, and 72 mM PABA dissolved in 70% ethanol were placed on tryptic soy agar covered thoroughly with Pst, incubated at room temperature, and assessed for zones after three days. ................................. 52

Table 2.2 Foliar, root, and total dry weight of six tomato cultivars treated with para-aminobenzoic acid (PABA). Plants were coated with 18 mM PABA 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of P. syringae pv. tomato 20 DAS. Dry weight of plants five days post inoculation is shown. ........................................................................................................ 57

Table 2.3 Foliar, root, and total dry weight of tomato cv. H5108 and cv. TSH33 treated with zero, one, two, or three applications of para-aminobenzoic acid (PABA). One, two, or three foliar PABA treatments (18mM) were applied by coating a fine mist on plants 10, 12, and/or 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of P. syringae pv. tomato five days after the last PABA application. Dry weight of plants five days post inoculation is shown. ................................................................................... 59

Table 2.4 Foliar, root, and total dry weight of tomato cv. H5108 inoculated with P. syringae pv. tomato (Pst) five, seven, or 10 days after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 days after seeding (DAS)). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of Pst five, seven, or 10 days after the second PABA application. Dry weight of plants five days post inoculation is shown. ........................................................................................................... 61

Table 2.5 Incidence of bacterial speck symptoms, population of P. syringae pv. tomato (Pst) per cm², and per lesion on the third youngest terminal leaflet of tomato cv. H5108 after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 days after seeding (DAS)). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of Pst five days after the second PABA application. .................................................................................. 62

Table 2.6 Area under the disease progress curve (AUDPC) for early and late disease in tomato cv. H5108. Early season disease was measured by calculating the percentage of leaves with disease symptoms and late season disease was measured by estimating defoliation in plants treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with P. syringae pv. tomato, Ridgetown, ON, in 2014. .......................... 67

Table 2.7 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. H5108, harvested from plots treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with P. syringae pv. tomato, Ridgetown, ON, 2014. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated. ............................................................................................................ 68

Table 2.8 Total, red, green, and rotten fruit yield in a 2m section of tomato cv. H5108 treated with CuOH and para-aminobenzoic acid (PABA) and inoculated with P. syringae pv. tomato, Ridgetown, ON, in 2014. .................................................................................................................. 70

Table 3.1 Treatment descriptions and application timings for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with P. syringae pv. tomato and X. gardneri, Ridgetown, ON, 2011-2013. Further details on applications methods are described in the text. .................................................................................................................. 89

Table 3.2 Treatment descriptions and application timings for tomato cv. TSH4 and H9909 treated with uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with P. syringae pv. tomato and X. gardneri, Ridgetown, ON, 2011-2013. Further details on applications methods are described in the text. ........................................................................................................ 90
Table 3.3 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated. ........................................................... 105

Table 3.4 Total, red, green, and rotten fruit yield in a 2m section of tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. ........................................................ 107

Table 3.5 Soluble solids, Agtron colour readings, and juice pH of ripe tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was processed. .......................................................... 108

Table 3.6 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated. ..................................... 113

Table 3.7 Stem diameter and foliar and root dry weight of tomato seedlings, cvs. TSH4 and H9909, treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse, Ridgetown, ON, 2011-2013. ....................................................................................................................................... 115

Table 3.8 Soluble solids, Agtron colour readings, and juice pH of ripe tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2012. A subsample of 50 red fruit harvested in a 2m section of each plot was processed. .................................... 118

Table 4.1 Source and colony description of endophytes isolated from roots of *S. lycopersici*, *S. arcanum*, *S. chmielewskii*, *S. cheesmaniae*, and *N. benthamiana* after growth on tryptic soy agar at room temperature (~22°C) for two days. Endophytes were isolated from macerated roots of plants growing outdoors in the field at Ridgetown Campus, in media containing 40 to 50% pasteurized field soil mixed with sand under controlled conditions (Ridgetown ‘R’ isolates), or in a 1:1 mixture of pasteurized soil from the Guelph Turfgrass Institute (Guelph, ON) and potting mix (Guelph ‘G’ isolates)...................... 145

Table 4.2 Disease incidence of bacterial speck on non-fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes listed in Table 4.1, Serenade Max (*B. subtilis* QST713) or Promix PGX (*B. subtilis* MBI600). Endophytes were inoculated following the Valenzuela-Soto et al. (2010) method using a solution of each endophyte with A600=1.000 then diluted 1:10 in 10 mM MgCl₂, except for MBI600 which was included in the potting mix (1 x 10⁷ CFU/mL) and QST713 which was applied at 1 x 10⁶ CFU/mL. Plants receiving 10 mM MgCl₂ were used as a control. Plants were inoculated with *P. syringae* pv. *tomato* at 20 days after seeding, and disease incidence assessed at seven days post inoculation. Disease was assessed on all leaflets of each plant. .......................................................................................................................... 145

Table 4.3 Growth parameters (relative chlorophyll, plant height, and root and foliage weight) of non-fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes listed in Table 4.1, Serenade Max (*B. subtilis* QST713) or Promix PGX (*B. subtilis* MBI600). Endophytes were inoculated following the Valenzuela-Soto et al. (2010) method using a solution of each endophyte with A600=1.000 that was diluted 1:10 in 10 mM MgCl₂, except for MBI600 which was included in the potting mix (1 x 10⁷ CFU/mL) and QST713 which was applied at 1 x 10⁶ CFU/mL. Plants receiving 10 mM MgCl₂ were used as a control. Plants were assessed at 27 days after seeding on the same day as assessment of disease incidence. .......................................................................................................................... 148

Table 4.4 Disease incidence of bacterial speck on non-fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes R9, R17, R19, R20 and R21. Endophytes were inoculated using the seed
soak + seed drench + seedling drench inoculation method (A600=1.000 then diluted 1:10 in 10 mM MgCl₂). Plants receiving 10 mM MgCl₂ were used as a control. Plants were inoculated with Pst at 20 days after seeding, and disease incidence assessed at seven days post inoculation on all leaflets of each plant.

Table 4.5 Growth parameters (relative chlorophyll, plant height, and root and foliage weight) of non-fertilized tomato cv. TSH4 following inoculation with the endophytes R9, R17, R19, R20 and R21. Endophytes were inoculated using the seed soak + seed drench + seedling drench inoculation method (A600=1.000 diluted by a factor of 10 in 10 mM MgCl₂). Plants receiving 10 mM MgCl₂ were used as a control. Plants were assessed at 27 days after seeding on the same day as assessment of disease incidence.

Table 4.6 In vitro inhibition of P. syringae pv. tomato (Pst) strain DC06T2-4 by co-incubation with endophytes R17 and R21 using a cross streaking assay. Each endophyte was streaked in a single line across the centre of a plate of tryptic soy agar and incubated for two days. Pst was then streaked in a straight line from the edge of the plate to the endophyte streak at a 90° angle. Plates were assessed two days after streaking Pst.

Table 4.7 In vitro inhibition of P. syringae pv. tomato (Pst) strain DC06T2-4 by co-incubation with endophytes R17 and R21 using an agar overlay assay. The overlay was tryptic soy agar (TSA) (1.50% agar) (50°C) that was spiked with 0.1 mL of 2 x 10⁷ CFU/mL Pst. This was poured over a 2-cm diameter circle of endophyte that had grown for 48 hours on a hard TSA agar base (1.50% agar), incubated for 48 hours, and assessed for the presence of a zone of inhibition.

Table 4.8 Disease incidence of bacterial speck and growth parameters (relative chlorophyll, plant height, and root and foliage weight) of fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes R17 and R21. Plants were fertilized 10, 15 and 21 days after seeding (DAS) with 20 (three experiments) or 80 mL (one experiment) of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Endophytes were inoculated using the seed soak + seed drench + seedling drench inoculation method at concentrations of 1 x 10⁷ CFU/ml (R17) or 1 x 10⁸ CFU/ml (R21). Plants receiving 10 mM MgCl₂ were used as a control. Plants were inoculated with P. syringae pv. tomato (Pst) 20 DAS. The incidence of bacterial speck lesions on all leaflets of the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with Pst.

Table 4.9 The effect of seed soak, seed drench and/or seedling drench application methods of R17 and R21 on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were inoculated with the seed soak, seed drench, seedling drench, seed soak + seed drench, seed soak + seed drench + seedling drench methods using 1 x 10⁷ CFU/ml (R17) or 1 x 10⁸ CFU/ml (R21) in 10 mM MgCl₂. Plants receiving 10 mM MgCl₂ were used as a control. Plants were fertilized 10, 15 and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Plants were inoculated with P. syringae pv. tomato (Pst) 20 DAS. The incidence of bacterial speck lesions on all leaflets of the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with Pst.

Table 4.10 The effect of raw and pelleted seed with seed soak and/or seed drench application methods of R17 treatment on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were inoculated using the seed soak, seed drench, or seed soak + seed drench method using 1 x 10⁷ CFU/ml in 10 mM MgCl₂. Plants receiving 10 mM MgCl₂ were used as a control. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Plants were inoculated with P. syringae pv. tomato (Pst) at 20 DAS. The incidence of bacterial speck lesions on all leaflets of the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with Pst.
Table 4.11 Effect of different doses of R17 as a seed drench treatment on the incidence of bacterial speck disease incidence and growth parameters (relative chlorophyll, plant height, foliar dry weight, and root dry weight) of fertilized tomato cv. TSH4. R17 was applied as a seed drench at zero days after seeding (DAS) at 0, 1x10^5, 1x10^6, 1x10^7, 1x10^8 or 1x10^9 cfu/mL in 10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. Plants were fertilized 10, 15 and 21 DAS with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. Plants were inoculated with \textit{P. syringae} pv. \textit{tomato} at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves.

Table 4.12 The effect of R17 seed drench treatment on the incidence of bacterial speck lesions, \textit{P. syringae} pv. \textit{tomato} (\textit{Pst}) population (LOG CFU) per cm^2 and \textit{Pst} population (LOG CFU) per lesion on terminal leaflets of the third youngest leaves of fertilized tomato cv. TSH4. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. R17 was applied as a seed drench at 0 DAS at 1 x 10^7 CFU/mL in 10 mM MgCl2. Plants were inoculated with \textit{Pst} 20 DAS, and disease incidence assessed at five days post inoculation.

Plants receiving 10 mM MgCl2 were used as a control.

Table 4.13 Comparison of R17 seed drench treatments with wt and rifampicin-resistant strains on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench at 0 DAS at 1 x 10^7 CFU/mL in 10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. Plants were inoculated with \textit{P. syringae} pv. \textit{tomato} (\textit{Pst}) 20 DAS, and disease incidence assessed at five days post inoculation (DPI) on all leaflets of the second and third youngest leaves. The relative chlorophyll content, plant height, dry root weight and foliar dry weight were also determined at five DPI.

Table 4.14 Populations of rifampicin resistant R17 mutant R17-RfpC in leaves, roots and rhizosphere of tomato cv. TSH4. Plants were fertilized at 10, 15 and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17-RfpC was applied as a seed drench at 0 DAS at 1 x 10^7 CFU/mL in 10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. R17-RfpC populations in rhizosphere growing media, roots and cotyledon leaves at 10 DAS or the rhizosphere growing media, roots and terminal leaflet on the second and third youngest leaves at 25 DAS. Samples were from 10 (10 DAS) or five (25 DAS) plants per replicate.

Table A.1 Effect of different para-aminobenzoic acid (PABA) concentrations and application volumes on the relative chlorophyll content in tomato cv. H9909 inoculated with \textit{P. syringae} pv. \textit{tomato} (\textit{Pst}). PABA was applied to the root zone with a pipette 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of \textit{Pst} 20 DAS. Relative chlorophyll was measured at five days post inoculation.

Table A.2 Effect of different PABA concentrations on the relative chlorophyll content in tomato cv. H9909 inoculated with \textit{P. syringae} pv. \textit{tomato} (\textit{Pst}). Plants were coated with 0, 0.01, 0.1, 0.5, 1, 4, 9, or 18 mM para-aminobenzoic acid (PABA) 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of \textit{Pst} 20 DAS. Relative chlorophyll was measured at five days post inoculation.

Table A.3 The incidence of bacterial speck and bacterial spot on green tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae} pv. \textit{tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. A subsample of 50 green fruit harvested in a 2m section of each plot was evaluated.

Table A.4 Incidence and severity of anthracnose symptoms on red tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae} pv. \textit{tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. A subsample of 50
red fruit was collected from all red fruit harvested in a 2m section of each plot, stored for three days at room temperature, and then assessed for anthracnose symptoms.

Table A.5 Number of days after transplanting to begin inflorescence, fruit set, and ripening for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae pv. tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. Five plants per plot were monitored at seven to 12 day intervals after transplanting.

Table A.6 The incidence of bacterial speck and bacterial spot on green tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with \textit{P. syringae pv. tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. A subsample of 50 green fruit harvested in a 2m section of each plot was evaluated.

Table A.7 Incidence and severity of anthracnose symptoms on red tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with \textit{P. syringae pv. tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit was collected from all fruit harvested in a 2m section of each plot, stored for three days at room temperature, and then assessed for anthracnose symptoms.

Table A.8 Number of days after transplanting to begin inflorescence, fruit set, and ripening for tomato cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with \textit{P. syringae pv. tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. Five plants per plot were monitored at seven to 12 day intervals after transplanting.

Table A.9 Response of commercial processing tomato cvs. H2401 (all variables) and H9553 (relative chlorophyll) for the effect of endophyte R17 treatment on the incidence of bacterial speck lesions, relative chlorophyll, plant height, dry root weight, and foliar dry weight. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. R17 was applied as a seed drench 0 DAS at 1 x 10^7 CFU/mL in 10 mM MgCl₂. Plants receiving 10 mM MgCl₂ were used as a control. Plants were inoculated with \textit{P. syringae pv. tomato} at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves.
LIST OF FIGURES

**Figure 1.1.** Symptoms of bacterial speck on a) tomato foliage, b) ripe fruit, and c) peeled processing tomato, and bacterial spot on d) tomato foliage, b) ripe fruit, and c) peeled processing tomato. .......... 10

**Figure 2.1** Effect of different para-aminobenzoic acid (PABA) concentrations and application volumes on the systemic acquired resistance response in tomato cv. H9909 inoculated with *P. syringae pv. tomato (Pst)*. A pipette was used to apply 10 ( ), 20 ( ), or 40 ( ) mL PABA to the root zone 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *Pst* 20 DAS. Disease incidence five days post inoculation is shown. Data points with the same letter are not significantly different at P <= 0.05, Tukey’s HSD. NS = no significant difference. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. .................................................................................................................................................. 53

**Figure 2.2** Phytotoxicity symptoms on tomato a) true leaf, and b) and c) cotyledon leaves coated to runoff with 18 mM PABA at 10 and 15 days after seeding. .................................................................................. 54

**Figure 2.3** Effect of different para-aminobenzoic acid (PABA) concentrations on the systemic acquired resistance response in tomato cv. H9909 inoculated with *P. syringae pv. tomato (Pst)*. Plants were coated with 0, 0.01, 0.1, 0.5, 1, 4, 9, or 18 mM PABA (LOG + 1 = 1, 0.0043, 0.0412, 0.1761, 0.3010, 0.6990, 1.000, 1.2787 mM PABA) 10 and 15 days after seeding (DAS), and then plants were coated with a fine mist of 2 x 10^7 CFU/ml of *Pst* 20 DAS. Disease incidence five days post inoculation is shown. Errors bars represent standard error of the mean. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. ........................ 55

**Figure 2.4** Incidence of bacterial speck symptoms on the second and third youngest leaves of six tomato cultivars treated with para-aminobenzoic acid (PABA). Plants were coated with 18 mM PABA 10 and 15 DAS. Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *P. syringae pv. tomato (Pst)* 20 days after seeding (DAS). Disease incidence five days post inoculation is shown for the nontreated control ( ) and PABA ( ). Errors bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at P <= 0.05, Tukey’s HSD. Data from three independent trials for cvs. H2401, H9553, TSH33, and TSH4 with five replications of each treatment, and five independent trials for cv. H5108 and cv. H9909 with five or four replications of each treatment, was pooled together because ANOVA showed no treatment x trial interaction. One outlier was removed each data set for cv. H9909 and cv. H2401. ................................................................................................................................ 56

**Figure 2.5** Incidence of bacterial speck symptoms on the second and third youngest leaves of tomato cv. H5108, and b) cv. TSH33 treated with zero, one (15 days after seeding (DAS)), two (10 and 15 DAS), or three (10, 12, and 15 DAS) applications of para-aminobenzoic acid (PABA). Foliar PABA treatments (18mM) were applied by coating a fine mist on plants. Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *P. syringae pv. tomato* five days after the last PABA application. Disease incidence five days post inoculation is shown. Errors bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at P <= 0.05, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Data for cv. H5108 was log transformed to meet assumptions of ANOVA. ................................................................................................................................. 58

**Figure 2.6** Incidence of bacterial speck symptoms on the second and third youngest leaves of tomato cv. H5108 inoculated with *P. syringae pv. tomato (Pst)* five, seven, or 10 days after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *Pst* five, seven, or 10 days after the second PABA application. Disease incidence five days post inoculation is shown for the nontreated control ( ) and PABA ( ). Errors bars represent standard error
of the mean. Data points with the same letter for the same inoculation timing are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. One outlier was removed from the analysis for the data from seven days after the last PABA application.

**Figure 2.7** The effect foliar applications of para-aminobenzoic acid (PABA) on a) the incidence of bacterial speck symptoms, b) mean lesion size, c) mean lesion circumference, and d) percent leaf area with lesions on the third youngest terminal leaflet of tomato cv. H5108. Foliar PABA treatments (18mM) were applied 10 and 15 days after seeding (DAS) by coating a fine mist on plants. Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *P. syringae pv. tomato* five days after the last PABA application. Disease incidence and mean lesion size of 10 to 15 lesions is shown for the nontreated control (■) and PABA ( ). Error bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from two independent trials with 10 (trial 1) and seven (trial 2) replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

**Figure 2.8** Early season disease progress of bacterial speck symptoms in tomato cv. H5108 treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato, Ridgetown, ON*. The percentage of leaves with disease symptoms in the nontreated control ( ), eight applications of CuOH at 7-day intervals ( ), eight applications of PABA at 7-day intervals ( ), two applications of PABA at 5-day intervals ( ), nine applications of PABA at 5-day intervals ( ), and seedlings soaked in PABA for one hour before transplanting ( ) in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Table 2.6. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference.

**Figure 2.9** Late season disease progress of bacterial speck symptoms in tomato cv. H5108 treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato, Ridgetown, ON*. Defoliation in the nontreated control ( ), eight applications of CuOH at 7-day intervals ( ), eight applications of PABA at 7-day intervals ( ), two applications of PABA at 5-day intervals ( ), nine applications of PABA at 5-day intervals ( ), and seedlings soaked in PABA for one hour before transplanting ( ) in whole plots is shown. The corresponding area under the disease progress curve is shown in Table 2.6. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference.

**Figure 2.10** Relative chlorophyll measured 24, 30, and 36 days after transplanting in tomato cv. H5108 treated with CuOH and para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato in Ridgetown, ON, 2014*. SPAD readings in the nontreated control ( ), eight applications of CuOH at 7-day intervals ( ), eight applications of PABA at 7-day intervals ( ), two applications of PABA at 5-day intervals ( ), nine applications of PABA at 5-day intervals ( ), and seedlings soaked in PABA for one hour before transplanting ( ) in whole plots is shown. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference.

**Figure 3.1** Daily maximum ( ) and minimum ( ) temperatures, and total daily rainfall ( ) for a) 2011, b) 2012, c) 2013, and d) 10-year average (2004-2013) at Ridgetown Campus, University of Guelph.

**Figure 3.2** Mean monthly a) maximum temperature, b) minimum temperature, and c) monthly rainfall in 2011 ( ), 2012 ( ), 2013 ( ) and 10-year average ( ) (2004-2013) at the Ridgetown Campus, University of Guelph.

**Figure 3.3** Early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P.
Early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (22 June-27 July), b) 2012 (8 June-5 July), c) 2013 (22 June-8 July). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.

---

Late season progress of bacterial spot and speck on tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (27 July-23 Aug), b) 2012 (4 July-27 Aug), c) 2013 (29 June-19 Aug). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.

---

Area under the disease progress curve (AUDPC) for early season disease. Early season disease was measured by the number of leaves with disease symptoms (shown in fig. 2) for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011, b) 2012, c) 2013. Defoliation in the nontreated control ( ), UNI ( ), ASM ( ), and ASM + UNI ( ) treatments in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Figure 3.4. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.

---

Area under the disease progress curve (AUDPC) for late season disease. Late season disease was measured by the percent defoliation (shown in fig. 4) for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (27 July-23 Aug), b) 2012 (4 July-27 Aug), c) 2013 (29 June-19 Aug). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.

---

SPAD chlorophyll readings measured 18, 36, and 56 days after transplanting in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in the nontreated control ( ), UNI ( ), ASM ( ), and ASM + UNI ( ) treatments a) 2011, b) 2012, c) 2013. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.

---

Area under the disease progress curve (AUDPC) for early season disease. Early season disease was measured by the number of leaves with disease symptoms (Figure 3.8) for tomato cv. TSH4 (black bars) and cv. H9909 (grey bars) treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (22 June-27 July), b) 2012 (8 June-5 July), c) 2013 (22 June-8 July). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference. An ‘*’ indicates differences among treatments which are discussed in the text.

---

Late season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and influe

---

*xvii
inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Defoliation in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments in whole plots is shown. The corresponding area under the disease progress curve is shown in Figure 3.11. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey's HSD. NS = no significant difference. .............. 111

**Figure 3.11** Area under the disease progress curve (AUDPC) for late season disease. Late season disease was measured by the percent defoliation (shown in fig. 8) for tomato cv. TSH4 (black bars) and cv. H9909 (grey bars) treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (27 July-29 Aug), b) 2012 (5 July-8 Aug), c) 2013 (1 July-23 Aug). Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference. ...................... 112

**Figure 3.12** Height of tomato seedlings cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference. ........................................ 114

**Figure 3.13** SPAD chlorophyll readings measured 18, 37, and 56 days after transplanting in tomato cv. TSH4 and cv. H9909 treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference. ...................... 116

**Figure 3.14** Total ( ), red ( ), green ( ), and rotten ( ) fruit yield in a 2m section of tomato cv. TSH4 and cv. H9909 treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. ........................................ 117

**Figure 4.1** Comparison of four commercial processing tomato cultivars for the effect of R17 seed drench on a) incidence of bacterial speck lesions, b) relative chlorophyll, c) plant height, d) dry root weight, and e) foliar dry weight in fertilized tomato cv. TSH4. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench at 0 DAS at $1 \times 10^7$ CFU/mL in 10 mM MgCl2. Control plants received 10 mM MgCl2. Plants were inoculated with *P. syringae* pv. *tomato* at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves. Data for each variable is shown for the nontreated control ( ) and R17 ( ). Errors bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with six replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Results for cv. H2401 (most variables) and cv. H9553 (relative chlorophyll) were inconsistent and are presented in Table A.9........................................... 157

**Figure 4.2** The duration of the plant response to R17 ( ) or 10 mM MgCl2 ( ) seed drench treatment as measured by the a) incidence of bacterial speck lesions, b) relative chlorophyll, c) plant height, d) dry root weight, and e) foliar dry weight in fertilized tomato cv. TSH4. Plants were fertilized 10, 15, 21 and 26 DAS (plants inoculated with *P. syringae* pv. *tomato* at 25 days after seeding (DAS) only) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench 0 DAS at $1 \times 10^7$
CFU/mL in 10 mM MgCl₂. Control plants received 10 mM MgCl₂. Plants were inoculated with Pst 15, 20 or 25 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves. Data points with the same letter at the same time point are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials with six replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Error bars represent standard error of the mean. .......................................................... 160

Figure 4.3 The effect of R17 seed drench treatment on a) the incidence of bacterial speck symptoms, b) mean lesion size, c) mean lesion circumference, and d) percent leaf area with lesions on the terminal leaflet of the third youngest leaf of fertilized tomato cv. TSH4. Disease incidence and mean lesion size of 10 to 15 lesions is shown for the nontreated control (■) and R17 (■). Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench 0 DAS at 1 x 10⁷ CFU/mL in 10 mM MgCl₂. Control plants received 10 mM MgCl₂. Plants were inoculated with P. syringae pv. tomato 20 DAS, and disease incidence assessed at five days post inoculation. Errors bars represent standard error of the mean. Bars with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from two independent trials with seven (experiment 1) and six (experiment 2) replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. .......................................................... 163

Figure 4.4 Neighbour joining tree of partial 16S sequences (1160 bp) from representative members of the E. cloacae complex, other Enterobacter sp., closely related Enterobacteracea, and R21.................. 167

Figure 4.5 Neighbour joining tree of partial rpoB sequences (934 bp) from representative members of the E. cloacae complex, other Enterobacter sp., closely related Enterobacteracea, and R21.................. 168

Figure 4.6 Neighbour joining tree of partial 16S sequences (1234 bp) from representative members of the B. cereus group, other Bacillus sp., and R17................................................................. 169

Figure 4.7 Neighbour joining tree of partial gyrB sequences (696 bp) from representative members of the B. cereus group, other Bacillus sp., and R17................................................................. 170

Figure A.1. Lesion size was determined by a) taking a photo (12.2 MB, 4288 x 2824 pixels) of each leaflet was taken using a Nikon D300s camera with a ruler for reference, b) uploading, enlarging and printing each photo in colour, c) tracing lesion circumference using a fine point marker on acetate, and d) scanning lesion circumference images and analyzing using Image J. .......................................................... 239

Figure A.2 Leaf counts and early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with P. syringae pv. tomato and X. gardneri, Ridgetown, ON, in 2013. The a) total number of symptomless and symptomatic leaves, and b) % of leaves with symptoms in the nontreated control (●), UNI (●), ASM (●), and ASM + UNI (●) is shown for a 1.24 m² area. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference. .......................................................... 240

Figure A.3 Leaf counts and early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with P. syringae pv. tomato and X. gardneri, Ridgetown, ON, in 2013. Figures represent a) the total number of symptomless and symptomatic leaves in cv. TSH4, b) the % of leaves with symptoms in cv. TSH4, c) the total number of symptomless and symptomatic leaves in cv. H9909, and d) the % of leaves with symptoms in cv. H9909, in the nontreated control (●), CuOH (●), CuOH + UNI (●), ASM (●), ASM + UNI (●), and UNI (●) treatments is shown for a 1.24 m² area. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference. .......................................................... 241

Figure A.4 Standard dilution curves of a) R17 and b) R21 endophytes of A600 versus CFU/ml. A
standard growth curve was developed for R17 and R21 by plating dilutions of the bacteria grown
overnight in tryptic soy broth and then diluted in sterile distilled water to 0.2, 0.4, 0.6, 0.8, and 1.0
absorbance (OD = 600). Serial dilutions at each absorbance level were plated onto tryptic soy agar and
counted at 48 hours after incubation at approximately 22°C. From this, a formula was developed for R17:
population (CFU/mL) = [(9x10^7) (absorbance)] - 1x10^7 (R^2 = 0.93) and for R21: population (CFU/mL) =
[(2x10^9) (absorbance)] - 2x10^8 (R^2 = 0.89). Values at 1.0 absorbance for R21 were omitted because using
the values from 0.200 to 0.800 provided a curve with a higher R^2 value. Second values for R21 at 0.4
absorbance are missing due to missing plots because of plate contamination........................................... 243
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-AA</td>
<td>4-aminobenzoic acid</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylate</td>
</tr>
<tr>
<td>AHO</td>
<td>3-acetyl-3-hydroxyindole</td>
</tr>
<tr>
<td>AM</td>
<td>Vesicular arbuscular mycorrhiza</td>
</tr>
<tr>
<td>AON</td>
<td>Autoregulation of nodulation</td>
</tr>
<tr>
<td>AUDPC</td>
<td>Area under the disease progress curve</td>
</tr>
<tr>
<td>ASM</td>
<td>Acibenzolar-s-methyl</td>
</tr>
<tr>
<td>BABA</td>
<td>β-aminobutyric acid</td>
</tr>
<tr>
<td>BIT</td>
<td>1, 2-benzisothiazol-3 (2H)-one 1,1-dioxide</td>
</tr>
<tr>
<td>BSX</td>
<td>Bacterial spot causing Xanthomonads</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CMPA</td>
<td>3-chloro-1-methyl-1Hpyrazole-5-carboxylic acid</td>
</tr>
<tr>
<td>CMV</td>
<td><em>Cucumber Mosaic Virus</em></td>
</tr>
<tr>
<td>DAPG</td>
<td>2,4-diacetylphloroglucinol</td>
</tr>
<tr>
<td>DAS</td>
<td>Days after seeding</td>
</tr>
<tr>
<td>DAT</td>
<td>Days after transplanting</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post inoculation</td>
</tr>
<tr>
<td>DPT</td>
<td>Days post treatment</td>
</tr>
<tr>
<td>EBDC</td>
<td>ethylenebis(dithiocarbamate)</td>
</tr>
<tr>
<td>ECM</td>
<td>Ectomycorrhiza fungi</td>
</tr>
<tr>
<td>efl18</td>
<td>Translational elongation factor-Tu</td>
</tr>
<tr>
<td>EIN3-like</td>
<td>ET-insensitive 3-like</td>
</tr>
<tr>
<td>ET</td>
<td>Ethylene</td>
</tr>
</tbody>
</table>

xxi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETI</td>
<td>Effector-triggered immunity</td>
</tr>
<tr>
<td>ETS</td>
<td>Effector triggered susceptibility</td>
</tr>
<tr>
<td>flg22</td>
<td>Bacterial flagellin</td>
</tr>
<tr>
<td>GA</td>
<td>Gibberellins</td>
</tr>
<tr>
<td>HR</td>
<td>Hypersensitive response</td>
</tr>
<tr>
<td>hrc</td>
<td>HR and conserved genes</td>
</tr>
<tr>
<td>hrp</td>
<td>HR and pathogenicity genes</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole acetic acid</td>
</tr>
<tr>
<td>ICS</td>
<td>Isochorismate synthase</td>
</tr>
<tr>
<td>INA</td>
<td>2,6-dichloro isonicotinic acid</td>
</tr>
<tr>
<td>IPM</td>
<td>Integrated pest management</td>
</tr>
<tr>
<td>ISR</td>
<td>Induced systemic resistance</td>
</tr>
<tr>
<td>JA</td>
<td>Jasmonic acid</td>
</tr>
<tr>
<td>JA-Ile</td>
<td>JA-isoleucine</td>
</tr>
<tr>
<td>JAZ</td>
<td>jasmonate ZIM-domain</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
</tr>
<tr>
<td>MAMP</td>
<td>Microbe associated molecular pattern</td>
</tr>
<tr>
<td>MeSa</td>
<td>Methyl salicylate</td>
</tr>
<tr>
<td>MTI</td>
<td>Microbe associated molecular pattern triggered immunity</td>
</tr>
<tr>
<td>NPR1</td>
<td>Nonexpressor of PR genes</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonia lyase</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PGPB</td>
<td>Plant growth promoting rhizobacteria</td>
</tr>
<tr>
<td>PGPF</td>
<td>Plant growth promoting fungi</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PGR</td>
<td>Plant growth regulator</td>
</tr>
<tr>
<td>PTI</td>
<td>PAMP-triggered immunity</td>
</tr>
<tr>
<td>PR</td>
<td>Pathogenesis-related</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PR1a</td>
<td>Acidic PR1</td>
</tr>
<tr>
<td>PR1b</td>
<td>Basic PR1</td>
</tr>
<tr>
<td>Pst</td>
<td><em>Pseudomonas syringae</em> pv. <em>tomato</em></td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAR</td>
<td>Systemic acquired resistance</td>
</tr>
<tr>
<td>SGT1</td>
<td>Suppressor of G2 allele of skp1</td>
</tr>
<tr>
<td>SIR</td>
<td>Systemic induced resistance</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>TMV</td>
<td><em>Tobacco mosaic virus</em></td>
</tr>
<tr>
<td>TSA</td>
<td>tryptic soy agar</td>
</tr>
<tr>
<td>UNI</td>
<td>Uniconazole</td>
</tr>
<tr>
<td>VBTar</td>
<td>Vogel Bonner-tartrate media</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>Xcv</td>
<td><em>Xanthomonas campestris</em> pv. <em>vesicatoria</em></td>
</tr>
<tr>
<td>Xe</td>
<td><em>Xanthomonas euvesicatoria</em></td>
</tr>
<tr>
<td>Xv</td>
<td><em>Xanthomonas vesicatoria</em></td>
</tr>
<tr>
<td>Xg</td>
<td><em>Xanthomonas gardneri</em></td>
</tr>
<tr>
<td>Xp</td>
<td><em>Xanthomonas perforans</em></td>
</tr>
</tbody>
</table>
Chapter 1: Literature Review

1.1 Introduction

The use of biological and chemical plant defense activators offer novel approaches for disease management, but their use has been limited because of inconsistent results and yield reductions associated with plant growth fitness costs due to the reallocation of plant metabolites to defense compound production from normal growth and development (Byrne et al., 2005, Heil, 2001, Heil et al., 2000, Walters & Heil, 2007, Louws et al., 2001). Further testing and optimization of novel defense activators as well as better understanding of the factors that may improve plant response to existing activators could lead to strategies involving defense activators that provide consistent results with acceptable levels of resistance induction and limited fitness costs. Factors include selection of highly responsive genotypes, activator application method and concentration, the duration of host response to activators, and synergies among activators and PGRs associated with stress tolerance. This approach could be applied to reduce losses in processing tomato (*Solanum lycopersicum* L. (*Lycopersicon esculentum*)) from plant diseases such as bacterial speck caused by *Pst* (Okabe) Young, Dye, & Wilkie and bacterial spot caused by one of four species of bacterial spot-causing Xanthomonads (BSX).

1.2 Processing tomatoes in Ontario

Tomatoes are the second most popular vegetable in the world, and the most popular vegetable for home gardeners (Foolad & Panthee, 2012). Processing tomatoes are used to produce a variety of processed goods including crushed, diced, and whole pack tomatoes, pastes used in ketchup and other sauces, and tomato juice. Globally, average annual production of processing tomatoes averaged over 39 million metric tonnes per year from 2014 to 2016, 1.0 % of which was grown in Ontario (WPTC, 2017). The farm gate value of the Ontario processing tomato production from 2014 to 2016 was approximately $55 million (OPVG, 2017b). The industry is primarily located in Essex and Kent counties in southwestern Ontario, and is economically important in those areas.

Processing tomato plants, unlike greenhouse and some fresh market cultivars, have a determinant growth habit. In Ontario, processing tomatoes are grown from approximately 6-week old transplants that are planted in a twin-row system. Rainfall is often supplemented with overhead or drip irrigation (OMAFRA, 2010). It is also common for growers to apply Ethrel (ethepon) to trigger fruit ripening of mature green fruit near harvest and maximize the number of red fruit harvested (OMAFRA, 2010).

Important features of processing tomato fruit quality include colour, low pH, and high soluble solids. Low fruit pH is necessary to prevent spoilage, and a range of 4.1 to 4.3 is recommended (Gould, 1992).
Higher levels of soluble solids in the fruit are desirable for production of tomato paste (S. Loewen, University of Guelph, *pers. comm.*). Growers delivering tomatoes with poor colour rating, as defined by an Agtron colour rating greater than 37 units, receive a lower price for their tomatoes and risk having loads rejected by processors.

Abiotic stresses can greatly damage tomatoes. Temperatures less than 10°C can have detrimental effects on tomato growth and development because of a sharp reduction in cellular activities (Breidenbach & Waring, 1977, Lyons, 1973). Tomatoes are sometimes subject to these temperatures in Ontario in May during the weeks immediately after transplanting. Tomato seedlings exposed to chilling at 5°C for three days exhibited loss of cell turgor, vacuolization, reduction in cytoplasm and vacuolar protein bodies, disorganization of plastids, loss of cytoplasmic structure, and cell death (Ilker et al., 1976). Other abiotic stresses often found for field tomatoes in Ontario are heat stress that can result in blossom drop when temperatures exceed 30°C daytime or 21°C nighttime, puffiness, and sunscald, drought that can result in poor growth and development and exacerbate blossom end rot, and excess water that causes water wilt as a lack of oxygen limits respiration and alters phytohormone concentrations (Scott, 2014). A tomato crop can often be exposed to several of these in the same growing season. Growth and development outcomes for tomato exposed to multiple abiotic stresses can be positive, negative or neutral, but prolonged exposure to abiotic stresses most often results in greater susceptibility to biotic stresses, like plant diseases (Suzuki et al., 2014).

Diseases are a major source of biotic stresses for Ontario tomatoes. The major field tomato bacterial diseases in Ontario are bacterial spot and bacterial speck, which are sometimes found together and are difficult to distinguish based on symptoms alone (Pitblado & Tartier, 1994, Tartier & Pitblado, 1994, Miller & Jones, 2014, Jones, 1991b). Bacterial canker is also present but is now rare in its damaging systemic form of infection (Jones et al., 1991, OMAFRA, 2010, LeBoeuf et al., 2009). Most of the other diseases in field tomatoes in Ontario causing economic losses are caused by fungi, including Verticillium wilt (*Verticillium dahliae; Verticillium albo-atrum*), early blight (*Alternaria solani*), Septoria leaf spot (*Septoria lycopersici*) and anthracnose (*Colletotrichum coccodes*) (Jones et al., 1991, OMAFRA, 2010). The fungal diseases gray mold (*Botrytis cinerea*) and white mold (*Sclerotinia minor, Sclerotinia sclerotiorum*) are occasionally seen in Ontario, but are not usually economically important (Jones et al., 1991, OMAFRA, 2010). Diseases of significance caused by Oomycetes in Ontario include late blight (*Phytophthora infestans*) and Phytophthora root rot (*Phytophthora capsici*). For viral diseases, *Cucumber Mosaic Virus* (CMV), tomato mosaic virus and tomato spotted wilt virus are all known in Ontario, although sightings are sporadic and rarely result in economic losses (OMAFRA, 2010).
1.3 Tomato genetics

Tomato (S. lycopersicum) is a member of the Solanaceae, which also includes agriculturally significant crops such as potato (S. tuberosum), pepper (Capsicum annuum), eggplant (S. melongena), tobacco (Nicotiana tabacum), petunia (Petunia ×hybrida), and a number of weed species including eastern black nightshade (S. ptycanthum) (Knapp et al., 2004). The cultivated tomato originated from Central and South America but the time of domestication is unknown (Bai & Lindhout, 2007). Wild tomatoes, such as S. chilense and S. peruvianum have greater genetic diversity than S. lycopersium, but only S. lycopersium has been domesticated (Bai & Lindhout, 2007). Open-pollinated cultivars once dominated the processing tomato market, but increasingly hybrid cultivar seed, which is produced using hand pollination, is used to produce processing tomatoes because of perceived benefits with tomato quality and yield (Bai & Lindhout, 2007).

Tomato has a set of 12 chromosomes and is diploid, which is similar to the majority of other Solanaceae species (Olmstead et al., 1999). An international consortium of researchers started sequencing the entire tomato genome in 2004 (Mueller et al., 2009) and it was publically released in 2012 (Consortium, 2012). The tomato genome is predicted to contain more than 30,000 genes (Consortium, 2012).

1.4 Bacterial endophytes of tomato

Bacterial endophytes are culturable or non-culturable organisms that are detected in plant tissues following surface sterilization and have no adverse effects on the host (Hallmann et al., 1997, Bulgarelli et al., 2013, Gaiero et al., 2013). The soil environment is considered the most important source of plant endophytes, but other sources include seeds, propagative material, and the phylloplane (Hallmann et al., 1997, Hardoim et al., 2008, Rosenblueth & Martinez-Romero, 2006). Bacterial endophytes are diverse with members of Actinobacteria, Bacteroidetes, Firmicutes and alpha, beta and gamma Proteobacteria. However, bacterial endophyte communities are typically less diverse than the rhizosphere communities (Berg et al., 2005, Bulgarelli et al., 2013, Gottel et al., 2011, Marquez-Santacruz et al., 2010). Higher diversity of endophytes in roots, shoots, and seeds were found in corn, tomato, melon and pepper grown in organically cultivated soil compared to conventionally managed soil (Xia et al., 2015), possibly because of differences in the soil environment, such as higher levels of soil carbon and nitrogen, and a different C:N, which can affect bacterial diversity and abundance (Trivedi et al., 2016).

For tomato, an examination of the diversity of culturable endophytes found 32 endophyte species in five phyla among 336 bacterial isolates from roots, shoots and seeds of an unspecified tomato cultivar (Xia et al., 2015), and 27 endophyte species among 34 bacterial isolates from roots of cvs. Arka Abha and Arka Vikas (Upreti & Thomas, 2015). Xia et al. (2015) found that the predominant species were members
of the Firmicutes, while Upreti & Thomas (2015) found gamma-Proteobacteria were the most abundant. Using non-culturing dependent methods of PCR-restriction fragment length polymorphism and 16S rDNA sequencing, 41 isolates in seven genetic groups were identified from tomato cv. Xiaihong-1 roots and stems and 38 isolates in four genetic groups were identified in cv. Baoshi-5 roots and stems (Feng et al., 2013). The sequences matched those of *Sphingomonas yanoikuya, Pseudomonas pseudoalcaligenes, Serratia marcescens, Bacillus megaterium, Paenibacillus polymyxa, B. pumilus, B. cereus, Pseudomonas fluorescens* and *Arthrobacter globiformis* in Xianhong-1 and *S. yanoikuyae, P. fluorescens, Arthrobacter globiformis* and *P. polymyxa* in Baoshi-5. Furthermore, 80 operational taxonomic units from tomato cv. Platense leaves were found using mass sequencing of 16S-ribosomal RNAs (Romero et al., 2014). Most were members of the Proteobacteria (>94%) with most of the rest belonging to the Actinobacteria, Planctomycetes, Verrucomicrobia and Acidobacteria. In contrast, more than 1700 operational taxonomic units from the tomato cv. Platense rhizosphere soil were identified with members of twelve different taxonomic groups being most common, among which the Acidobacteria, Verrucomicrobia, Proteobacteria and Gemmatimonadetes comprised 22, 24, 17 and 16% of the population, respectively. Romero et al. (2014) concluded that the diversity of leaf endophytes in tomato is less than the rhizosphere and dominated by Proteobacteria. Thus, similar to other crops, all tissues of tomato appear to contain both culturable and non-culturable bacterial endophytes, which have a considerable diversity but less than that found in the environment around tomato plants.

### 1.5 Bacterial speck pathogen of tomato

Bacterial speck is caused by the hemibiotrophic bacterium *Pst* (Preston, 2000, Young et al., 1978). *Pst* was formerly classified as *Pseudomonas tomato* (Okabe) prior to being renamed *Pst* (Hirano & Upper, 1990, Young et al., 1978). *Pst* is a gram-negative, rod-shaped bacterium classified in phylum Proteobacteria, class Gammaproteobacteria, and family Pseudomonadaceae (Kado, 2010a). *Pst* is only reported to be of economic importance in tomato, although it has been isolated from numerous other crop and weed species as an epiphyte (Preston, 2000). *Pst* strain DC3000 is commonly used in molecular research studies as it also infects the model plant *Arabidopsis thaliana* (Cuppels & Ainsworth, 1995, Preston, 2000), and was originally isolated from the Channel Islands, Guernsey, United Kingdom, in 1960 (Cuppels & Elmhirst, 1999). Its complete genome sequence was published in 2003 (Buell et al., 2003).

*Pst* can spread in the environment through the movement of water, especially during rain or overhead irrigation events that allow for splash dispersal onto leaves within the crop canopy, and infections can be exacerbated by heavy winds that may force the bacteria through natural openings like stomata, or create wounds that enable pathogen entry (Jones, 1991a, Pitblado & Tartier, 1994). There is potential that insects might also act as a vector of the pathogen; however, there are no reports of nonpersistent,
semipersistent, or persistent transmission of Pst by insects in the literature.

Once arriving on a leaf, Pst is similar to other P. syringae pathovars, in that it has two related phases. The first phase is epiphytic, on the surface of the leaf, and the second stage is endophytic and occurs in the leaf apoplast (Melotto et al., 2008). Bacteria such as Pst can survive on the leaf surface under a range of high UV, moisture, and temperature conditions (Hirano & Upper, 2000). The leaf surface contains a number of peaks, valleys, and cavities that enable survival, such as the area in and around the trichomes and epistomatal cavities (Hirano & Upper, 2000, Schneider & Grogan, 1977b). Furthermore, Pst possesses characteristics that enable epiphytic colonization. For example, DC3000 mutants deficient in type IV pili had lower populations on the leaf surface than the wild-type DC3000 under field conditions, possibly because type IV pili are responsible for aggregation of bacteria that may enhance UV tolerance, (Roine et al., 1998).

The relative importance of the epiphytic phase of Pst on bacterial speck epidemics under field conditions is not entirely clear. In a study in Ontario greenhouses and fields in the early 1990s, Pst populations on healthy transplants did not correlate with disease severity in the field, even though epiphytic populations of Pst were detected on transplants from all three greenhouses included in the study (Cuppels & Elmhirst, 1999). The difference in severity among fields was largely attributed to environmental variables such as the number of intense rains that occurred during the field season, which would have created wounding sites that would easily facilitate pathogen entry (Cuppels & Elmhirst, 1999).

Pst shifts from an epiphytic to an endophytic phase when it enters the apoplast through stomata and other natural openings or wounds (Preston, 2000). Environmental conditions that favour invasion and infection of Pst include cooler temperatures (18 to 24°C) (Jones, 1991a, Smitley & McCarter, 1982) and extended periods of leaf wetness (Preston, 2000). Leaf morphology is thought to play a limited role in the ability of Pst to successfully invade plant tissues. In a study of the number of stomata, trichomes, and other natural openings on 15 tomato cultivars with a range of susceptibility to Pst, a positive correlation was observed between an increased number of openings and in increase in disease susceptibility; however it was felt that even in very tolerant cultivars, the number of natural openings would still allow for infection to occur (Bashan et al., 1985).

Previously, it was believed that bacterial pathogens such as Pst entered plants through purely passive means; however, recent evidence indicates that Pst may be able to actively induce the opening of stomata. Stomatal closure within one hour of exposure of leaves to bacteria is due to recognition of pathogen associated molecular patterns (PAMPs), also known as microbe associated molecular patterns in nonpathogenic bacteria (Melotto et al., 2008). These have been observed as a resistance mechanism against plant pathogenic bacteria in A. thaliana and tomato. Melotto et al. (2006) reported that exposure
to live *Pst* or the purified PAMPs lipopolysaccharides (LPS) and flagellin triggered stomatal closure in *A. thaliana* within one hour, but the phytotoxin coronatine and the type III secretion system (T3SS) effector *avrRpt2* suppressed stomatal closure and resulted in re-opening of stomata. Inoculation with wild type (wt) DC3000, a coronatine mutant DC3000 mutant, and the non-pathogenic human pathogen *Escherichia coli* O157:H7 also resulted in stomatal closure, but in the case of the wt DC3000, the stomata reopened later. This effect was also explored in tomato, where similar to *A. thaliana*, LPS-induced stomatal closure was overcome by *Pst* DC3000 (Melotto et al., 2006). Thus, coronatine appears to be an important molecule that helps suppress the PAMP-triggered immunity (PTI) responsible for stomatal closure in tomato.

Coronatine production by *Pst* field strains is common, as all 244 *Pst* isolates collected in a survey of Ontario tomato fields in 1989 were able to produce coronatine (Cuppels et al., 1990). An additional 6-year survey completed from 1997 to 2002 found that 68 of 70 isolates collected reacted positively with the COR1/2 primer, which was derived from the bacterial speck probe TPRI. The TPRI probe is derived from a gene cluster in *Pst* DC3000 that is responsible for the production of coronatine, indicating that the vast majority of bacterial speck symptoms occurring in Ontario are associated with coronatine producing strains of *Pst* (Cuppels et al., 2006).

Once inside the leaf, various PAMPs of *Pst* are rapidly detected by membrane-bound receptor kinases known as pattern recognition receptors (PRRs) (Abramovitch et al., 2006). This form of resistance is known as PTI and results in a mitogen-activated protein kinase signaling cascade that induces the expression of genes responsible for the activation. This triggers a number of integrated defenses including deposition of callose for cell wall fortification, production of antimicrobial secondary metabolites such as the alkaloid tomatine (Friedman, 2002) and phytoalexin rishitin (Le Floch et al., 2005), pathogenesis-related (PR) proteins such as PR3 and PR4 (chitinase), PR2 (glucanase) and PR5 (osmotin or thaumatin-like proteins) (Jia & Martin, 1999, Ren et al., 2011, Van Loon & Van Strien, 1999), and production of antimicrobial defensins and defensin-like peptides (Stotz et al., 2009, Pieterse et al., 2009a). In addition, expression of a number of other plant hormones, such as the auxin indole acetic acid (IAA) and gibberellins (GA) can also be induced or repressed as a result of PTI (Pieterse et al., 2009a). A key step in the activation of PTI is salicylic acid (SA) production, which is related to reactive oxygen species (ROS) production and controlled by an increase in cytoplasmic calcium levels (Nicaise et al., 2009). Furthermore, stomatal closure in *A. thaliana* against *Pst* was accelerated for 48 h following exposure to rhizobacterium *B. subtilis* FB17, indicating that stomatal resistance can be triggered systemically (Kumar et al., 2012). Interestingly, recent evidence in *A. thaliana* indicates that PTI against *Pst* varies according to the plant circadian clock, with *A. thaliana* having greater immunity to *Pst* challenge in the morning than the evening (Bhardwaj et al., 2011). The implications for this discovery on
bacterial speck management in the field are not yet clear.

*Pst* can overcome PTI defenses by releasing effectors through its T3SS that interfere with tomato PRRs or the downstream signaling of resistance (Kado, 2010b). This is defined as effector triggered susceptibility (ETS) since the release of these effector proteins interact with a variety of targets inside plant cells undergoing PTI resulting in the suppression of PTI and colonization of the plant by *Pst* and leading to the development of bacterial speck symptoms. The pathogenicity of *Pst* is reliant on the T3SS, which is encoded by the *hrp* (*HR* and pathogenicity)/*hrc* (*HR* and conserved) genes (Kado, 2010b). *hrp* genes are located in a highly conserved region in many bacteria, whereas *hrc* genes are common only in group I T3SS pathogenic bacteria (Preston, 2000). For *Pst* DC3000, there are seven weakly expressed effectors and 28 fully active effector proteins introduced into host cells using this system. Two effectors, AvrPto and AvrPtoB, are implicated in interfering with PRRs such as FLS2 and BAK1 that are part of the PTI response in tomato (Munkvold & Martin, 2009, Segonzac & Zipfel, 2011, Xiang et al., 2011). In the case of FLS2, AvrPto and AvrPtoB prevent it from binding with the PAMP bacterial flagellin (flg22) peptide (Abramovitch et al., 2006). This interference results in suppression of PTI and enables infection and colonization of the tomato host. AvrPtoB can also degrade some PRRs, suppress kinase activation, and suppress PAMP-induced gene expression and microRNA production (Munkvold & Martin, 2009). Similar effects are also reported for AvrPto (Munkvold & Martin, 2009). In addition to interfering or suppressing the actions of PRRs and PAMPs-mediated response, effectors released by *Pst* also play other roles in overcoming PTI. For AvrPto, these host processes include inducing ethylene (ET) production, and for AvrPtoB, inducing both abscisic acid (ABA) and ET production. These hormones are thought to antagonize SA-dependent defense responses in the host (Munkvold & Martin, 2009). Furthermore, the effectors HopQ1-2 and Hopl1 are implicated in directly reducing plant levels of SA (Cunnac et al., 2009).

Tomatoes may successfully recognize these effector proteins resulting in effector-triggered immunity (ETI) and a hypersensitive response (HR) (Preston, 2000). Tomato cultivars with the *Pto* gene are able to recognize the effectors AvrPto and AvrPtoB in *Pst* race 0. Both AvrPto and AvrPtoB physically interact with *Pto* to activate plant immunity (Kim et al., 2002, Pedley & Martin, 2003). A second gene, *Prf*, is also required for *Pto*-mediated resistance to *Pst* in tomato (Pedley & Martin, 2003).

Previously, it was thought that *Pto* does not recognize *Pst* race 1 because it lacks the AvrPto effector (Pedley & Martin, 2003). However, in an analysis of *Pst* race 1 strains collected over 13 years in California, a functional *avrPto* with a key amino acid polymorphism that prevents its interaction with *Pto* was identified in four isolates (Kunkeaw et al., 2010). *avrPtoB* was still expressed in race 1 strains, but AvrPtoB protein expression was maintained at low levels using an unknown mechanism, and this enables race 1 strains to overcome *Pto*-mediated resistance. *Pto*-mediated resistance for bacterial speck management has been used commercially for approximately 20 years, and over 90 percent of processing
tomato cultivars grown in California carry the Pto and Prf genes. Therefore, it is possible that Pst race 1 have evolved to overcome this resistance (Kunkeaw et al., 2010).

In addition to its role in overcoming PTI, coronatine is also important as a phytotoxin in the later stages of pathogenesis. Coronatine production in DC3000 mutants deficient in expression of hrp/hrc genes and their hrpFGCTV operon still occurred, but typical speck lesions did not develop and the mutated bacteria were unable to reproduce (Penaloza-Vazquez et al., 2000). However, some hrp mutations resulted in overexpression of coronatine, indicating the existence of cross-talk between hrp and cor gene clusters (Penaloza-Vazquez et al., 2000).

Coronatine also mimics the action of the phytohormone jasmonic acid (JA), and this plays a key role in the virulence of Pst. Coronatine induces the expression of JA-dependent wound-related genes associated with herbivory leading to suppression of SA related genes (Zhao et al., 2003). JA-insensitive mutants are resistant to DC3000, and the expression of JA-dependent wound-related genes is reduced while PR gene expression increases. In contrast, in wild-type plants, both coronatine and the T3SS repress expression of PR genes (Zhao et al., 2003). Coronatine is also implicated in suppression SA-dependent defense responses in tomato (Uppalapati et al., 2007).

If Pst overcomes PTI-related defenses and is not detected by plant R proteins, it successfully infects tomato tissues and continues colonization in a biotrophic fashion. Tomato apoplastic fluid is a complete nutrient medium for Pst, and Pst uses both constitutive and plant-induced assimilation pathways to use nutrients available in the apoplast (Rico & Preston, 2008). The influence of environmental conditions on the nutrient profile in the apoplast and subsequent impact on Pst growth has not been explored, but might be an interesting avenue for future research (Rico & Preston, 2008).

The shift from biotrophy to necrotrophy and subsequent development of symptoms is not clearly understood, but a number of factors have been identified that contribute to symptom development. The chlorotic halo surrounding lesions is attributed to the phytotoxin coronatine, and is dependent on SGT1 (suppressor of G2 allele of skp1), a gene that is also required in programmed cell death associated with a HR (Uppalapati et al., 2011). Bender et al. (1987) observed a lack of chlorosis and a reduction in lesion size in Pst mutants deficient in coronatine production.

Furthermore, effectors also play a role in necrosis. Both AvrPto and AvrPtoB are implicated in increasing plant ET levels. High levels of ET are linked to plant stress response and can result in cell death which might improve Pst access to nutrients, but results in plant tissue necrosis (Abramovitch et al., 2006). Lesion formation by Pst was also reduced when the effector HopAA1-1 and the chlorosis-promoting factor PSPTO4723 were absent (Munkvold et al., 2009). The effector avrE also contributes to lesion formation more than bacterial growth (Badel et al., 2006). Undoubtedly, additional effectors probably play roles in necrosis and lesion development which are yet to be determined.
Bacterial speck symptoms are typically visible five days following inoculation under controlled conditions, and seven to 10 days after inoculation under field conditions. It is possible that tomato susceptibility to \( Pst \) is dependent on tomato developmental stage, since this phenomenon is reported in tomato and other plant species for other pathogens, and for \( Pst \) in \( A. \) thaliana (Develey-Rivière & Galiana, 2007, Kus et al., 2002, Shah et al., 2015, Panter et al., 2002, Sharabani et al., 2013). Bacterial speck symptoms may appear on all foliar plant parts, including tomato leaves, fruit, stems, and flowers. Small, round, dark brown to black lesions develop on leaves that often have a chlorotic yellow halo (Jones, 1991a) (Figure 1.1a). Eventually, lesions coalesce causing large areas of tissue to die. This may lead to defoliation, early fruit ripening, and sunscald, which in the case of processing tomatoes, negatively affects harvesting schedules and the quantity of fruit meeting acceptable standards. Small (<1mm) lesions develop on green fruit less than 3 cm in diameter, but do not develop on larger green fruit or on red fruit (Getz et al., 1983, Jones, 1991a, Preston, 2000). These specks reduce the quality of fresh market tomatoes, and can negatively affect the peeling process for processing tomatoes (Figure 1.1b & c). Increasing bacterial speck severity is normally positively correlated with \( Pst \) population in leaf tissues (Baysal et al., 2007, Vallad et al., 2003, Scarponi et al., 2001).

Following infection, \( Pst \) may survive as a saprophyte on crop residue for up to 30 weeks and in soil for less than 30 days (Jones, 1991a, Preston, 2000). \( Pst \) can also survive on infested seed for long periods (Bashan et al., 1982, Jones, 1991a, McCarter et al., 1983). In one instance, \( Pst \) was isolated from 20 year old tomato seeds in Israel (Bashan et al., 1982). Potential mechanisms of seed infection include internal colonization via the host xylem, colonization through the pistil, and external contamination after contact with symptomatic fruit tissues (Maude, 1996). Other potential sources of inoculum include volunteer tomato plants, infected weed hosts, crop residue, and contaminated equipment such as farm machinery, greenhouse structures, insects, animal and tools (Bashan, 1986, McCarter et al., 1983, Schneider & Grogan, 1977a).

### 1.6 Bacterial spot pathogen of tomato

Bacterial spot is also a common bacterial disease in Ontario (Cuppels et al., 2006, LeBoeuf et al., 2009). The bacterium causing this disease was previously classified as \( Xanthomonas campestris \) pv. \( vesicatoria \) (Doidge) Dye (Xcv) (Young et al., 1978). Xcv was first divided into Group A and Group B strains based on starch hydrolysis and pectolytic activity, and it was suggested that these groups should be reclassified as two separate species, where Group A is \( X. \) axonopodis pv. \( vesicatoria \) and Group B is \( X. \) vesicatoria (Vauterin et al., 1995). A second \( X. \) axonopodis pv. \( vesicatoria \) strain was later characterized
Figure 1.1. Symptoms of bacterial speck on a) tomato foliage, b) ripe fruit, and c) peeled processing tomato, and bacterial spot on d) tomato foliage, b) ripe fruit, and c) peeled processing tomato.
as Group C, along with *X. gardneri* in Group D (Potnis et al., 2015). Four groups known as A, B, C, and D are now distinguished based on DNA relatedness (Jones et al., 2004). DNA:DNA hybridization revealed that Group A and Group C have less than 70 percent DNA relatedness with each other, and Group D has less than 70 percent any of the other three groups (Jones et al., 2004). Groups A, B, C and D are referred collectively as BSX, and it has been proposed that these groups be renamed as separate species: Group A as *X. euvesicatoria* Jones, sp. nov. (*Xe*), Group B as *X. vesicatoria* (Doidge) Vauterin, sp. nov., nom. rev. (*Xv*), Group C as *X. perforans* Jones, sp. nov. (*Xp*), and Group D as *X. gardneri* (ex Sutic) Jones, nom rev., comb. nov. (*Xg*) (Jones et al., 2004). A fifth species, *Xanthomonas arboricola* Vauterin, sp. nov., is also reported to cause bacterial spot of tomato but so far this report is limited to Tanzania (Mbega et al., 2012). The pathogens are also classified into races based on different interactions with a standard group of tomato genotypes, which is based on the production of different T3SS effector proteins. The races that have been found thus far are T1 in *Xe*, T2 in *Xv*, *Xp* and *Xg*, T3 in *Xp*, and T4 in *Xp* (Jones et al., 2005).

*Xe* and *Xv* have a worldwide distribution, whereas *Xp* is limited to Mexico, Canada, USA, Thailand and the Seychelles, Comoros and Mauritius Islands in the southwestern Indian Ocean (Hamza et al., 2010, Jones et al., 2000). *Xg* is confirmed in Ontario, Pennsylvania, Michigan, Ohio, Brazil, the former Yugoslavia, Costa Rica, and the Reunion Island in the southwestern Indian Ocean (Cuppels et al. 2006). *Xg*, which is considered more aggressive than other strains, is the predominant strain in Southwestern Ontario (Cuppels et al., 2006, Abbasi et al., 2015). Previously *Xv* was the secondary species of importance in Ontario (Cuppels et al. 2006), but more recently *Xp* is more common (Abbasi et al., 2015).

Cuppels et al. (2008) reported that *Xv*, *Xp*, *Xg* were detected on crop residue after one winter, but not after two winters, and detection of *Xp* and *Xg* on volunteer tomatoes, wheat, and weeds near tomato fields in Ontario was random. In addition to these potential inoculum sources, infested seed and equipment can also be sources of all four BSX (Bashan et al., 1982, Bashan, 1986, Koike et al., 2007, Tartier & Pitblado, 1994).

Similar to *Pst*, BSX have a hemibiotrophic lifestyle. Initial interactions with the host plant begin on the leaf surface; however, the epiphytic lifestyle may not be as great for BSX as *Pst*. Zhang et al. (2009b) reported congregations of *Xe* cells around leaf depressions, guard cells and stomatal openings 48 and 72 h after inoculation but found little extensive growth on the remaining tomato leaf surface. Instead, bacterial cells were found in sub-stomatal chambers and mesophyll tissues. The growth and development of the BSX is favoured by warm temperatures ranging from 24°C to 30°C, and similar to *Pst*, they are also favoured by wet conditions (Koike et al., 2007). These conditions encourage entry into the host plant through natural leaf openings or wounds.

Expression of *hrc* and *hrp* genes, which are responsible for the induction of the T3SS, are required
for BSX virulence. There is evidence that induction of these genes occurs early in the infection process, prior to establishment within leaf tissues. Fluorescence was observed on the surface of tomato leaves 24 h after inoculation with Xe cells carrying hrpG- and hrpX-gfp reporter constructs, which was prior to detection of bacteria within leaf tissues (Zhang et al., 2009b). HrgG begins a regulatory cascade that induces expression of hrc and hrp. Thus, these results may indicate that the T3SS is activated prior to pathogen entry. Because the HrpG/HrpX regulon includes other genes that are unrelated to the T3SS, activation on the leaf surface might also be related to enhanced survival on the leaf surface or priming for ETS, as opposed to immediate suppression of PTI (Zhang et al., 2009b). There is also evidence that X. campestris pv. campestris is capable of interfering with stomatal closure on A. thaliana induced by the bacteria on the leaf surface (Gudesblat et al., 2009a, Gudesblat et al., 2009b). This mechanism might also be important in the infection process of BSX.

Establishment of BSX in tomato tissues is also dependent on T3SS effector proteins. Seventeen different effector groups have been identified for BSX, most of which belong to the Xop and Avr protein families (Kay & Bonas, 2009). Ten of these effectors are fully or partially homologous to known effectors in P. syringae (Kay & Bonas, 2009). The effector XopD are present in Xanthomonas spp., Acidovorax spp., and Pseudomonas spp. and are known to suppress PTI (Kim et al., 2011). Effectors in the AvrBs3 family are unique in that they have only been detected in Xanthomonas spp. and Ralstonia solanacearum (Kay & Bonas, 2009). These effectors, which are known as transcription activator-like (TAL), induce plant gene expression by acting as transcription factors in planta, and have also been demonstrated to be important in dissemination in the field (Kay & Bonas, 2009).

Although the homology of effectors among the BSX is not clearly understood, it appears at least some T3SS effectors are homologous among the Xanthomonas spp. The effector protein XopE2 also has a role in virulence and suppression of the HR for Xe and Xv (Lin et al., 2011), and avrBs3 genes are common in BSX, X. campestris pv. campestris, and Xanthomonas oryzae pv. oryzae (Oh et al., 2011). However, recently avrHahl was identified as a novel member of the avrBs3 gene family in Xg (Schornack et al., 2008).

Once established within the host, BSX starts obtaining nutrients from the host. The enzymatic activity of Xg in A. thaliana revealed that the production of the cell wall degrading enzymes cellulase and a-arabinofuranosidase, but not pectinase, invertase and xylanase were involved in nutrient acquisition (Candido et al., 2008). Furthermore, BSX may also interfere with nutrient export from leaf tissues to plant sinks (Kocal et al., 2008).

Bacterial spot symptoms of chlorosis and necrosis are related to the action of T3SS effectors (Salomon et al., 2011). The production and release of T3SS effectors by BSX in yeast led to an attenuation of cell growth and toxicity, and these characteristics were associated with chlorosis and
necrosis in plants (Salomon et al., 2011). Both virulent and avirulent BSX bacteria induce ET- and SA-dependent defense responses in tomatoes, which are associated with a reduction in symptom development from secondary challenges with these pathogens (Block et al., 2005).

Foliar symptoms of bacterial spot on tomato are difficult to distinguish from bacterial speck (Figure 1.1d). They are lesions that are dark brown, circular to irregular and usually remain less than 5 mm in diameter. As the symptoms advance, lesions coalesce and premature defoliation can occur (Koike et al., 2007). Lesions on fruit begin as small brown blisters that eventually enlarge to 5 to 8 mm in diameter, which are distinct from those of bacterial speck because they are larger in size (Koike et al., 2007) (Figure 1.1e & f). Occasionally the initial lesions on green fruit are surrounded by a white halo, which can be confused with symptoms of bacterial canker caused by Clavibacter michiganensis subsp. michiganensis (Tartier & Pitblado, 1994).

1.7 Current bacterial speck and spot management practices in tomato

The current strategy for bacterial disease management in Ontario relies on several tenets of integrated pest management, including cultural, biological, and chemical controls. The use of clean seed and proper greenhouse sanitation for transplant production are critical to prevent early infections (Jones, 1991a, Jones, 1991b, Koike et al., 2007, Pitblado & Tartier, 1994, Tartier & Pitblado, 1994, Trueman, 2016, Trueman & LeBoeuf, 2015). Genetic resistance is used to some extent to help limit the severity of bacterial speck in Ontario, and processing tomato cultivars carrying the Pto, Prf genes, or both are available that confer resistance to Pst race 0, but not race 1 (Chang et al., 2000). Approximately 25 % of Pst isolates collected in Ontario during the early 1980s were identified as race 1, but extensive survey work has not been done since that time (Lawton & Macneill, 1986). In New York, a Pst phenotype that reaches an intermediate population size in Pto-expressing tomato leaves was recently reported, which the authors hypothesize means Pst is recognized by Pto but then suppressed by another factor (Kraus et al., 2017). There are no commercially available tomato cultivars with full resistance to the BSX (Pei et al., 2012). Some biological control agents, such as Bacillus subtilis QST 713 (Serenade Max) and Streptomyces lydicus WYEC 108 (Actinovate) are also reported to directly antagonize or outcompete bacterial pathogens (Roberts et al., 2008). The mechanisms of antagonisms by B. subtilis QST 713 are not fully explored (Fousia et al., 2016).

The antibiotic compound streptomycin is used in the USA for bacterial disease management, but bacterial populations are known to develop resistance to this compound (Ritchie & Dittapongpitch, 1991). The use of bacteriophages has also been explored, and these are used to some extent in the southeastern US, in combination with other control methods (Obradovic et al., 2009, Obradovic et al., 2004). Use of streptomycin and bacteriophages in Canada are hindered by regulatory obstacles. Kasugamycin (Kasumin
2L) is registered in Canada for suppression of BSX on tomato (PMRA, 2016a), but data on its efficacy in field trials is limited (Trueman, 2015). Kasugamycin has only been shown to be effective when mixed with other products like copper (Griffin et al., 2017). Thus, management of bacterial disease in the field is heavily reliant on copper bactericides.

Copper bactericides (copper hydroxide) are historically the most common tools used for management of bacterial spot and bacterial speck diseases of tomato (OMAFRA, 2010). Copper is a trace element that has important function in living organisms, including within the superoxide dismutases and within enzymes involved in the respiratory chain that produce energy to pump protons across cytoplasmic membranes. However, at high levels, the intracellular concentration of copper ions can no longer be controlled and toxic compounds are formed (Nies, 1999). These bactericides offer some benefit in suppressing levels of bacterial speck and bacterial spot, but do not always provide the level of control required to prevent yield or quality losses associated with these diseases (Lange & Smart, 2005, Lewis Ivey et al., 2004, Griffin et al., 2017). High levels of copper can also affect cellular processes in tomatoes and lead to toxicity symptoms (Iseri et al., 2011). Mixtures of copper with mancozeb or other ethylenebis(dithiocarbamate) (EBDC) fungicides may improve the activity of copper bactericides under field conditions, although in some reports no additional benefit over copper alone or unsprayed control treatments is reported (Damicone & Trent, 2003, Graves & Alexander, 2002, Lewis Ivey et al., 2004, Louws et al., 2001, Obradovic et al., 2004, Roberts et al., 2008).

One of the major limitations of the repeated use of copper bactericides is the development of resistance in populations of bacteria (Griffin et al., 2017, Abbasi et al., 2015). All Pst isolates tested since the late 1970s in Ontario have some level of tolerance to copper (Cuppels & Elmhirst, 1999). Bender and Cooksey (1986) demonstrated that the Pst copper sensitive strains PT12.2 and PT17.2 became copper insensitive after acquiring the plasmid pPT23C, which confers copper resistance by an undetermined mechanism, via horizontal gene transfer. However, another native plasmid in Pst, pT23D, also confers copper resistance, and contains four genes, copA, copB, copC, and copD that are periplasmic and outer membrane proteins that bind and sequester copper outside the cytoplasm as a copper-resistance mechanism (Cha & Cooksey, 1991, Cooksey & Azad, 1992, Cooksey et al., 1990). The copper resistant saprophytic species Pseudomonas putida, and a yellow Pseudomonas sp., as well as the pathogenic BSX and Pseudomonas cichorii, all contained plasmid DNA that was homologous with DNA encoded by pPT23D, suggesting that copper resistance genes can be transferred between bacterial species (Cooksey et al., 1990).

In an effort to improve management of bacterial disease, plant activators that induce or are presumed to induce systemic acquired resistance (SAR) or induced systemic resistance (ISR) have been registered in Canada. These include the SAR compound ASM (Actigard) (PMRA, 2011) and the ISR activator B.
*subtilis* QST 713 (Serenade Max, Serenade Opti, Serenade Soil, Cease) (Bayer, 2017, Bayer, 2014a, Bioworks, 2016, Fousia et al., 2016), which is available in Canada for suppression of BSX on field tomatoes and various other crop disease combinations (Bayer, 2017, Bayer, 2014b, Bayer, 2014a, Bioworks, 2016). However, field performance of these activators has been inconsistent for bacterial spot and speck control in field tomatoes (Roberts et al., 2008, Trueman, 2015). *B. mycoides* isolate J (LifeGard) (Certis, 2017b) and an extract from *Reynoutria sachalinensis* (giant knotweed) (Regalia Maxx) (PMRA, 2016d) are also registered for suppression or partial suppression of bacterial speck, bacterial spot or both on field tomatoes in Canada (PMRA, 2016d, OMAFRA, 2010, Syngenta, 2012). Applications of *B. mycoides* isolate J increase defense enzyme production in sugarbeet, but the defense signaling pathway in tomato has not been identified (Bargabus et al., 2002). Another potential SIR activator is *B. amyloliquefaciens* strain D747 (Double Nickle 55) (Certis, 2017a). It lacks evidence for SIR, although at least one other *B. amyloliquefaciens* strain induced resistance against *Pst* in tomato (Lanna-Filho et al., 2017). The possible modes of action of ASM, *B. subtilis* QST 713, and extract from *R. sachalinensis* is discussed later in this review.

### 1.8 SIR and its use in bacterial disease management

The ability of plants to suppress or resist plant pathogens depends on intruder recognition through PTI and ETI (Pieterse et al., 2009a). The activation of PTI or ETI also activates another plant immune response, known as systemic induced resistance (SIR). SIR is further subdivided into SAR and ISR (Pieterse et al., 2009a). Both SAR and ISR result in phenotypically similar reductions in disease severity, but the modes of action of the two systems are different.

Evidence in *A. thaliana* suggests that SAR generally activates defense mechanisms that are effective against biotrophic and hemibiotrophic pathogens, whereas ISR generally activates defense mechanisms effective against necrotrophic and hemibiotrophic pathogens, as well as insects (Ton et al., 2006). In other words, pests controlled through the SA-dependent defenses are generally suppressed by SAR, and pests controlled through JA- and ET-dependent defenses are generally suppressed through ISR (Pieterse et al., 2009a).

#### 1.8.1 SAR

##### 1.8.1.1 Mechanisms of SAR

SAR is a systemic defense response to preventatively suppress disease symptoms. A characteristic of SAR is that the defense system is activated systemically in distal plant parts as a result of a local activation, thus preventing the spread of disease. SAR was first reported in 1961 (Ross, 1961) where it was found that local inoculation of tobacco plants with *tobacco mosaic virus* (TMV) resulted in broad-
spectrum systemic resistance to TMV and other pathogens in distal leaves.

SAR is dependent on the generation of ROS in a cell, which triggers an accumulation of SA that changes the oxidation state of the regulatory protein nonexpressor of PRI gene (NPR1) (Durrant & Dong, 2004, Nicaise et al., 2009). Inactive NPR1 oligomers are reduced to active monomers that are translocated from the cytosol to the nucleus (Pieterse et al., 2009a, Spoel & Dong, 2012). Nuclear import is dependent on phosphorylation of NPR1 by the SNF1-RELATED PROTEIN KINASE2.8 (Birkenbihl et al., 2017) as well as thioredoxins TRX-h5 and TRX-h3 (Caarls et al., 2015). In A. thaliana, the NPR1 paralogues, NPR3 and NPR4, are SA receptors (Fu et al., 2012). NPR3 and NPR4 help modulate levels of NPR1 and SA in the nucleus by binding with SA and degrading NPR1 (Caarls et al., 2015). NPR3 has low binding affinity with SA, and SA promotes NPR1-NPR3 interaction, whereas NPR4 has high binding affinity with SA, but SA disrupts NPR4-NPR1 interaction (Withers & Dong, 2017). Intermediate levels of SA lead to interaction between NPR3 and NPR1, which results in accumulation of NPR1 and activation of SA-dependent defenses (Caarls et al., 2015). NPR1 interacts with transcription factor proteins in the nucleus, which results in the expression of PR genes and production of PR proteins associated with disease suppression (Spoel & Dong, 2012, Pieterse et al., 2009a) as well as WRKY transcription factor genes. Nuclear interactions with NPR1 also include sumoylation by SUMO3. This shifts NPR1 association from WRKY70 transcription factor to the transcription activator TGA3, which promotes PR gene expression and establishes SAR (Withers & Dong, 2017, Birkenbihl et al., 2017). For example, the expression of acidic PRI (PRIa) is induced during SAR in tomato and this gene has been used as a marker for SAR gene expression in this crop (Block et al., 2005, Herman et al., 2007, Tornero et al., 1997). Although NPR1-dependent SAR is well documented, there is also some evidence that SA can accumulate and induce a defense response in an NPR1-independent manner that still results in the accumulation of PR proteins (An & Mou, 2011), and this is dependent on accumulation of ROS and nitric oxide (Gao et al., 2015).

While SAR is dependent on the SA signaling pathway, local accumulation of SA is not necessarily required for the systemic nature of SAR to occur (Pieterse et al., 2009a, Spoel & Dong, 2012). The mechanism by which the SAR signal travels from the site of local infection to healthy plant parts is not entirely understood; however methyl salicylate (MeSA), JA, a glycerol-lipid factors, and various peptides are all implicated in the signaling process (Liu et al., 2011b, Vlot et al., 2008). Environmental factors such as the duration of light after pathogen inoculation can also influence the relative importance of MeSA in SAR activation (Liu et al., 2011a). It has been proposed that immune signals MeSA, lipid-derived glycerol-3-phosphate, and azelaic acid, might work cooperatively to induce SAR, and the particular signaling molecules involved might be pathogen-dependent (Spoel & Dong, 2012). Regardless of the signaling mechanism, the SAR signal travels through the phloem to healthy plant tissues (Spoel &
Dong, 2012, Vlot et al., 2008). In the case of MeSA, it must be hydrolised to SA in the distal plant cell prior to activating the SAR response (Vlot et al., 2008). SA may accumulate through mechanisms other than MeSA, but these are unknown (Spoel & Dong, 2012).

1.8.1.2 Biological activators of SAR

SAR is a natural defense response to localized infection by a pathogen; therefore, natural activators of SAR include virulent plant pathogens and non-pathogens. Activation of SAR in tobacco occurs against a variety of pathogens including *P. syringae* pv. *tabaci*, *Phytophthora parasitica*, and TMV after inoculation with *P. syringae* and TMV (Edreva, 2004). In another example, inoculation of tomato plants with virulent BSX resulted in accumulation of SA and ET in plant tissues, increased expression of PR marker genes for SAR, and decreased tissue damage for secondary challenges with BSX even though there was not a decrease in the BSX population (Block et al., 2005). Primary inoculation with *Pst* elicited decreased tissue damage due to secondary challenge by *Pst* and BSX, but primary inoculation by BSX result in a reduction in tissue damage from a secondary challenge from BSX but not *Pst* (Block et al., 2005). Inoculation of tomato plants with an avirulent strain of BSX and a virulent strain of BSX was also shown to reduce symptoms of bacterial spot after a subsequent challenge with virulent BSX by reducing plant cell death, as indicated by measurement of cellular ion leakage (Block et al., 2005). Thus, initial localized infections helped suppress further spread of the same or sometimes different pathogens, but clearly do not reduce disease severity of bacterial spot and bacterial speck to economically acceptable levels (Jones, 1991a, Jones, 1991b, Koike et al., 2007, Pitblado & Tartier, 1994, Tartier & Pitblado, 1994).

Avirulent mutants of plant pathogens, particularly those with *hrp* mutations, have also been reported to reduce disease severity in the greenhouse and field. The *Pst* DC3000 *hrpS*, *hrpH*, and *hrpA* mutants reduced severity of disease in tomatoes inoculated with *Pst* wild-type DC3000 in the greenhouse. However, only *hrpS* provided a significant reduction under field conditions (Wilson et al., 2002). Similarly, *hrpG*, *hrpX*, *hrpF*, and *hrpEI* mutants of *Xe* reduced the severity of bacterial spot in tomatoes grown under greenhouse conditions while only *hrpG* and *hrpF* mutants significantly reduced disease severity in the field (Moss et al., 2007). Although these mutants are useful for studying the mechanisms of SAR, their practical application is limited by regulatory bureaucracy and other obstacles associated with their commercialization (Wilson et al., 2002).

Non-pathogenic microorganisms are also associated with SAR, although this association is less frequent than with ISR, which is discussed later in this review. Living activators associated with SAR include isolates of *B. pumilus* and *B. amyloliquefaciens* that induced higher PAL activaty after *Pst* inoculation in tomato (Lanna-Filho et al., 2017). An increase in *PRIa, PDF1.2* and *PAL* gene expression
in *A. thaliana* after inoculation with Pepper mild mottle virus suggests that *B. amyloquefaciens* induces both SA and JA-pathways (Ahn et al., 2002), and an enhanced expression of SA, JA and ET-dependent genes in *A. thaliana* challenged with *Pst* suggests *B. cereus* AR156 primes host defenses for SAR and ISR (Niu et al., 2011). *Streptomyces* sp. from healthy wheat tissue are also reported to prime for SAR and ISR in *A. thaliana* against *Fusarium oxysporum* or *E. carotovora* subsp. *carotovora* (Conn et al., 2008).

### 1.8.1.3 PAMP activators of SAR

Harpin proteins promote the translocation of T3SS effectors and are associated with a number of effects on plants including induction of SAR (Chen et al., 2008, Dong et al., 1999). There are four harpin genes in the *Pst* DC3000 genome (Cunnac et al., 2009, Kvitko et al., 2007, Preston, 2000). The harpin protein produced by the *hrpN* gene of *Erwinia amylovora* (Wei et al., 1992) has been commercialized and is marketed in the United States under the brand name Messenger by the Eden Bioscience Corporation (Walters & Fountaine, 2009).

Additional PAMP activators of induced resistance include chitosan (such as Elexa marketed by SafeScience), sulfated laminarin, which induces SA accumulation and *PR1* expression, the flg22, and translational elongation factor-Tu (elf18) proteins, which are associated with ROS production and callose accumulation, and LPS, which are specific to gram-negative bacteria (Schreiber & Desveaux, 2008, Walters, 2009). Small proteins secreted by *Pythium* and *Phytophthora* spp, called elicitors, and the *Phytophthora* protein cellulose-binding elicitor lectin are also associated with induced resistance, although their dependence on SA-signaling is not clear (Schreiber & Desveaux, 2008).

### 1.8.1.4 Plant activators of SAR

A number of compounds extracted from a variety of plants can activate SAR. For example, SA is a phenolic compound and plant secondary metabolite that is produced in plants via the isochorismate synthase (ICS)-mediated isochorismate pathway and the phenylalanine ammonia lyase (PAL)-mediated phenylalanine pathway (An & Mou, 2011). Its accumulation in plant cells is usually necessary for the development of SAR (Durrant & Dong, 2004, Yasuda et al., 2003). Exogenous applications of SA to plants elicits SAR (Sticher et al., 1997). Presumably, these applications result in SA accumulation in plant cells that leads to the accumulation of PR proteins associated with SAR. However, SA does not translocate efficiently in plants, and can be phytotoxic to plants if the concentration applied is too high.

Members of the B vitamin group, including thiamine (vitamin B₁), riboflavin (vitamin B₂), and PABA are also associated with SAR (Song et al., 2013, Yang et al., 2011b, Azami-Sardooei et al., 2010, Boubakri et al., 2013a, Dong & Beer, 2000, Liu et al., 2010, Nie & Xu, 2016, Taheri & Tarighi, 2010, Zhang et al., 2009a, Tazhoor, 2014, Ahn et al., 2005, Ahn et al., 2007, Boubakri et al., 2013b). PABA, also known as 4-aminobenzoic acid (4-AA), vitamin H₁, B₆ and B₁₀, is a benzoic acid derivative.
synthesized by plants, fungi, bacteria and protistans as a folic acid precursor (Basset et al., 2004). Endogenous levels of PABA occur in tomato leaves and fruit at the nM level (Basset et al., 2004, Quinlivan et al., 2003). The ability of PABA to reduce symptoms is reported for diseases caused by *Puccinia striiformis* in wheat (Kelman & Cook, 1977), *Xanthomonas axonopodis* pv. *vesicatoria* (*Xe* or *Xp*) for soil drench applications in pepper for up to 77 days (Song et al., 2013), *Pectobacterium carotovorum* subsp. *carotovum* for growth media applications in tobacco seedlings (Yang et al., 2011b), and *Pst* in some tomato breeding lines using foliar applications (Tazhoor, 2014). The efficacy of suppression of *Xe/Xp* using PABA application to growth media was dependent on concentration (Song et al., 2013). PABA was associated with the induction of the SAR marker gene *PR1a* in tomato (Tazhoor, 2014). Likewise, an increase in SA-related gene expression but not JA or ET-dependent gene expression in PABA-treated pepper was observed after pathogen challenge (Song et al., 2013), suggesting PABA primes plants for SAR.

An ethanolic extract of giant knotweed (*R. sachalinensis*) (Milsana / Regalia marketed by Marrone Bio Innovations) is also implicated in SAR (Walters & Fountaine, 2009). The knotweed extract is implicated in induced resistance through induction of phytoalexin and phenolic compound production (Daayf et al., 1997), production of defense-related proteins (Schneider & Ullrich, 1994), accumulation of ROS (Vechet et al., 2005) and increased lignifications and papilla formation in cell walls in a variety of host-pathogen systems (Fofana et al., 2005, Wurms et al., 1999). However, it is not clear if extract from *R. sachalinensis* induces SAR, ISR or another plant defense pathway. Extract from fruit of *Azadirachta indica* also appears to induce resistance in cucumber against *Podosphaera xanthii*, as application resulted in an increase in PAL and tyrosine ammonia lyase activity and phytoalexin accumulation (Aboellil, 2007). Beta-aminobutyric acid (BABA) has also been implicated in SAR (Kunz et al., 1997), but is rarely found naturally in plants and there are conflicting reports on its ability to induce resistance through a SA-dependent manner consistent with other SAR activators (Jakab et al., 2001). Other plant derived activators include azelaic acid (Jung et al., 2009), humic acids (Abdel-Monaim et al., 2011), extracts of *Strobilanthes cusia* (Li et al., 2008) and β-1,4 cellodextrins (Aziz et al., 2007).

1.8.1.5 Synthetic activators of SAR

ASM is a functional analog of SA and has been commercialized as a plant activator by Syngenta Crop Protection (formerly Novartis Crop Protection) for a number of agricultural crops, and is marketed as Actigard in North America and Bion in Europe (Vallad & Goodman, 2004). ASM is a derivative of benzol [1,2,3] thiadiazol-7-carbothioic acid-S-methyl ester (Kunz et al., 1997). ASM was initially found to have the ability to induce plant resistance against a wide range of pathogens, including *Colletotrichum lagenarium* in cucumber, *Pseudomonas lachrymans* in cucumber, *Erysiphe graminis* in wheat, *P.*
infestans and Pst in tomato, Pyricularia oryzae in rice and Peronospora tabacina in tobacco (Kunz et al., 1997). ASM activates SAR by inhibiting the enzymes catalase and ascorbate peroxidase, which results in the generation of ROS within the cell (Wendehenne et al., 1998). Effects of ASM on induced resistance defense enzyme production or gene expression have been observed up to 10 to 22 days after application in tomato (Goodwin et al., 2017b, Cavalcanti et al., 2006), up to 20 days after application in tobacco (Friedrich et al., 1996) and 21 days after application in canola (Potlakayala et al., 2007). Bacterial speck lesion diameter decreases with increasing concentrations of ASM (Scarponi et al., 2001). 2,6-dichloroisonicotinic acid (INA) and its derivatives also function as analogs of SA in the SAR signaling pathway (Oostendorp et al., 2001). Commercialization of INA and SA has not occurred because of problems with phytotoxicity (Oostendorp et al., 2001). However, the widely used systemic neonicotinoid insecticide imidaclorpid breaks down into an analog of INA in planta and induces SAR (Ford et al., 2010).

The chemical 3-allyloxy-1,2-benzothiazole-1,1-oxide (probenazole) and its active metabolite 1,2-benzisothiazol-3 (2H)-one 1,1-dioxide (BIT; saccharin) are also synthetic activators of SAR (Schreiber & Desveaux, 2008, Yoshioka et al., 2001). Based on SA- and NPR1-dependent induced resistance, SAR was demonstrated as the mode of action of probenazole in A. thaliana using plants deficient in SA accumulation, insensitive to JA and ET, and an npr1 mutant (Yoshioka et al., 2001). Probenazole is thought to act upstream of SA accumulation because it failed to stimulate SAR or PR gene expression in NahG plants that cannot accumulate SA (Yoshioka et al., 2001), and a similar pathway was later confirmed in tobacco (Nakashita et al., 2002). Probenazole-dependent SA accumulation in rice may be dependent on plant growth stage, as probenazole increased SA and PR protein accumulation in rice at the 8-leaf stage but not at the 4-leaf stage (Iwai et al., 2007).

3,4-dichloro-N-(2-cyanophenyl)-1,2-thiazole-5-carboxamide (tiadinil), the tiadinil metabolite SV-03, 3-chloro-1-methyl-1H-pyrazole-5-carboxylic acid (CMPA), catechin, and 3-acetyl-3-hydroxyindole (AHO) are also associated with SAR (Schreiber & Desveaux, 2008). Other chemicals reported to induce resistance include phosphate compounds (Reuveni et al., 1997, Schreiber & Desveaux, 2008), micronutrient sprays (Reuveni et al., 1997), metal phthalocyanines (Vol’pina et al., 2000), adipic acid derivatives, sulfamethoxazole, oxalates, trehalose, cholic acid, ergosterol, and syringolin are also reported to induce resistance. However, the mechanisms of induced resistance of these compounds have not been thoroughly examined; therefore it is not clear if SAR or another induced resistance mechanism is implicated in the host response.

1.8.1.6 Plant fitness costs associated with SAR

The defense response induced by SAR depends on the production of PR proteins that require plant nutrients and energy that would otherwise be allocated to plant growth and development, thus SAR is
associated with some plant fitness costs, particularly in a pathogen-free environment (Durrant & Dong, 2004, Walters & Heil, 2007). Reductions in plant growth and development resulting from ASM applications have been reported in sunflower, tobacco, cauliflower, strawberry, melons, pepper and tomato (Walters & Heil, 2007, Lanna-Filho et al., 2017). A single application of the endophytes, B. pumilus and B. amyloliquefaciens, to tomato seeds also resulted in reductions in plant height and total dry weight five weeks after seeding, although the negative effects were not as severe as those of ASM (Lanna-Filho et al., 2017). However, in many other cases no negative effects of activators were found and reported yield was equivalent to the pesticide standard (Walters & Heil, 2007, Vallad & Goodman, 2004). PABA is reported to have various effects on plant growth including stimulation of seed germination in winter wheat and winter barley (Bekusarova et al., 2013), reduction in root length in A. thaliana, and changes in the number of lateral roots of A. thaliana (Crisan et al., 2014). The effects on root structure appear to be concentration dependent, since no effects are reported in another study (Hoang et al., 2007). PABA effects on plant growth may be related to its auxin-like root growth regulating activity (Crisan et al., 2014), but plant growth evaluations using concentrations reported for induced resistance activity have not been completed.

The presence of sufficient or excess resources for plant growth and development likely contributes to a reduction in fitness costs associated with SAR, as this improves the likelihood that plants can obtain the energy and nutrients required for defense responses (Walters & Heil, 2007). The production of the defense related enzymes chitinase, chitosanase and peroxidase in ASM-treated A. thaliana was lower in plants grown under limited nitrogen (Dietrich et al., 2004). ASM-induced A. thaliana initially had reduced rates of growth under the low, medium, and highest nitrogen fertilization regimes, but not the high regime (Dietrich et al., 2005). Later in the study period, plants compensated for this growth reduction by increasing growth rates in the highest nitrogen regime, but not the low and medium regimes. In addition, induced plants produced more seed than nontreated controls during a shortened growing period initiated by seizing watering one week after ASM treatment, but not during a full growing period that lasted 4.5 weeks after ASM treatment. Greater reductions in biomass, shoot, and ear production in ASM-treated wheat were also observed in plants growing in low nitrogen conditions, as compared to nontreated control plants growing in the same conditions (Heil et al., 2000). In A. thaliana, competition between intraspecific neighbours was not found to exacerbate fitness costs associated with SAR (Cipollini, 2002). It may also be possible that some plant species are able to compensate for the increase in inputs required for defense by increasing their rate of photosynthesis (Walters & Fountaine, 2009). However, ASM-dependent activation of the SAR regulatory protein NPR1 by creating monomeric NPR1 is demonstrated to downregulate expression of genes responsible for basic cellular processes such as photosynthesis in rice (Sugano et al., 2010).
Reports of yield losses associated with ASM in tomato under field conditions are limited to a few field trials in fresh market cultivars (Damicone & Trent, 2003, Lange & Smart, 2005, Louws et al., 2001). In those three studies of yield losses, application rates ranged from 26.3 to 35.0 g ASM/Ha, which is more than twice the Canadian label rate of 12.5 g/Ha (PMRA, 2011). In other studies where no statistically significant yield reductions were observed, the rates ranged from 17 to 26.5 ASM/Ha, 10.5 g ASM/Ha, 11.6 to 26.3 g ASM/Ha, 38 g ASM/Ha, 12 g ASM/Ha, and 9.5 g ASM/Ha (Alexander & Waldenmaier, 2003, Graves & Alexander, 2002, Lange et al., 2007, Lewis Ivey et al., 2004, Miller et al., 2002, Roberts et al., 2008, Trueman, 2015). A yield reduction in grafted tomato plants growing in *R. solanacearum* infested soil and treated with foliar ASM is also reported (Kunwar et al., 2017), and in processing tomatoes that received more than eight applications in a season (Pontes et al., 2016). Nevertheless, the observation that ASM-treated plants produced lower yields has probably resulted in a negative image for the use of plant defense activators as a disease management tools.

### 1.8.1.7 Variation in SAR response among cultivars

Plant response to SAR activators results in changes in gene expression, and therefore variation in response to activators among cultivars is not surprising. The severity of white mold symptoms caused by *S. sclerotiorum* was reduced in four soybean cultivars treated with INA or ASM, and the greatest reductions in disease severity were observed in the two highly susceptible cultivars, as compared to the two cultivars, which had greater tolerance to white mold (Dann et al., 1998). In contrast, the concentration of INA required to control powdery mildew in cucumber using INA was lower in a partially resistant cultivar than two susceptible cultivars (Hijwegen & Verhaar, 1994). In pepper, five cultivars with no or partial resistance to bacterial spot (*Xe*) all responded to ASM with equivalent reductions in disease, although yield reductions associated with ASM application varied among cultivars (Romero et al., 2001). The levels of induced resistance by validamycin A and PABA to *F. oxysporum* f. sp. *lycopersici* and *Pst*, respectively, also varied among tomato genotypes (Ishikawa et al., 2007, Tazhoor, 2014). The conclusion from this is that response to SAR activators can vary by plant cultivar, but that may or may not be related to the resistance level of a cultivar to a particular pathogen.

Attempts have been made to relate the degree of response to SAR activators with the level of plant defense gene expression. The expression of tomato *PR1a*, a marker gene for induction of SAR, increased in cv. Supersonic one day after treatment but returned to baseline level three days after treatment, while in cv. Rutgers, there was a similar but relatively greater response, and for cv. Rio Grande, expression also rose after one day, declined more slowly than in the other two cultivars and fell below the nontreated control after seven days (Herman et al., 2007). In all three cultivars, *PR1a* gene expression was higher after a second application of ASM, with cv. Supersonic and cv. Rio Grande having
a similar PR1a response to the first ASM application, but cv. Rutgers having peak PR1a expression two days after the second ASM application and then falling sharply the next day. ASM also activated PR1b gene expression, which is associated with ET-dependent ISR, and followed similar expression patterns as PR1a but at lower levels (Herman et al., 2007). This study did not include a challenge of the plants with Pst or BSX after induction by ASM, thus variation in the field effectiveness of ASM among cultivars was not reported.

Furthermore, Cipollini (2002) observed variation in peroxidase activity among five A. thaliana lines treated with exogenous applications of SA under pathogen-free conditions. Tazhoor (2014) also reported a differential response in tomato breeding lines to applications of PABA, and this was related to a faster and stronger increase in SlPR1a expression after Pst inoculation in the responsive breeding line. These results indicate that although SAR is a common response in plants, genotype differences within plant species may dictate the speed and intensity of a SAR response.

The genetic variation in response to defense activators can be made more complicated by an epigenetic effect, defined as the control of gene expression based on chromatin organization instead of primary DNA sequence information (Bender & Scholz-Schroeder, 2004). Chromation structure can be influenced by the activities of different enzymes that can modify DNA or disrupt its interactions with histones which occur as a response to stress (Alvarez et al., 2010). The induction of SAR might have effects on the plant immunity in subsequent plant generations. Progeny of A. thaliana plants activated for SAR by Pst DC3000 had lower levels of colonization by Hyaloperonospora arabidopsidis and Pst than those that had mock-inoculated parents (Luna et al., 2012). This effect was also observed for second generation progeny inoculated with H. arabidopsidis. Gene expression analysis revealed that PR1a gene expression in the DC3000-inoculated progeny was faster and stronger than in control plants and was NPR1-dependent. Similar results are also reported by Slaughter et al. (2012) who successfully primed A. thaliana progeny against attack by H. arabidopsidis and Pst by applying BABA and an avirulent strain of Pst to a parental line. Furthermore, priming treatment on the first generation progeny resulted in an even stronger priming effect in second-generation progeny challenged with H. arabidopsidis.

1.8.1.8 SAR in disease management

The potential for SAR as a disease management tool has been explored on a variety of economically important plants. For example, ASM reduced the severity of several diseases including downy mildew (Peronoscleropora sorghii) and powdery mildew (Blumeria graminis f. sp. tritici) in wheat, bacterial wildfire (Pseudomonas syringae pv. tabaci) and blue mold (Perenospora hyoscyami f. sp. tabacina) in tobacco, bacterial spot (Xcv) in pepper, white mold in soybean, bacterial blight (Xanthomonas campestris pv. malvacearum) in cotton, white rust (Albugo occidentalis) in spinach, fire blight (E. amylovora) in
apple, and rust (*Gymnosporangium asiaticum*) and scab (*Venturia nashicola*) in pear (Vallad & Goodman, 2004). ASM also reduced post-harvestrots in rockmelon caused by *Fusarium* spp., *Alternaria* spp., and *Rhizopus* spp. (Bokshi et al., 2006), and the severity of rust (*Uromyces viciae-fabae*), ascochyta blight (*Ascochyta fabae*), and broomrape (*Orobanche crenata*) in faba bean (Sillero et al., 2012). However, it did not reduce levels of late leaf spot (*Cercosporidium personatum*) in peanut (Vallad & Goodman, 2004), and its effectiveness at reducing citrus canker on grapefruit trees was dependent on the application rate, interval and timing (Graham & Myers, 2011). The combination of ASM, BABA, and cis-jasmone induced resistance in barley against *Rhynchosporium secalis*, but there was no effect on *PR1* expression, defense-related enzyme production, and the ability of the host to prevent new infection when the elicitor combination was applied to plants with *R. secalis* infections on lower leaves (Walters et al., 2011b).

Probenazole, which is marketed as Oryzemate, is a plant activator used in Asia against rice blast and bacterial leaf blight caused by *Magnaporthe grisea* and *X. oryzae* pv. *oryzae* (Walters & Fountaine, 2009, Yoshioka et al., 2001). Probenazole suppressed symptoms of southern corn leaf blight (*Cochliobolus heterostrophus*) in corn with no adverse effects on plant growth or yield (Yang et al., 2011a). Probenazole also reduced symptoms of tea gray blight (*Pestalotiopsis longiseta*) and anthracnose (*Colletotrichum theae-sinensis*) in field grown tea (Yoshida et al., 2010).

Fewer field studies are available in the literature regarding the field efficacy of INA, imidacloprid and exogenous SA. INA reduced levels of rust (*Uromyces appendiculatus*) in bean, white mold in soybean, alternaria leaf spot, and Verticillium wilt in cotton, but not late leaf spot in peanut (Vallad & Goodman, 2004). Exogenous SA reduced levels of rust but not ascochyta blight and broomrape in faba bean (Sillero et al., 2012). In addition, one or two soil applications of imidacloprid were effective at reducing the severity of citrus canker in grapefruit trees, except during conditions of high disease pressure (Graham & Myers, 2011).

In tomato, ASM reduced the severity of bacterial wilt in greenhouse experiments under conditions of low disease pressure (Anith et al., 2004), but did not reduce symptoms in a later unrelated field study (Hong et al., 2011). In Florida, drip applications of ASM reduced bacterial wilt incidence in non-grafted plants in two trials, but drip ASM applications to grafted plants with tolerant rootstock provided no additional benefits to grafting alone (Kunwar et al., 2017). ASM reduced the severity of leaf mold (*Fulvia fulva*), early blight, anthracnose, and target spot (*Corynespora cassiicola*) (Abbasi et al., 2002, Vallad, 2010, Vallad & Goodman, 2004), but resulted in only a slight reduction in Fusarium crown and root rot (*Fusarium oxysporum* f. sp. *radicis-lycopersici*) (Myresiotis et al., 2012).

### 1.8.1.9 SAR in management of bacterial speck and spot of tomato
The effectiveness of SAR activators to control bacterial spot and speck on tomato has yielded mixed results. Bacterial speck lesion diameter decreases with increasing concentrations of ASM (Scarponi et al., 2001). In a series of efficacy trials across North America in fresh and processing tomato cultivars, ASM reduced the severity of bacterial spot on tomato foliage below that observed in control plots and the standard copper + EBDC treatment in 13 of 14 and 4 of 14 experiments (Louws et al., 2001). Furthermore, ASM reduced the severity of bacterial speck on foliage in six of six and two of six experiments compared to nontreated control plots and the standard bactericide treatment. However, the degree of disease reduction ranged from 17 to 85% for bacterial spot with a mean of 51%, and 23 to 100% for bacterial speck with a mean of 56%. The application rates and application intervals of ASM varied among experiments and ranged from 10.5 to 70.0 g/Ha, and every seven days to every 14 days depending on the location. Another report showed that ASM reduces bacterial spot and speck severity; however disease severity was not always statistically lower than the control and rarely better than standard copper + EBDC treatments (Obradovic et al., 2004, Roberts et al., 2008, Wilson et al., 2002, Trueman, 2015).

Application rates and intervals of ASM may be important in determining its efficacy. In Florida, weekly applications of ASM using rates ranging from 75 to 200 µM effectively reduced disease severity, and increasing rates were inversely related to disease severity against Xp (Huang et al., 2012). Bi-weekly applications of ASM using the same rates were not as effective, and some phytotoxicity symptoms were observed at the high rate of 200 µM. Similarly, in a study in processing tomato and Xg and Xp in Brazil, the ideal application interval for ASM was identified to be between eight and 10 days (Pontes et al., 2016). The current Canadian label dictates that Actigard be applied up to eight times at 7-day intervals at a rate of 12.5 g ASM/Ha, in 280 to 655 L water/Ha, which is equivalent to 91 to 212 µM (PMRA, 2011). The Actigard label for the United States differs from the Canadian label in that it dictates that ASM be applied at 7-day intervals at a rate equivalent to 95 to 158 µM starting to two weeks after transplanting, then 103 to 120 µM at three to four weeks after transplanting, and finally at 108 to 154 µM at five to eight weeks after transplanting (CDMS, 2016).

Although Huang et al. (2012) found a significant inverse relationship between area under the disease progress curve (AUDPC) and ASM concentration in tomato against Xp, this only accounted for 37 and 35 percent of the variation observed, suggesting that other factors also influence the effectiveness of ASM and other defense activators in the field. Plants are subject to a variety of biotic and abiotic stresses, which could influence the degree that SAR can be induced, resulting in priming for a stronger defence response or negatively affecting the defence response due to excessive stress or cross-talk with other signaling pathways such as ISR (Walters, 2009). For example, the efficacy of ASM for reducing B. cinerea infection in tomato was also reduced in tomatoes exposed to wounding, water, and N deficiency stress.
Excessive stress is also indicated by reports of phytotoxicity with ASM. For example, tobacco treated with increased concentrations of ASM had greater SAR against tomato spotted wilt virus, but the highest rate of ASM also caused temporary foliar spotting and stunting of the plants (Mandal et al., 2008). Chlorotic phytotoxicity symptoms were also observed in tobacco treated with ASM, although this problem was corrected with top dressing calcium nitrate fertilizer (Cole, 1999). Therefore, the additional stress due to an SAR activator coupled with biotic and abiotic stresses could reduce the field effectiveness of SAR.

1.8.2 ISR

1.8.2.1 Mechanisms of ISR

ISR is a systemic defense response to suppress pathogen infections after colonization by non-pathogenic root-associated microbes, such as mycorrhizal fungi, plant growth promoting fungi (PGPFs), plant growth promoting rhizobacteria (PGPRs) and bacterial endophytes. ISR is phenotypically similar to SAR (Pieterse et al., 2009a). ISR was first reported by Alstrom (1991) who found a reduction in halo blight on beans after seed treatment with the PGPR, P. fluorescens S97; however, later studies that used root inoculations that spatially separated the beneficial microorganism from a foliar pathogen are more conclusive, as they eliminated the potential for direct antagonism between the organisms and confirmed the systemic nature of the response (van Loon et al., 1998).

A hallmark characteristic of ISR is that the defense system is primed systemically in distal plant parts as a result of root colonization, thus preventing the spread of disease in roots or leaves. Priming is defined as the sensitization of plant tissues to express basal defense mechanisms more rapidly and more strongly following pathogen challenge, as opposed to induction soon after application (Conrath et al., 2006). Priming has been studied in SAR and BABA-induced resistance and is most likely due to the accumulation of inactive protein kinases following treatment with an activator (Beckers et al., 2009). The accumulation of inactive unphosphorylated MPK3 and MPK6 resulted after ASM application in A. thaliana, however, after pathogen challenge MPK3 and MPK6 were phosphorylated. This activation enabled their interaction with downstream targets, thus initiating plant defense gene expression. Furthermore, low levels of SA-dependent transcription factors were induced by BABA, resulting in priming but not expression of SA-inducible defense genes. Priming by riboflavin in A. thaliana is associated with ROS- and Ca2+-signaling dependent pathways involving MPK3 and MPK6 activation (Nie & Xu, 2016).

Unlike in SAR, SA does not accumulate in plants cells during ISR, and NPR1 remains in its oligomer state in the cytoplasm (Van Wees et al., 2008) because of the presence of intermolecular disulfide bonds (Caarls et al., 2015). NPR1 is still required for ISR to occur but its exact role is not
currently understood (Pieterse et al., 2009a). In the absence of JA, JA-responsive genes are repressed within the cell by the formation of a complex between the E3 ubiquitin ligase SCFCOI1 complex, jasmonate ZIM-domain (JAZ) proteins, the adapter protein NINJA, or HDA6 (Caarls et al., 2015, Pieterse et al., 2009b). This represses transcription factors like MYC2, EIN3, and EIL1 (Caarls et al., 2015). When JA accumulates in the cell during induction of ISR, JA-isoleucine (JA-Ile) binds to the F-box protein COI1 in the SCFCOI1 complex, which eventually results in degradation of the JAZ proteins, and activation of JA-responsive genes. There are two JA-dependent signaling pathways. The first pathway is regulated by MYC2, which activates marker genes $VSP2$ and $LOX2$, and is also dependent on ABA. The second pathway is regulated by the transcription factors ET-insensitive 3-like (EIN3), EIL1, and ERF, which activates the marker gene $PDF1.2$, and is also regulated by ET(Caarls et al., 2015). The MED25 enhancer-bound transcription factor in the Mediator complex regulates JA signaling for both pathways (Birkenbihl et al., 2017).

Marker genes for ISR include $PR1b$ for ET-dependent gene expression in tomato (Herman et al., 2007, Block et al., 2005), the plant defensin genes, $PDF1.2$ ($PR12$), $PR3$ and $PR4$, for ET- and JA-dependent gene expression in $A. thaliana$ (Pieterse & van Loon, 1999, Ahn et al., 2007), and the thionin gene, $Thi2.1$ ($PR13$), for JA-dependent gene expression in $A. thaliana$ (Pieterse & van Loon, 1999, van Loon et al., 2006). $Lox1$, $Pal1$, $ETR1$, and $CTR1$ have also been used as markers of JA and ET-signaling in cucumber (Shoresh et al., 2005). The dependence of ET, JA and SA on different PR protein induction can vary by plant species (van Loon et al., 2006). Gene expression is monitored to determine if the priming phenomenon associated with ISR can be identified. In other words, ET- and JA-dependent marker genes are often used to look at the difference in expression before application of the ISR activator, before pathogen infection, and after pathogen infection in activated, inoculated and control plants.

The dependence of ISR on JA and ET signaling was demonstrated in the $A. thaliana$ - $P. putida$ LSW17S system against $Pst$ DC3000 (Ahn et al., 2007). Monitoring the expression of defense-related genes before and after inoculation with DC3000 confirmed that LSW17S primed the system to respond faster to pathogen attack, as demonstrated by the difference in gene expression in LSW17S- and DC3000-only inoculated plants, and mock-inoculated plants. Furthermore, disease suppression remained intact in a $NahG$ plant deficient in SA accumulation, suggesting that induced resistance was not dependent on the SA-signaling pathway (Ahn et al., 2007). Disease suppression was compromised in the mutant $npr1$ which does not accumulate PR1, the $etr1$ mutant with altered perception of ET, and the $jar1$ mutant with reduced sensitivity to JA, indicating dependence on the JA- and ET-signaling pathways (Ahn et al., 2007). A similar priming response by LSW17S was also observed for $Pst$ in tomato, but not for $R. solanacearum$ (Ahn et al., 2011). While most ISR signaling occurs through JA- and ET-dependent pathways, there are a few reports of activation through other signaling pathways by PGPRs (Conn et al., 2011).
The *MYB72* transcription factor gene in *A. thaliana* is also responsive to root colonization by *P. fluorescens* WCS417r and is required in early signaling for ISR (Van der Ent et al., 2008). Transgenic *A. thaliana* lines with constitutive expression of *MYB72* do not express ISR, potentially because at least one other component is required upstream or in parallel with MYB72 transcription factor (Van der Ent et al., 2008). The transcription factor ethylene-insensitive 3, which is linked to the ET-signaling pathway, is one possible candidate, as it was found to interact in vitro with MYB72 (Van der Ent et al., 2008). MYB72 is also required for ISR activation by the beneficial fungi *Trichoderma asperellum* T34, indicating that MYB72 may be an essential transcription factor for ISR for a wide variety of microorganisms (Segarra et al., 2009, Van Wees et al., 2008). The signaling components required to communicate the MYB72 signal to distal plant parts signal are currently unknown.

### 1.8.2.2 Biological activators of ISR

Induced resistance is often associated with PGPRs including *Bacillus* spp., *Pseudomonas* spp., and members of Actinobacteria, such as *Streptomyces*, *Saccharopolyspora* and *Micromonospora* spp. (Bent, 2006, Hallmann & Berg, 2006). *Bacillus* spp. are gram-positive, aerobic, and endospore forming bacteria in the phylum Firmicutes and are ubiquitous in rhizospheric soils (Govindasamy et al., 2010, Kloepper et al., 2004). Common PGPR species that activate ISR include *B. subtilis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Bacillus cereus* (Bent, 2006). Direct antimicrobial effects against pathogens and insects are also reported for certain strains of *Bacillus* spp. (Govindasamy et al., 2010, Kloepper et al., 2004). *Pseudomonas* spp. and *Pseudomonas*-like genera (*Burkholderia*, *Ralstonia*, *Acidovorax*, *Comamonas*) are also ubiquitous in the rhizosphere (Haas & Defago, 2005, Weller, 2007). *Pseudomonas* spp. are members of class Gammaproteobacteria and are gram-negative and aerobic (Haas & Defago, 2005). Common PGPR species that induce ISR are *Pseudomonas aureofaciens*, *Pseudomonas chlororaphis*, *P. fluorescens*, *P. putida*, *P. brassicacearum*, *P. thevervalensis*, and nonpathogenic strains of *P. syringae* (Bent, 2006, McSpadden-Gardener, 2007). Similar to *Bacillus* spp., some *Pseudomonas* strains also directly antagonize plant pathogens and insects (Haas & Defago, 2005, Saveetha et al., 2010, Sevim et al., 2012, Weller, 2007). The Actinobacteria PGPRs that induce ISR are *Streptomyces*, *Saccharopolyspora*, and *Micromonospora* spp. that are filamentous, gram-positive bacteria that are ubiquitous in soils and can also cause direct antagonism against plant pathogens and insects (Conn et al., 2008, El-Tarabily et al., 2010, Pan et al., 2011, Qin et al., 2011, Verma et al., 2011).

The plant defense activation by *Bacillus* spp. is most commonly associated with ISR and is independent of SA and dependent on JA, ET, and NPR1 (Govindasamy et al., 2010, Kloepper et al., 2004). However, specific activation mechanisms differ between strains, host plant and pathogen. In some
cases, induced resistance from *Bacillus* spp. is associated with the SA pathway (Govindasamy et al., 2010, Kloeper et al., 2004, Lanna-Filho et al., 2017). These bacteria may also be antagonistic toward microorganisms and certain insects via the production of antibiotics (Govindasamy et al., 2010).

Many, but not all, PGPRs can invade roots to become endophytes, and these bacteria can also activate ISR. *Rhizobium* spp., which belong to class Alphaproteobacteria and form nodules with plants fixing atmospheric nitrogen (Kado, 2010a), are the best studied bacterial endophytes of plant roots (Zamioudis & Pieterse, 2012). At least one species, *Rhizobium etli*, is capable of inducing ISR in tomato (Martinuz et al., 2012). In an incompatible *Rhizobium*-legume interaction, host resistance (R) proteins recognize *Rhizobium* effectors and activate ETI and nodulation fails to occur. However, in susceptible legume roots, *Rhizobium* secrete microbe associated molecular patterns (MAMPs) which are detected by PRRs (leucine-rich repeat receptor-like-kinases) triggering PTI (also known as microbe-associated molecular pattern triggered immunity; MTI). However, surface polysaccharides and protein effectors released by *Rhizobium* spp. counteract PTI, perhaps resulting in a response analogous to ETS. The recognition of Nod factors secreted by the bacteria results in symbiotic plant gene expression reprogramming, which further prevents PTI. *Rhizobium* peptides produced at infection sites are transported to the shoot and perceived by leucine-rich repeat receptor-like kinases resulting in the production of plant molecules that are transported back to the roots via the phloem that restrict nodule formation. This process is known as autoregulation of nodulation (AON) (Oka-Kira & Kawaguchi, 2006, Staehelin et al., 2011). AON shares similarities with ISR and SAR as all three reactions are triggered by local infection of an organism and result in a systemic response that limits colonization in distal plant parts (Zamioudis & Pieterse, 2012). Studies of *Rhizobium* spp. indicates that the interactions among endophytes and PGPRs that elicit ISR are similar to the way plants interact with pathogens, and involve the recognition of PAMPs and the elicitation of PTI, ETS and ETI (Zamioudis & Pieterse, 2012, Van Wees et al., 2008). Interactions between PGPFs and plants appear to share similarities with endophytes and PGPRs (Zamioudis & Pieterse, 2012).

In tomato, reports of ISR by bacterial endophytes include *Acinetobacter johnsonii*, *S. marcescens*, *Sinorhizobium* sp. and *B. megaterium* that induced resistance against early blight (*A. solani*) and bacterial speck applied to cut stems (Barretti et al., 2009), and *Bacillus pumilus* SE34 that induced resistance against *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Benhamou et al., 1998). For the later, the endophyte induced ultrastructural changes in tomato, such as a the production of root wall appositions and electron-dense substances that are associated with resistance. Inoculation of tomato with endophytes from other plant species can also have positive effects on reducing disease. For example, bacterial endophytes from oilseed rape, grape, watermelon, and papaya reduced wilt symptoms caused by *F. oxysporum* f. sp. *lycopersici* (Nejad & Johnson, 2000) or *R. solanacearum* (Thomas & Upreti, 2014). In most cases, ISR
by bacterial endophytes is assumed; however, in some cases, SAR is suggested based on higher PAL activity (Lanna-Filho et al., 2017), as discussed earlier in this review.

In addition to bacteria, some fungi can also activate ISR. *Trichoderma* spp. colonize the root surface and are also endophytes of roots (Drzużhinina et al., 2011, Shoresh et al., 2010). Induced resistance in *T. asperellum*–cucumber system implicated JA- and ET-dependent signalling in the defence response, which is consistent with ISR (Shoresh et al., 2010). However, gene expression analysis of tomato inoculated with *T. harzianum* 382 revealed upregulation of 36 genes including the SAR marker, *PR5*, but not the ISR markers, *Lox1*, *ETRI*, or *CTR1* (Alfano et al. 2007). Another group of ISR-related fungi are the Sebacinales fungi, such as *Piriformospora indica* (Shoresh et al., 2010, Zamioudis & Pieterse, 2012). *P. indica* is a root colonizing filamentous Basidiomycete that colonizes a wide range of plants, including *A. thaliana* (Zuccaro et al., 2011). Like *R. etli*, *P. indica* may also initially activate plant defenses during root colonization but then suppress PTI and ETI through as of yet not well understood mechanisms (Zamioudis & Pieterse, 2012). A third group of ISR-related fungi are the vesicular arbuscular mycorrhiza (AM) and ectomycorrhiza fungi (ECM) (Brundett, 2006). Reports of ISR associated with AM and ECM include reduction in the number of penetration sites by root-knot nematode (*Meloidogyne incognita*) in tomato in plants treated with the AM fungus, *Glomus mosseae* (Vos et al., 2012), induction of PR genes in potato growing in the presence of AM fungus, *Glomus irregular*, and inoculated with *Fusarium sambucinum* (Ismail & Hijri, 2012), and a reduction in the severity of poplar canker in poplar trees growing in the presence of *G. mosseae* and the ECM *Boletus luridus* (Zhan et al., 2010).

### 1.8.2.3 PAMP activators of ISR

In addition to living cells, bacterial PAMPs can also elicit ISR. The volatile organic compound (2R,3R)-butanediol, released by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a acts as a PAMP to trigger ISR in the *A. thaliana* - *Erwinia carotovora* subsp. *carotovora* system (Ryu et al., 2003). Seedlings exposed to transgenic *B. subtilis* that emitted low levels of (2R,3R)-butanediol had higher disease levels than non-transgenic lines, and had no disease suppressing effect in ET insensitive line but not an JA insensitive line and an *NahG* line, indicating that (2R,3R)-butanediol elicits ISR in *A. thaliana* via an ET-dependent pathway (Ryu et al., 2004). Exogenous applications of (2R,3R)-butanediol, which is produced by some *Bacillus* spp., induced ET-dependent ISR in *A. thaliana* against *E. carotovora* subsp. *carotovora*, in *Nicotiana benthamiana* against *Colletotrichum orbiculare*, and in *Agrostis stolonifera* against *Microdochium nivale*, *Rhizoctonia solani*, and *Sclerotinia homoeocarpa* (Ryu et al., 2004, Cortes-Barco et al., 2010a). The volatile organic compound acetoin (3-hydroxy-2-butanone), also released by *B. subtilis*, is implicated in ISR against *Pst* in *A. thaliana*, and when released by *B. amyloliquefaciens* and *B. subtilis* induced ISR against *E. carotovora* subsp. *carotovora* in *A. thaliana* (Rudrappa et al., 2010, Ryu et
PAMPs are also important characteristics of *Pseudomonas* spp. with the ability to induce resistance in plants, and include flagellins, the main protein of flagella, LPS, iron-regulated metabolites (siderophores), N-alkylated benzylamine derivative, and the phenolic compound 2,4-diacetylphloroglucinol (DAPG) (Bakker et al., 2007, Schreiber & Desveaux, 2008). However, many of these mechanisms are not universal triggers among *Pseudomonas* spp, and the exact mode of action of a *Pseudomonas* spp. may depend on the specific strain, host and pathogen. For example, the flagellins of *P. putida* WCS358 triggered ISR against *Pst* in *A. thaliana* but not in bean or tomato, and a mutant WCS358 lacking flagellin also induced resistance in *A. thaliana* (Gal et al., 2003). Siderophores, which are low-molecular weight Fe^{3+} chelators, also directly compete with plant pathogens for iron acting in another mode for disease suppression (Duffy & Defago, 1999, Weller, 2007). In addition, DAPG is produced by some fluorescent *Pseudomonas* spp. and is an antibiotic that is also associated with induction of ISR, but also acts directly as an antagonist of plant pathogens (Cronin et al., 1997, Notz et al., 2001).

PAMPs produced by fungi that activate ISR are not as well understood as those of PGPRs and bacterial endophytes, but are thought to include chitin, cellulose, cutinase, glucans, xylanase, and the hydrophobin-like elicitor Sm1, peptaibols, swollenin and cerato-platanins (Druzhinina et al., 2011, Schreiber & Desveaux, 2008). Extracts from other organisms may also elicit ISR, as demonstrated by the treatment of *A. thaliana* roots with extracts from the brown microalga, *Ascophyllum nodosum*, which induced resistance against *Pst* and *S. sclerotiorum* in a JA-dependent manner (Subramanian et al., 2011). The plant compounds, galactinol, ET precursor 1-aminocyclopropane-1-carboxylate (ACC), and methyl jasmonate (MeJA) are all linked to ISR (Kim et al., 2008, Pozo et al., 2004, van Wees et al., 1999). Exogenous applications of JA can induce ISR, such as treatment of tomato seeds with JA resulting in increased resistance to *B. cinerea*, which was JA-, ET- and abscisic acid dependent (Worrall et al., 2012).

### 1.8.2.4 Synthetic activators of ISR

The isoparaffin mixture PC1, which is marketed in North America as Civitas, is a mixture of food-grade synthetic isoparaffins and food-grade emulsifiers and does not have direct antimicrobial effects (Cortes-Barco et al., 2010a). Gene expression studies have revealed that reductions in disease severity in *N. benthamiana* against *C. orbiculare*, and in *A. stolonifera* against *M. nivale, R. solani*, and *S. homoeocarpa*, are consistent with ISR, although the mechanisms of priming and gene expression differed from (2R,3R)-butanediol (Cortes-Barco et al., 2010a, Cortes-Barco et al., 2010b). Thus far, the mode of action of Civitas is unknown.

### 1.8.2.5 Plant growth effects associated with ISR

The priming that results from ISR activation has advantages over induction of gene expression and
production of PR and other proteins that occurs with SAR (Pieterse et al., 2009a, Van Wees et al., 2008). van Hulten et al. (2006) reports that fitness costs associated with ISR are not as high as those observed through induction of SAR in *A. thaliana*, because the system is primed and thus no energy is diverted to the production of plant defenses in the absence of a pathogen. Priming may be advantageous in both natural and agricultural systems by reducing the net plant fitness cost associated with defense. In *A. thaliana*, exogenous applications of 10 mg/L and 25 mg/L of BABA, which activates a type of resistance is not controlled by the same signalling pathway as ISR, only primed *PRI* gene expression, while ASM or a higher concentration of BABA (60 mg/L) activated *PRI* gene expression immediately. At the lower levels of BABA, the plants produced the same number of seeds as the water control under disease-free conditions, and the 25 mg/L treatment produced more seeds than the water control when challenged with *P. syringae* or *Hyaloperonospora parasitica* (van Hulten et al., 2006). In contrast, direct induction of *PRI* with ASM or 60 mg L⁻¹ BABA resulted in lower seed production than the water control in disease-free conditions, and no increase in seed production under pathogen challenge. It is tempting to speculate that similar advantages of priming would be extended to ISR.

Repeated applications of *T. harzianum* T39 under disease-free conditions resulted in no negative effects in plant growth or chlorophyll content in greenhouse-grown grapevines, which is in contrast to repeated applications of ASM that negatively affected these parameters (Perazzolli et al., 2011). *T. harzianum* T39 appears to prime the expression of some but not all PR genes. In grapevine treated with T39, systemic expression of *PR2* and *PR10* increased above levels of the nontreated control after inoculation with *Plasmopora viticola*, but systemic expression of *PR4* and *LOX9* increased slightly above levels in control plants prior to pathogen challenge. T39 also upregulated the JA- and ET-dependent signaling pathways, which is also consistent with priming (Perazzolli et al., 2011). However, it is possible that fitness costs could occur with ISR. The effectiveness of mycorrhiza-induced resistance against *B. cinerea* in tomato by the arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, which is similar to ISR, was reduced under low nitrogen (Sanchez-Bel et al., 2016) indicating that host nitrogen status may affect the response to colonization by PGPR and bacterial endophytes.

For bacterial endophytes that induce ISR, there are no reports of fitness costs, and reports of plant growth promotion are frequent (Hardoim et al., 2015), including tomato (Pillay & Nowak, 1997, Barretti et al., 2009, Amaresan et al., 2012, Nava-Diaz, 2006). Plant growth promotion by endophytes is associated with increases in the availability of nitrogen and phosphorous and modulating levels of phytohormones such as IAA, cytokinins, and ET (Hardoim et al., 2015). Plant growth promotion may also be due to competition with plant pathogens, such as the production of antimicrobial compounds, nutrient competition, and siderophore production (Hallmann et al. 1997; Lugtenberg and Kamilova 2009; Rosenblueth and Martinez-Romero 2006). For example, *Bacillus* and *Arthrobacter* species from tomato
caused plant growth promotion and this was associated with IAA and siderophore production, and solubilized inorganic phosphate (Amoresan et al., 2012).

1.8.2.6 Factors affecting ISR

Induction of ISR by competent microorganisms occurs in diverse plant species, but specific plant-microbe interactions are dependent on gene-for-gene interactions among the microorganism and its potential host. The importance of host genotype differences was demonstrated by Ton et al. (1999) who observed that *P. fluorescens* WCS417r failed to trigger ISR in two of 10 *A. thaliana* ecotypes challenged with *Pst*. Reciprocal crosses from the ISR-responsive Col-type and the ISR-nonresponsive RLD revealed that the F₂ segregation pattern for susceptibility to *Pst* in plants growing in *P. fluorescens* WCS417r amended soil was 3:1. Further analysis revealed that inducibility of ISR and relatively high basal resistance against the pathogen were genetically linked at the same locus, *ISR₁*, and the 3:1 segregation is likely the result of the same dominant gene. This gene was later found to cosegregate with a trait for reduced sensitivity to ET but not JA, and to be involved in WCS417r mediated resistance against *Xanthomonas campestris* pv. *amoraciae* and *Phytophthora parasitica* (Ton et al., 2001, Ton et al., 2002).

Cultivar-dependent expression of transcripts encoding defense-related enzymes and PR10a is also reported in wheat (Maketon et al., 2012). The mean expression ratios of eight transcripts were greater in cv. Buchanan than cv. Tara within the first 24 hours after inoculation with the *P. fluorescens* Q8rl-96. Although the study period was relatively short and the gene expression of the wheat cultivars was not monitored after challenge with a pathogen, the results still indicate that host reaction to rhizosphere colonization is dependent on the combination of the host genotype and the bacterial strain (Maketon et al., 2012).

The influence of plant genotype on ISR against *B. cinerea* was also evaluated after *T. harzianum* T22 and *T. atroviride* P1 seed treatment using two inbred processing tomato lines M82 and TA209, the landrace Corbarino, the advanced breeding line SM26, and the Peruvian accession of the wild tomato species *Solanum habrochaites* LA1777 (Tucci et al., 2011). There was a significant interaction between the tomato lines and the *Trichoderma* spp. with regards to disease severity, and further analysis of gene expression revealed that upregulation of marker genes for both SA- and JA-pathways differed among lines.

Root colonization by PGPRs and tissue colonization by endophytes can be affected by genotype. *Pseudomonas* sp. PsJN colonized the tomato root surface more for cultivar Celebrity than for cultivars Blazer, Scotia and Mountain Delight (Pillay & Nowak, 1997). Root surface populations increased linearly with increasing endophyte inoculation concentration from $4.6 \times 10^7$ to $8.8 \times 10^8$ CFU/mL, when seedlings were inoculated by suspending seedlings with trimmed root tops in the endophyte solution for 15 minutes.
Induced resistance was not examined in this study, but there was no relationship between root surface colonization and plant growth promotion. Endophytic colonization of inner root tissues was also highest at a similar inoculum density (4 x 10^1 CFU/mL) but did not vary with tomato genotype. Inoculum levels (3 x 10^6 to 7 x 10^6 CFU/mL) that promoted endophytic colonization were also the best for promoting tomato seedling growth. The ability of the rhizobacteria and endophyte *B. pumilus* SE34 and rhizobacteria *P. fluorescens* 89B61 to induce tomato seedling growth and resistance against *P. infestans* was linearly related to increasing inoculum (ranging from 10^3 to 10^9 CFU/g soil mix) (Yan et al., 2000). The diversity of endophytes colonizing the bacterial wilt resistant tomato cv. Arka Abha was greater than the susceptible cv. Arka Vikas (Upreti & Thomas, 2015), and colonization of the spermosphere by over 20 seed inoculated *B. cereus* strains differed among inbred tomato lines (Simon et al., 2001).

The relationship between microbial populations and the induction of resistance have been contradictory. Pillay and Nowak (1997) proposed that there is a biostimulation threshold within tissues of inoculated tomato plants, which could also apply to PGPRs or bacterial endophytes for ISR induction. Endophyte concentrations ranging from seedling soil drench at 1 x 10^9 CFU/ml for *Azospirillum* sp. 510 (Fujita et al., 2017), seed treatment at 1 x 10^9 CFU/ml for *B. pumilus* SE34 and *B. amyloliquifaciens* IN937a (Ji et al., 2006), and soil drench at 1 x 10^7 CFU/ml for *B. pumilus* SE34 and *B. amyloliquifaciens* IN937a (Ji et al., 2006) induced resistance against *Pst* in tomato. However, it is not clear to what extent host colonization is required to achieve induced resistance (Hardoim et al., 2015). Colonization by the PGPR *S. marcescens* 90-166 and *P. putida* 89B-27 resulted in a greater reduction in *C. orbiculare* disease symptoms in older leaves even though root colonization by these bacteria dropped over time. Endophyte colonization may also be influenced by the timing and method of inoculation. In tomato, seed soak or seed drench (Lanna-Filho et al., 2017, Ji et al., 2006), seedling drench (Fujita et al., 2017), and combinations of seed treatment and soil drenches (Ji et al., 2006) have been used successfully.

Plant developmental stage also influences colonization of plants by microorganisms, although the relationship of this with ISR has not been explored. For example in vetiver, diazotrophic bacterial populations were lower 1 month after transplanting compared to 3, 6, or 12 months (Monteiro et al. 2011). In soybean, the vegetative stage of soybean had higher populations of phosphate solubilizing bacteria compared to the flowering and senescence stages (Kuklinsky-Sobral et al., 2004). *Pseudomonas* spp. and *Actinobacteria* populations were lower at the senescence stage in potato compared to flowering and vegetative stages (van Overbeek and van Elsas 2008), and populations of *B. subtilis* peaked before heading whereas populations of other endophytes in the same wheat plants remained constant throughout all developmental stages (Comby et al., 2016). Thus, endophyte community composition and diversity is dynamic and appears to be dependent on the host developmental stage. This may be due to changes in host defense (Andreote et al., 2010), the level of soluble carbohydrates, calcium and phenolic compounds...
(Hunter et al., 2010), or starch content (Inceoglu et al., 2010).

### 1.8.2.7 ISR as a disease management tool

There are numerous reports from greenhouse and field-level research that implicate ISR in economically viable levels of disease suppression. Many *Bacillus* spp. reduce the severity of a range of diseases and a range of plants species (Govindasamy et al., 2010, Kloepper et al., 2004). Specific examples from greenhouse and field studies using either foliar applications, soil drenches, or seed treatments include *B. pumilus* and *B. mycoides* and cercospora leaf spot (*Cercospora beticola*) on sugarbeet, *Bacillus* spp. and CMV, *B. pumilus* and angular leaf spot (*P. syringae* pv. *lachrymans*) on cucumber, and *B. pumilus* and late blight on tomato (Kloepper et al., 2004). The level of disease reduction is variable within individual *Bacillus* species, with some strains appearing more effective than others (Kloepper et al., 2004). Other examples of ISR-related disease suppression by PGPRs in the field include sheath blight (*R. solani*) of rice by two strains of *P. fluorescens*, bacterial wilt (*Erwinia tracheiphila*) of cucumber by *Serratia marcescens*, angular leaf spot of cucumber by *S. marcescens*, *P. putida*, *Flavomonas oryzae* and *Curtobacterium flaccumfaciens*, anthracnose (*C. orbiculare*) of cucumber by *Burkholderia gladioli* and *C. flaccumfaciens*, blue mold (*P. tabacina*) of tobacco by *P. fluorescens* and *S. marcescens*, botrytis (*B. cinerea*) of beans by *P. aeruginosa*, and leaf spot (*X. campestris* pv. *armoraciae*) of radish by *Panteo agglomerans* (Bent, 2006, Vallad & Goodman, 2004).

For endophytic bacteria, there are also many reports of disease suppression. For examples, screening of 150 isolates revealed that two endophytes closely related to *Pseudomonas rhodesiae* and *Pantoea ananatis* promoted plant growth and induced resistance to *Xe* in one pepper cultivar, although the mechanism of resistance was not explored further (Kang et al., 2007). Similarly, four of 190 bacterial endophytes isolated from watermelon roots in Vietnam were associated with reducing gummy stem blight because of increases in hydrogen peroxide and peroxidase levels (Nga et al., 2010). The ability of endophytes to induce resistance in harvested fruit is also documented in *P. putida* MGY2 and papaya against *Colletotrichum gloeosporioides* because of decreases in fruit symptom and increases in phenylalanine ammonia-lyase, catalase, peroxidase, and phenolics, and expression of *PAL1*, *CAT1*, and *POD* genes (Shi et al., 2011).

Some PGPRs and endophytes have been developed as commercially available products. For endophytes, there is YieldShield (*B. pumilus* strain INR7) (Kloepper et al., 2004, Govindasamy et al., 2010, Kloepper & Ryu, 2006). For PGPRs, there is Ecoguard (*Bacillus licheniformis*), Kodiak (*B. subtilis* GB03), Subtilex (*B. subtilis* MBI600), HiStick (*B. subtilis* MBI600 and *Rhizobium* sp.) (Govindasamy et al., 2010), Bio-Save (*P. syringae* ESC-10), (PMRA, 2010a) and BlightBan A506 (*P. fluorescens* A506) (PMRA, 2009). However, it is possible that several of these PGPRs could also be endophytes. BioYield is
a combination product containing endophyte *B. amyloliquefaciens* IN937a and PGPR *B. subtilis* GB03 (Kloepper et al., 2004, Govindasamy et al., 2010). Although *Pseudomonas* spp. possess a number of positive characteristics such as rapid growth in vitro and a good ability to compete well with other microorganisms, they do not produce endospores like *Bacillus* spp. This has led to challenges in developing formulations that have long shelf lives (Weller, 2007). For example, Biosave can be stored up to one year at 4°C and BlightBan can be stored up to one year at -27°C (PMRA, 2010a, PMRA, 2009).

Commercially available PGPFs are *T. asperellum* T34, *T. harzianum* ATCC 20476, *T. harzianum* Rifai T-22, *T. harzianum* T-39, and *Trichoderma polysporum* ATCC 20475 (EPA, 2012), and *T. harzianum* Rifai strain KRL-AG2, which is marketed in Canada as Rootshield (PMRA, 2010b). *Trichoderma* spp. have issues related to shelf life. For example, Rootshield must be refrigerated and used within twelve months of manufacturing.

### 1.8.2.8 ISR as a disease management tool in tomato

For tomato fungal disease control, twelve PGPF *Trichoderma* spp. isolates reduced the leaf area affected by early blight (*A. solani*) when added to the growing media seven days before pathogen inoculation (Fontenelle et al., 2011). Inoculation of tomato growing media with the bacterial endophytes *Acinetobacter johnsonii*, *S. marcescens*, *Sinorhizobium* sp., and *Bacillus megaterium* also reduced the severity of disease symptoms caused by *A. solani* (Fontenelle et al., 2011). For tomato viral diseases, the endophyte *B. pumilus* SE34 and *B. amyloliquefaciens* IN937a and PGPR *B. subtilis* IN937b reduced severity of CMV and tomato mottle virus (Vallad & Goodman, 2004). For tomato nematode control, soil drench of four endophytic bacteria, *P. agglomerans* MK-29, *Cedeca davisae* MK-30, *Enterobacter* spp. MK-42 and *P. putida* MT-19, reduced juvenile penetration and the number of root galls by *M. incognita* (Valenzuela-Soto et al., 2010, Munif et al., 2001). It thus appears that PGPFs, PGPRs, and endophytes in tomatoes can suppress a variety of diseases and pests, although ISR has not been clearly demonstrated in all cases.

For tomato bacterial disease control, greenhouse experiments demonstrated that seed treatment followed by drenching transplant media with the rhizobacteria *P. putida* 89B61 reduced the severity of bacterial wilt in two experiments. The effectiveness of a formulation containing the rhizobacteria *B. subtilis* GB03 and endophyte *B. amyloliquefaciens* IN973a, and another formulation containing the endophyte *B. pumilus* SE34 was inconsistent (Anith et al., 2004). For *Pst* control, the PGPRs, *P. syringae* TLP2, *P. syringae* Cit7, and *P. fluorescens* A506, were applied to foliage in greenhouse screening and field trials in North America (Wilson et al., 2002). *P. syringae* Cit7 was the most consistent biological control agent evaluated and reduced disease symptoms by an average of 28% in field trials, possibly because of preemptive competitive exclusion on leaf surface (Wilson et al., 2002). However, due to the
numerous reports in the literature reporting the induction of resistance by various *Pseudomonas* spp., it may be possible that these agents also act through ISR mechanisms.

In addition, seed treatment and soil drenching with the PGPRs, *P. putida* 89B61, *B. pasteurii* M38, *B. cereus*, 83-6, *Burkholderia gladioli* IN26, *Stenotrophomonas maltophilia* IN287, *B. cereus* M-22, and endophytes *B. pumilus* SE34, *B. amyloliquifaciens* IN937a, and *B. megaterium* reduced the incidence of *Pst* infection in greenhouse screening assays (Barretti et al., 2009, Ji et al., 2006). Combining 89B61 with *P. syringae* Cit7 resulted in better disease control in one greenhouse experiment, but not a second, compared to 89B61 alone (Ji et al., 2006). In field experiments using the same application methods, Cit7 reduced *Pst* severity in three of three trials, 89B61 was effective in two of three trials, and SE34 was not consistently effective (Ji et al., 2006). The effectiveness of seed treatment following by soil drench with these microorganisms at reducing the severity of BSX was also reported, where SE34, 89B61, and Cit7 all reduced disease severity in two trials. For both *Pst* and BSX control evaluations, the bacteria rarely provided disease control that was equivalent or better than a copper + mancozeb standard (Ji et al., 2006). The disease severity and *Pst* population *in planta* was also reduced in tomato following seedling drench inoculation of the endophyte *Azospirillum* sp. B510 from rice (Fujita et al., 2017).

In a series of field experiments across North America, the efficacy of weekly foliar applications of *P. syringae* Cit7 and *P. putida* B56 were also evaluated for BSX control (Byrne et al., 2005). The severity of BSX symptoms on foliage and fruit was reduced in Cit7 treated tomatoes in five of nine experiments and one of five experiments (Byrne et al., 2005). Disease suppression ranged from 28 to 44% for foliage and 77% for fruit. Likewise, weekly foliar applications of B56 reduced the severity of BSX on foliage and fruit in seven of nine experiments and one of five experiments (Byrne et al., 2005). B56 reduced the severity of BSX from 16 to 34% on foliage, and 67% on fruit. Applications of *B. subtilis* QST 713 reduced the severity of BSX below that of the notreated control in two of four field trials completed in Florida (Roberts et al., 2008). In both cases where it reduced the severity of BSX, it was also equivalent to a copper + mancozeb standard.

In addition to PGPRs, application of a PGPF *Trichoderma* spp. to the growing media tomatoes growing under greenhouse conditions successfully reduced the severity of BSX reducing disease from 24 to 95% for the twelve isolates tested (Fontenelle et al., 2011). Amendments with one isolate successfully reduced disease severity when applied three, seven, 14, or 21 days prior to inoculation with *Xe*.

There are currently no commercially available biocontrol agents marketed as bacterial endophytes for tomato disease management. However, further research in this area may yield benefits as endophytes are well adapted to colonize plants and there may be secondary benefits of endophytes such as plant growth promotion (Mercado-Blanco & Lugtenberg, 2014).
1.8.3 Interactions and relationships between SAR and ISR pathways

A significant level of cross-talk exists between SA/JA, JA/ET, and ET/SA signaling pathways may further complicate the interactions between phytohormone levels and activation of SAR and ISR (Pieterse et al., 2009a). SA is antagonistic toward JA-responsive genes in *A. thaliana* (Pieterse et al., 2009a, Gimenez-Ibanez & Solano, 2013). Suppression of JA/ET defense signaling is linked to an increase in disease symptoms by the necrotrophic fungi *Alternaria brassicola* and *Ramularia collo-cygni* in *A. thaliana* after prior inoculation with *Pst* and in barley after prior application with ASM, BABA, and cis-jasmone, respectively (Spoel et al., 2007, Walters et al., 2011a). In addition, SA and JA are considered positive and negative regulators of stomatal defense, respectively (Melotto et al., 2017). On the other hand, ET-SA signaling and ET-JA signaling are often synergistic, and in the case of ET and JA there is often an integrated response with the same transcription factors mediating a response (Pieterse et al., 2009a).

There is also evidence linking ABA, IAA, GA and cytokinins to the SA, JA and ET defense signaling pathways, and these additional phytohormones can also be modulated by some PGPRs and bacterial endophytes (Hardoim et al., 2008, Pieterse et al., 2009a, Zamioudis & Pieterse, 2012). For example, ABA is reported to weaken JA/ET-dependent gene expression and have a role in JA biosynthesis during the response of *A. thaliana* to attack by *Pythium irregular*, but ABA increased susceptibility of *A. thaliana* to *Pst* by suppressing the induction of SAR (Adie et al., 2007, Mohr & Cahill, 2007). ABA is also considered a positive regulator of stomatal defense (Melotto et al., 2017). GA may interact with the SA-JA-ET network by modulating levels of proteins responsible for promoting susceptibility to biotrophs and resistance to necrotrophs (Navarro et al., 2008).

Combinations of SAR and ISR activators may also lead to a synergistic or antagonistic response. Koornneef et al. (2008) demonstrated that SA is capable of suppressing JA-responsive genes at certain concentrations and timings, suggesting possible antagonisms between the SAR and ISR signaling pathways. Conversely, induction of SAR and ISR in *A. thaliana* resulted in a lower disease index for *Pst* than SAR or ISR plants alone (van Wees et al., 2000) and application of the PGPR *P. putida* 89B61 to amaranthus limited fitness cost effects by ASM (Nair et al., 2007). The complex and not yet fully understood mechanisms of cross-talk between SAR and ISR pathways no doubt contribute to the mixed results of combining SAR and ISR activators.

Combinations of SAR and presumed ISR activators have also been tested under field conditions in tomato. Combinations of ASM + *P. fluorescens* A506 resulted in a reduction in the incidence of *Pst* infections on tomato leaves that was greater than ASM or *P. fluorescens* alone, but the severity of *Pst* on foliage was not lower than ASM alone (Wilson et al., 2002). In a separate experiment, ASM combined
with *P. syringae* Cit7 resulted in lower disease severity than ASM alone on one assessment date, but combinations of ASM + *P. syringae* TLP2 and ASM + *P. fluorescens* A506 provided the same level of disease control as ASM alone (Wilson et al., 2002). Combinations of ASM and a mixture of the PGPR *B. subtilis* GB03 and endophyte *B. amyloliquefaciens* IN937a for *Pst* control in tomato resulted in synergies in only one of three experiments (Herman et al., 2008).

### 1.9 GA-related PGRs

PGRs are chemical compounds that increase or decrease levels of phytohormones in plants upon application resulting in changes in plant growth. Several PGRs have been commercialized and are used in the production of food and ornamental crops. These include the triazole group growth regulators, UNI and paclobutrazol, which are marketed as Sumagic and Bonzi, respectively, to inhibit GA formation, and there are also GA products, marketed as Activol and Falgro.

The triazole-type PGRs are involved in inhibiting the formation of GA in plants (Rademacher, 2000). GAs are implicated in longitudinal shoot growth, germination, bolting, and fruit set and development, thus application of GA inhibitors are applied to food and ornamental crops to prevent stem elongation (Rademacher, 2000). For example, paclobutrazol and UNI prevent stem elongation in greenhouse tomato transplants, which prevents the production of over-sized plants that are difficult to ship and transplant into the field (Zandstra et al., 2006, Agehara & Leskovar, 2017). UNI and paclobutrazol are structurally similar, and both inhibit GA biosynthesis by blocking the cytochrome P-450-dependent monooxygenases, which prevents the oxidation of ent-kaurene into ent-kaurenoic acid in the GA biosynthesis pathway (Rademacher, 2000).

UNI causes many plant physiological responses, including those related to reductions in plant stress. UNI reduced symptoms of chilling injury in tomato, salt stress in *Datura* spp., and drought stress in soybean and wheat (Al-Rumaih & Al-Rumaih, 2007, Duan et al., 2008, Senaratna et al., 1988, Zhang et al., 2007). These stress tolerances are linked to an increase in antioxidant levels, protein content, photosynthetic rate, and chlorophyll content, as well as changes in other phytohormone levels. These effects may be related to the fact that some enzymes involved in GA biosynthesis also cause inhibition of ABA, inhibition of ET, and changes in sterol production which serve multiple functions in plant physiology (Fletcher et al., 2000, Rademacher, 2000, Todoroki et al., 2008). The effects of UNI can be long term, as treatment at the seedling stage increased days to flower in Argyranthemum and scaevola and reduced flower number in calibrochoa, scaevola, and verbana at high concentrations (Blanchard & Runkle, 2007). Thus, PGRs such as UNI can have multiple beneficial effects on plant growth and development.

Conversely, GAs are applied to some agricultural crops to delay ripening, increase shoot
development, reduce tuber size, or avoid long vernalization periods. GA increase fruit size, weight and firmness in cherry, which can enable later harvest without the loss of fruit quality (Canli & Orhan, 2009, Cline & Trought, 2007). Application of GA to potatoes results in smaller tuber size, as tuber initiation is delayed in favour of shoot growth (Lovell & Booth, 1967). Furthermore, applications of GA to rhubarb crown can result in earlier emergence and higher yield in some cultivars (Maynard, 1990).

There is only one report on the effect of a GA-PGRs on the effectiveness of induced resistance against a plant disease. Applications of paclobutrazol with the plant activator ASM reduced symptoms of Pst on tomato leaves under greenhouse conditions to a greater extent than ASM alone, although the mechanism of this synergy was not examined (Mahesaniya, 2002). This is not surprising given the known effects of PGRs on phytohormones, and the association of phytohormones either directly or indirectly with the plant defense system.

In summary, plant genotype, GA-PGRs, activator application method, activator concentration and novel chemical and biological activators all have the potential to increase the effectiveness of induced resistance of tomato against pathogens, such as Pst and BSX. These factors can be explored using controlled conditions and field studies to better understand induced resistance at the whole plant level.

1.10 Hypothesis

Induced resistance of tomato against Pst and BSX can be achieved by using alternative activators, such as PABA and endophytes from tomato, and the level of effectiveness of an activator requires optimization for factors such as application method, concentration and host genotype. In addition, induced resistance of tomato against Pst and BSX by the well-established activator ASM can be improved by combining it with the PGR, UNI, to reduce possible stresses created by ASM.

1.11 Objectives

1. Evaluate the effects of PABA on inducing resistance against Pst and optimize for its effectiveness by evaluating the impact of application method, timing, number of applications, concentration and plant genotype.

2. Examine combinatorial effects of UNI and ASM on reducing the severity of bacterial foliar diseases of tomato under field conditions, and examine changes in plant growth and development associated with the combination.

3. Screen tomato root endophytes for their ability to induce resistance against Pst and optimize their effectiveness by examining inoculation timing, inoculation method, endophyte concentration and host genotype.
Chapter 2: Effects of para-aminobenzoic acid on the incidence of bacterial speck disease, *P. syringae* pv. *tomato* growth, and plant growth in processing tomato

2.1 Introduction

The folic acid precursor PABA, also known as 4-AA, is a benzoic acid derivative synthesized by plants, fungi, bacteria and protistans (Basset et al., 2004). PABA is also known as vitamin H₁, Bₓ and B₁₀ as it is a member of the B vitamin group which also includes thiamine (vitamin B₁) and riboflavin (vitamin B₂). PABA is a vitamin because it is a folate precursor and folates are essential nutrients that cannot be synthesized by animals (Bailey & Gregory, 1999). Synthesis of PABA by plants occurs in chloroplasts, and it is then exported to the cytosol where it is reversibly esterified and then taken up by the mitochondria for folate synthesis (Quinlivan et al., 2003, Basset et al., 2004). Folates are essential cofactors for one-carbon reactions in plants, but endogenous levels of PABA are low, such as at the nM level in tomato leaves and fruit (Quinlivan et al., 2003, Basset et al., 2004).

PABA, as well as the related compounds thiamine and riboflavin, are also associated with SIR in plants, which is broadly defined as enhanced resistance of a plant to a pathogen after being stimulated by a biotic or abiotic agent (Van Loon, 1997). Induced resistance is an attractive alternative to conventional pesticides for plant disease management because many conventional pesticides have negative environmental and human health impacts and there are challenges with effectiveness due to the evolution of pesticide resistance by pathogens (Walters & Fountaine, 2009, Osborn, 2012). The two major types of induced resistance in plants are SAR and ISR. They are distinguished by the phytohormone signaling pathways, which SA for SAR versus ET and JA for ISR (Pieterse et al., 2009a). The two types also differ as biotrophic pathogens tend to be suppressed by SAR whereas necrotrophic pathogens suppressed by ISR (Pieterse et al., 2009a, Ton et al., 2006). Hemibiotrophic pathogens such as those that cause bacterial leaf spots may be suppressed by both SAR and ISR (Ton et al., 2006).

The first report of applying PABA for management of plant diseases was for wheat stripe rust (*Puccinia striiformis*) in China (Kelman & Cook, 1977); however, details on its use pattern and mode of action were not described and so it is not clear whether the researchers considered the control to be due to induced resistance. More recently, soil drench applications of PABA to pepper seedlings reduced the severity of the hemibiotroph *Xe/Xp* in leaves in greenhouse experiments and one field experiment (Song et al., 2013). Induced resistance by PABA was inferred from an increase in expression of defense related genes of pepper by PABA application after inoculation compared to control plants. The form of induced resistance was considered to be SAR rather than ISR since the expression of the SA signalling marker defense genes *CaPR4* and *CaPR9* were primed by PABA, but not the expression of *Pin2*, which is related to JA signalling, or *Tin1*, which is related to ET signalling. Yang et al. (2011) showed that root applied
PABA reduced the severity of the necrotroph, *Pectobacterium carotovorum* subsp. *carotovorutrum*, in tobacco seedlings. PABA also reduced the incidence of the hemibiotrophic bacterial speck pathogen, *Pst* in tomato, which was associated with induction of the SAR marker gene *SlPR1a* after pathogen inoculation but not the induction or priming of ISR marker genes, *SlPin2* and *SlPR2b* (Tazhoor, 2014).

Induction of SAR is sometimes associated with a plant fitness cost (i.e., reduced growth and yield) since the defense response requires nutrients and energy that would otherwise be used for plant growth and development (Durrant and Dong 2004; Walters and Heil 2007). It is unknown if this occurs with PABA. Soaking seeds in a PABA solution stimulated seed germination in winter wheat and winter barley (Bekusarova et al., 2013). *A. thaliana* seeds sown on PABA-amended agar media had reduced root length with increasing PABA concentration, an increase in lateral roots at low concentrations, and a decrease in lateral root growth at higher concentrations possibly due to its auxin-like root growth regulating activity at 0.02 to 2 mM (Crisan et al., 2014). However in another study, *A. thaliana* seeds grown on 0.1 mM PABA amended agar media had no effect on root length (Hoang et al., 2007). Thus, PABA may have effects on the growth and development of plants but these effects appear to be dependent on concentration and may be more related to its plant growth hormone activity. Also, such studies have not tested PABA at the often higher concentrations used for SAR, such as 0.1 to 10 mM in pepper, 1 and 18 mM in tobacco, and 18 mM in tomato (Song et al., 2013, Yang et al., 2011b, Tazhoor, 2014).

Although there have been three studies on the mode of action of PABA and its use under controlled conditions, only one study (Song et al., 2013) tested it under field conditions showing that a single soil drench application of PABA reduced disease severity caused by the hemibiotrophic pathogen *Xe/Xp* and the biotrophic pathogen CMV resulting in increased yield. However, this was done only for one field season. If PABA is to be used in the field, a number of factors should be assessed. The objective of this study was to evaluate these factors including; PABA concentration, plant cultivar, application method, number of applications and pathogen inoculation timing on disease incidence of bacterial speck (*Pst*) on tomato, as well as examine plant growth of PABA-treated plants under growth chamber and field conditions.

### 2.2 Materials & Methods

#### 2.2.1 Direct antimicrobial effects of PABA on *Pst*

For the filter paper antimicrobial assay, 0, 1.0, 9.0, 18.0, and 27.0 mM PABA, and 0, 18.0, and 72.0 mM PABA were prepared by dissolving in distilled water or 70% ethanol, respectively. Filter discs (1.2 or 2.5 cm qualitative P5, Fisher Scientific, Pittsburg, PA) were then soaked in each PABA solution, blotted to prevent dripping, and placed on a TSA plate covered with 100 µL of *Pst* from an overnight TS
broth culture. Plates were incubated for three days and assessed for the presence of a zone of inhibition. The zone of inhibition at three points was measured for each disc and the mean calculated. The experiment was repeated three times, with 10 plates per experiment, each containing one disc of each PABA concentration.

2.2.2 Growth room evaluation of foliar applications of PABA against bacterial speck

2.2.2.1 Optimal concentration

Tomato cv. H9909 was sown in 3.5-inch square pots filled with Fafard germination mix (Fafard et Frères, St. Bonaventure, PQ), covered with vermiculite, and placed in a growth chamber at 24°C, 16 h photoperiod, and with light intensity of approximately 95 µmol/m²/s. The pots were arranged in a randomized complete block with five replications per treatment and one plant per pot per replicate. PABA (MP Biomedicals, Solon, OH) at 0.01, 0.1, 0.5, 1, 4, 9 or 18 mM was applied to the upper side of the leaves until just before runoff at 10 and 15 days after seeding (DAS) using a hand-held mist applicator. Tomatoes were at the cotyledon stage at 10 DAS and the two-true leaf stage at 15 DAS. PABA was mixed in distilled water using a magnetic stir bar for approximately 10 minutes prior to transfer to the applicator. Concentrations greater than 18 mM were not tested because they could not be dissolved. Each pot was fertilized with 80 mL of Plant-Prod Ultimate fertilizer (Plant Products Co Ltd, Brantford, ON) at 10, 15, and 21 DAS using 1.26 g/L.

Plants were inoculated with approximately 2 x 10⁷ CFU/mL of Pst 06T2-4 with 0.01% Sylgard 309 (Dow Corning Canada Inc., Georgetown, ON) at 20 DAS (five days after the second PABA treatment) when the third and fourth compound leaves were developing. Pst 06T2 was originally isolated by Dr. Diane Cuppels from processing tomatoes in southwestern Ontario in 2006 (Cuppels, pers. comm.). Bacterial speck symptoms include foliar lesions, defoliation, early fruit ripening and sunscald (Preston 2000; Young et al. 1978). The upper and lower leaf surfaces of each plant were covered with inoculum until just before runoff using a hand-held mist applicator and covered with a translucent plastic container for 24 hours. The number of bacterial speck lesions on the second and third youngest leaves of each plant was recorded five days post inoculation (DPI). Leaf area was determined by detaching compound leaves, tracing the circumference of each leaflet on a transparency, scanning the image, and determining the number of pixels using Image J v.1.47 (http://imagej.nih.gov/ij/). Pixel number was converted to area (cm²) using the following equation: area = ((0.0171*no. of pixels) + 5.3828) * 0.01. The equation was developed by measuring the numbers of pixels in circles with known areas and creating a standard curve using the number of pixels. The number of lesions per cm² on the whole plant, and the second and third youngest leaves on each plant was calculated. The second youngest leaf was identified using the definition of the youngest leaf as the first developed leaf from the apical meristem with a terminal leaflet.
midrib length of 1.5 cm or longer. Relative chlorophyll measurements were taken from the terminal leaflet of the youngest leaf using a Minolta SPAD-502 Chlorophyll Meter (Konica Minolta, Osaka, Japan).

2.2.2.2 Host genotype effect, foliar PABA application number, foliar PABA response duration and soil PABA application

To evaluate host genotype effects, tomato cvs. H2401, H5108, H9553 and H9909 (H.J. Heinz, Leamington, ON), and cvs. TSH33 and TSH4 (Tomato Solutions, Chatham, ON) were grown, treated with 18 mM PABA and disease evaluated as described previously, except foliar and root dry weights were recorded after fresh samples were placed in a greenhouse for 1 to 2 weeks until dry. Three experiments with five replications per treatment were completed for cv. H2401, cv. H9553, cv. TSH33, and cv. TSH4, and five experiments with five replications per treatment for three of the experiments or four replications per treatment for two of the experiments were completed for cv. H5108 and cv. H9909. The effect of the number applications of PABA on bacterial speck suppression was determined with tomato cvs. TSH33 and H5108. Plants were grown, 18 mM PABA was applied zero, once (15 DAS), twice (10 and 15 DAS) or three times (10, 12, and 15 DAS) and disease was evaluated as described previously. Three experiments were completed with five replications per treatment. The duration of tomato response to PABA was determined with tomato cv. H5108. Plants were grown, treated with 18 mM PABA at 10 and 15 DAS, and inoculated with *Pst* 06T2-4 at five, seven, or 10 days after the second PABA treatment as described previously. Three experiments were completed with five replications per treatment. To evaluate the effect of soil drench applications of PABA, tomato cv. H9909 was grown as described previously, and 10, 20 or 40 mL of 0.0, 1.0, 4.5 and 9.0 mM PABA was applied to the growing media at 10 and 15 DAS. Two experiments were completed with five plants per treatment.

2.2.2.3 *Pst* populations in tomato leaves

Tomato cv. H5108 plants were grown and treated with 18 mM PABA at 10 and 15 DAS and then inoculated at 20 DAS as previously described, except on the day of disease assessment (five DPI), the third terminal leaflet of control and PABA treated plants was collected to determine *Pst* population. Leaf area for each terminal leaflet was determined as previously described. Samples were wrapped in paper towel and stored in plastic bags until processing, which occurred on the same day as sampling. Each sample was weighed, then surface sterilized in 3.1% sodium hypochlorite solution for 90 seconds, 70% alcohol for 60 seconds, and then rinsed in sterile distilled water for 30 seconds. The leaflet sample was then blot dried on filter paper, and homogenized in 2 mL of sterile distilled water using a mortar and pestle. Serial dilutions were then performed. The diluted homogenized leaf solution (100 µL) from dilutions 10⁻² through 10⁻⁶ were plated on Vogel Bonner-tartrate media (VBTar), a semi-selective media
for Pst (Cuppels & Elmhirst, 1999), with two plates per dilution. The rinse water from each sample was also plated on tryptic soy agar (TSA) to confirm the efficacy of the surface sterilization method. Plates were incubated at room temperature, and the number of colonies on each plate recorded after four days. The number of colony forming units (CFU) per cm² of leaf tissue and per lesion was then calculated. The number of CFU per lesion was calculated by dividing the population per cm² by the number of lesions per cm². The trial was repeated three times with five plants per treatment in each trial. To compare Pst populations between symptomatic and asymptomatic regions of tomato leaflets, 0.78 cm² leaf tissue discs were excised from leaflets with bacterial speck lesions and from apparently healthy tissue at least 5 mm away from any visible lesions in plants not treated with PABA. There were five replications per treatment and five leaf discs per replication.

2.2.2.4 Pst lesion sizes in tomato leaves

Tomato cv. H5108 was grown, treated with PABA at 10 and 15 DAS and inoculated with Pst as previously described, except on the day of disease assessment (five DPI), the third terminal leaflet of control and PABA treated plants was collected to determine mean lesion size (Figure A.1). A photo (12.2 MB, 4288 x 2824 pixels) of each leaflet was taken using a Nikon D300s camera (Nikon Canada Inc., Mississauga, ON) set to JPEG fine quality. A ruler was also included within each image. Photos were uploaded to PowerPoint 2010 (Microsoft, Redmond, Washington, U.S.A.), increased in size to allow for tracing of the lesion circumference, printed in colour, and the circumference of 12 to 15 lesions per leaflet traced using a fine point permanent marker. Lesions were selected by tracing all lesions within a randomly placed 225 mm² quadrant, and then 12 to 15 lesions were randomly selected. The lesion circumferences were then scanned and the number of pixels determined as previously described for leaf area, except the size of each image was standardized by calibrating each image. This was completed in Image J by using the line tool to draw a straight line representing a distance of 2 cm on the ruler included in the original image, clicking ‘analyze’ and ‘set scale’, setting the distance in pixels to 40 and the known distance to 2 cm. The experiment was repeated twice with 10 (experiment 1) and seven (experiment 2) plants per treatment.

2.2.3 Field evaluation of foliar applications of PABA

Tomato cv. H5108 seedlings produced using normal grower practices were acquired from a local commercial transplant grower and transplanted into twin-rows in the field using a mechanical transplanter on 26 May 26 2014. Plants were spaced 33 cm within rows, and twin-rows were spaced 75 cm apart on 1.5 m centers. Each plot consisted of one 7 m twin-row. Treatments were arranged in a randomized complete block design with four replications per treatment. Treatments were a nontreated control, Kocide
2000 (E.I. du Pont Canada Co, Missisauga, ON; 53.8% CuOH) with eight foliar applications of 69 mM CuOH for first four applications using 200 L per Ha of water and 46 mM for final four applications using 200 L per Ha on a 7-day interval beginning 3 days after transplanting (DAT), 1 mM PABA with one soil drench application applied 0 DAT, 18 mM PABA with two foliar applications on a 5-day interval beginning 3 DAT, 18 mM PABA with eight foliar applications on a 7-day interval beginning 3 DAT and 18 mM PABA with nine foliar applications on a 5-day interval beginning 3 DAT, and. For PABA treatments of two foliar applications, 200 L water per Ha was applied, whereas for PABA treatments of eight and nine foliar applications, 200 L water per Ha for the first four applications and 300 L/Ha for remaining applications. Foliar CuOH and PABA treatments were applied using a hand-held CO2 sprayer (40 psi) with ULD 120-02 (Pentair Ltd., New Brighton, MN, USA) nozzles for the first four applications and ULD 120-03 (Pentair Ltd., New Brighton, MN, USA) nozzles for the remaining applications. For soil drench PABA application, application water volume was 3 L in transplant bottom trays and then plug cells were incubated in the solution of PABA or water for 60 minutes so that roots, but not stems or foliage, were exposed to the PABA solution. Mean absorption per cell was 3.1 mL. Foliar applications were made on 29 May, 5, 12, 19, 26 June, 3, 10, and 17 July for eight applications at 7-day intervals, on 29 May and 3 June for the two application at 5-day intervals, on 29 May, 3, 8, 12, 18, 25, 30 June, 4 and 9 July for the nine application at 5-day intervals. Soil drench application was on 29 May 2014.

2.2.4 Statistical analysis

Statistical analysis was completed using SAS v9.4 (SAS Institute, Cary, NC, USA). Data were tested for normality using the Shapiro-Wilk statistic. Outliers were identified using Lund’s test of standardized residuals (Lund 1975). Analysis of variance on plant growth and disease incidence data was completed using Proc Mixed with treatment as a fixed effect and replication as a random effect. Data from repeated experiments was pooled together when statistical analysis showed no treatment x experiment interaction ($P \leq 0.05$), except where noted in some tables and figures. Experiment was treated as a fixed effect. Means comparisons were performed when $P \leq 0.05$, and means were separated using Tukey’s HSD. Regression analysis for PABA concentration was completed using Proc Reg to determine model significance and Proc GLM to obtain parameter estimates. The LOG of each PABA concentration tested + 1 was used for regression analysis to obtain a linear curve and allow the calculation of LOG values for all concentrations tested from 0 to 18 mM.

2.3 Results

2.3.1 PABA direct antimicrobial effect
A filter disc assay with a distilled water control and 1.0, 9.0, 18.0 or 27.0 mM PABA dissolved in water did not result in any measurable zone of inhibition of \textit{Pst} (Table 2.1). However, higher PABA concentrations were not able to dissolve completely in distilled water but could be dissolved in ethanol (data not shown). A zone of inhibition was observed with the ethanol control in the filter disc assay, indicating that the ethanol inhibited the growth of \textit{Pst}. Concentrations of 18.0 and 72.0 mM PABA in ethanol resulted in significantly higher zones of inhibition than the ethanol control at 15 and 50\%, respectively. The presence of a zone of inhibition by 18.0 mM PABA greater than the ethanol control but not the water control implies that PABA may have been able to be taken up more readily by \textit{Pst} when associated with ethanol than only distilled water. The larger zone of inhibition for 72.0 mM PABA compared to 18.0 mM PABA in ethanol shows that the antimicrobial effect of PABA was dose dependent. These results indicate that PABA would have no direct antimicrobial effect on \textit{Pst} at 18.0 mM or less in water, and thus PABA at concentrations 18.0 mM or less in water was used in all subsequent experiments.

2.3.2 PABA soil drench application

Soil drench application of 18 mM PABA to cv. H9909 seedlings in growth chambers showed sufficient toxicity to the plants that disease assessment was not possible because the plants were stunted or died (data not shown). Therefore, 1.0, 4.5, and 9.0 mM PABA with each concentration applied at 10, 20, or 40 mL per pot, was used as a soil drench. There was no difference among treatments for any PABA concentration (Figure 2.1). There was no effect of soil applications of PABA on relative chlorophyll (Table A.1) compared to the control.

2.3.3 PABA foliar application

Foliar application to cv. H9909 seedlings in growth chambers with 9.0 and 18.0 mM PABA resulted in symptoms of phytotoxicity approx. three days post treatment (DPT) on true leaves and cotyledon leaves (Figure 2.2 a-c). Foliar application of 0.01, 0.1, 0.5, and 1.0 did not result in phytotoxicity, and symptoms were less severe at 4.0 mM. The number of bacterial speck lesions per cm\(^2\) of leaf tissue on the second and third youngest leaves on each plant was evaluated, which developed after the PABA applications, and thus had not come in direct contact with PABA and had no phytotoxicity symptoms. Results of an ANOVA analysis indicated significant effects of two PABA applications at 10 and 15 DAS on disease incidence at concentrations of 9 or 18 mM. Two applications of PABA to the same leaves were chosen based on preliminary results. Regression analysis of bacterial speck lesion number versus PABA dose indicated a dose response that could be explained by the equation disease incidence = 3.40 –
0.32(\text{\scriptsize LOG10 (PABA concentration)}) (R^2 = 0.53, p < 0.0001) (Figure 2.3). Based on these results, 18 mM PABA was selected for additional experiments since this provided the greatest reduction in disease. There was no effect of PABA concentration on relative chlorophyll readings (Table A.2).

### 2.3.4 Effect of plant genotype on PABA response

Among the six commercial processing tomato cultivars evaluated, PABA reduced bacterial speck lesions per cm\(^2\) of leaf tissue by 28, 27, and 24% in cvs. H5108, H9553 and H9909, but there was no reduction in bacterial speck in cvs. H2401, TSH33 and TSH4 (Figure 2.4). Phytotoxicity to PABA similar to that observed in Figure 2.2a-c was observed on the PABA sprayed leaves for all the cultivars. Foliar and dry root weight was not affected by PABA application in cv. H2401, H5108, H9553 or TSH4, but cv. TSH33 had lower foliar dry weight and cvs. H9909 and TSH33 had lower root dry weights than control plants (Table 2.2). Based on these results, cv. H5108 was used in subsequent experiments because PABA application reduced disease incidence without affecting plant growth, and thus was considered to show increased resistance due to PABA without negative growth effects. Also, cv. TSH33 was used in subsequent experiments because PABA application did not reduce disease incidence but did reduce foliar and root dry weight, and thus was considered to show no change in resistance due to PABA but with growth negatively affected.

### 2.3.5 PABA applications

To determine the effect of the number of foliar PABA applications on bacterial speck suppression, PABA was applied once (15 DAS), twice (10 and 15 DAS) or three times (10, 12 and 15 DAS) before pathogen inoculation. One, two, or three applications of PABA to tomato cv. H5108 all resulted in significant reductions in bacterial speck compared to the control (Figure 2.5a). In contrast, one, two, or three applications of PABA to tomato cv. TSH33 did not reduce the incidence of bacterial speck (Figure 2.5b). Thus, the number of applications did not alter whether a tomato cultivar showed increased disease resistance with PABA treatment.

Detrimental growth effects of PABA were observed for cv. H5108 (Table 2.3). One, two or three PABA applications resulted in a reduction in root dry weight compared to the control, and one or three PABA applications resulted in lower foliar dry weight than the control (Table 2.3). This was unlike the results of the previous experiment with two foliar applications of PABA for root dry weight with this cultivar (Table 2.2). In contrast, no detrimental growth effects of PABA were observed for cv. TSH33 with any number of PABA applications (Table 2.3). Once again, this was inconsistent with previous results that showed lowered foliar and dry root weights with two PABA applications (Table 2.2). It
appears that the effect of PABA treatment on plant growth is variable.

2.3.6 PABA response duration

For the 10 and 15 DAS PABA application method, inoculation of tomato cv. H5108 at five, seven, or 10 days after the second PABA application (15 DAS) showed that bacterial speck lesions per cm² of leaf tissue was reduced when inoculation occurred at five and seven but not 10 days after the second PABA application (Figure 2.6). The effect at five days after the 15 DAS PABA application was consistent with that observed in previous experiments (Figure 2.4 and Figure 2.5). This indicates that the protection provided by PABA had been lost by 10 days after treatment. However, bacterial speck incidence in control plants varied over time increasing between five and seven days and then dropping between seven and 10 days (Figure 2.6). The increased growth of the plants between seven and 10 days versus between five and seven days for both PABA-treated and control plants can be seen in the changes in foliar and root dry weights (Table 2.4). There were no negative effects of PABA on foliar or root dry weight, which is consistent with the results for cv. H5108 from the host genotype evaluation (Table 2.2), but different from the results of the evaluations that assessed the effects of the number of PABA applications on host response (Table 2.3).

2.3.7 PABA effect on Pst populations and lesions

Even though bacterial speck lesions per cm² of leaf tissue in PABA treated plants was significantly lower than the non-treated control, the Pst population per cm² of leaf tissue in PABA treated plants was equivalent to the non-treated control (Table 2.5). Thus, the fewer number of lesions was not reflected in the presence of fewer cells of the pathogen when Pst population in whole leaflets was assessed. When the population was examined on a per lesion basis, the Pst population per lesion was 38% higher in PABA treated plants compared to the non-treated control. This indicates that the average lesion in PABA treated leaves contained more bacteria than the average lesion in the control leaves. One possibility is that Pst populations with lesions are similar between PABA and no PABA treated leaves, but Pst populations outside of the lesions (i.e., asymptomatic tissue) could be different. A comparison of Pst populations in no PABA treated inoculated leaves between symptomatic (i.e., bacterial speck lesions) and asymptomatic leaf tissue indicated that symptomatic tissue had Pst populations three orders of magnitude higher (5.12 x 10⁸ CFU per cm² leaf tissue) than asymptomatic tissue (1.12 x 10⁵ CFU per cm² leaf tissue) from the same leaflets (p = 0.0076, sem = 0.645). Thus, lesions made the greatest contribution to total Pst population in a leaflet.

Even though PABA treatment reduced the incidence of bacterial speck lesions per cm² of leaf
tissue (Figure 2.7a), mean lesion size in PABA treated plants was 94% larger than those on control plants (Figure 2.7b). However, the fewer number of these larger lesions resulted in no significant difference in the percentage of total leaf surface area with lesions between the PABA and control treatments (Figure 2.7d). Lesion circumference was also calculated since population growth of \( \textit{Pst} \), which is a hemibiotroph, is likely highest near lesion edges. Mean lesion circumference was 28% greater when PABA was applied compared to lesions from control plants (Figure 2.7c). These results suggest that PABA is suppressing initial infections so that fewer lesions develop, but after initial infection, the lesions of PABA treated plants reached a larger size by five DPI.

2.3.8 Field evaluation

In 2014, PABA was applied to foliage 2, 8 or 9 times, or one time as a plug seedling soak, to tomato cv. H5108. No symptoms of phytotoxicity were observed when PABA was applied to foliage, but phytotoxicity was observed after drench applications of 1 mM PABA. Affected plants were randomly distributed within treatment plots and had a noticeably purple colour with stunting.

Early season bacterial speck incidence was measured from mid-June to early July as the percentage of symptomatic leaves in a 1.24 m\(^2\) area in each plot. None of the PABA treatments reduced disease incidence during the early season assessment period compared to the non-treated control on any of the assessment dates (Figure 2.8), nor was there any significant effect on early season AUDPC (Table 2.6). The CuOH standard also showed no differences from non-treated control on any assessment date (Figure 2.8) or early season AUDPC (Table 2.6). Late season disease severity was measured as the percent defoliation per plot from mid-July to late-Aug. Similar to the results with early season disease severity, there was no effect of PABA on late season disease severity compared to the non-treated control on any assessment date (Figure 2.9), or for late season AUDPC (Table 2.6). The CuOH standard was also ineffective.

While bacterial speck incidence on tomato fruit with all the methods of foliar PABA applications were numerically lower than the non-treated control, there were no significant differences among treatments (Table 2.7). The CuOH standard was also ineffective compared to the non-treated control. Bacterial spot (\( \textit{X. gardneri} \)) symptoms on tomato fruit were also detected, and even though the nine PABA applications resulted in 55% of the disease incidence of the control, there were no significant differences among PABA treatments and the non-treated control. This was related to high variability among plots. The CuOH standard was once again ineffective. The presence of bacterial spot on fruit is a strong indicator that some of the bacterial disease symptoms on tomato foliage in the trial were caused by \( \textit{X. gardneri} \) as well as \( \textit{Pst} \).

Relative chlorophyll was measured 24, 30 and 36 DAT. None of the PABA treatments had any
effect on relative chlorophyll content compared to the non-treated control, and the CuOH standard treatment also did not affect relative chlorophyll content compared to the non-treated control (Figure 2.10). Similarly, there was no significant effect of any PABA treatments on total, red, green or rotten tomato fruit yield compared to the non-treated control (Table 2.8). There was a trend toward higher green fruit yield compared to the non-treated control with the PABA seedling soak, but this was likely due to symptoms of phytotoxicity in the PABA seedling soak treatment that delayed growth. The CuOH standard was also ineffective in altering total, red, green or rotten tomato fruit yield compared to the non-treated control.
Table 2.1 Effect of PABA concentration on growth of *P. syringae* pv. *tomato* (*Pst*) in a filter disc assay. Filter discs soaked in 0, 1, 9, 18, and 27 mM para-aminobenzoic acid (PABA) dissolved in water, and 0, 18, and 72 mM PABA dissolved in 70% ethanol were placed on tryptic soy agar covered thoroughly with *Pst*, incubated at room temperature, and assessed for zones after three days.

<table>
<thead>
<tr>
<th>PABA (mM)</th>
<th>Water</th>
<th>Ethanol (70%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0 a</td>
<td>2.0 c</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0 a</td>
<td>-</td>
</tr>
<tr>
<td>9.0</td>
<td>0.0 a</td>
<td>-</td>
</tr>
<tr>
<td>18.0</td>
<td>0.0 a</td>
<td>2.3 b</td>
</tr>
<tr>
<td>27.0</td>
<td>0.0 a</td>
<td>-</td>
</tr>
<tr>
<td>72.0</td>
<td>-</td>
<td>3.0 a</td>
</tr>
</tbody>
</table>

\[ \text{sem}^b \]

0.0  0.11

\[ ^a \text{Means in the same column followed by the same letter are not significantly different at P \leq 0.05, Tukey’s HSD. Data from three independent trials with 10 replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.} \]

\[ ^b \text{sem = standard error of the mean for all ls means in the same column.} \]
Figure 2.1 Effect of different para-aminobenzoic acid (PABA) concentrations and application volumes on the systemic acquired resistance response in tomato cv. H9909 inoculated with *P. syringae pv. tomato* (*Pst*). A pipette was used to apply 10 (−−), 20 (−−−), or 40 (−−−−) mL PABA to the root zone 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* 20 DAS. Disease incidence five days post inoculation is shown. Data points with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Figure 2.2 Phytotoxicity symptoms on tomato a) true leaf, and b) and c) cotyledon leaves coated to runoff with 18 mM PABA at 10 and 15 days after seeding.
Figure 2.3  Effect of different para-aminobenzoic acid (PABA) concentrations on the systemic acquired resistance response in tomato cv. H9909 inoculated with *P. syringae* pv. *tomato* (*Pst*). Plants were coated with 0, 0.01, 0.1, 0.5, 1, 4, 9, or 18 mM PABA (LOG + 1 = 1, 0.0043, 0.0412, 0.1761, 0.3010, 0.6990, 1.000, 1.2787 mM PABA) 10 and 15 days after seeding (DAS), and then plants were coated with a fine mist of 2 x 10^7 CFU/ml of *Pst* 20 DAS. Disease incidence five days post inoculation is shown. Errors bars represent standard error of the mean. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Figure 2.4 Incidence of bacterial speck symptoms on the second and third youngest leaves of six tomato cultivars treated with para-aminobenzoic acid (PABA). Plants were coated with 18 mM PABA 10 and 15 DAS. Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *P. syringae pv. tomato* (*Pst*) 20 days after seeding (DAS). Disease incidence five days post inoculation is shown for the nontreated control (■) and PABA (□). Errors bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials for cvs. H2401, H9553, TSH33, and TSH4 with five replications of each treatment, and five independent trials for cv. H5108 and cv. H9909 with five or four replications of each treatment, was pooled together because ANOVA showed no treatment x trial interaction. One outlier was removed each data set for cv. H9909 and cv. H2401.
Table 2.2 Foliar, root, and total dry weight of six tomato cultivars treated with para-aminobenzoic acid (PABA). Plants were coated with 18 mM PABA 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of 2 x 10^7 CFU/ml of *P. syringae* pv. *tomato* 20 DAS. Dry weight of plants five days post inoculation is shown.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Foliage</th>
<th></th>
<th>Roots</th>
<th></th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No PABA</td>
<td>PABA sem a</td>
<td>No PABA</td>
<td>PABA sem</td>
<td>No PABA</td>
<td>PABA sem</td>
</tr>
<tr>
<td>H2401</td>
<td>167.7 a b</td>
<td>152.6 a 18.6</td>
<td>67.2 a 62.3 a 9.2</td>
<td>234.9 a 214.9 a 26.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H5108</td>
<td>228.2 a 199.5 a 19.5</td>
<td>93.0 a 75.2 a 9.3</td>
<td>321.3 a 275.0 a 26.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9553</td>
<td>206.7 a 192.9 a 19.0</td>
<td>82.0 a 75.5 a 9.4</td>
<td>288.7 a 268.4 a 27.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H9909</td>
<td>229.2 a 215.0 a 20.5</td>
<td>81.1 a 59.0 b 7.4</td>
<td>310.6 a 274.0 a 26.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH33</td>
<td>191.4 a 123.8 b 15.0</td>
<td>79.7 a 45.9 b 7.5</td>
<td>271.1 a 169.7 b 22.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH4</td>
<td>314.3 a 281.8 a 17.2</td>
<td>145.5 a 115.2 a 11.4</td>
<td>459.8 a 396.9 a 26.6</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a sem = standard error of the mean for all ls means in the same row for the same variable.

b Means in the same row and group followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials for with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Figure 2.5 Incidence of bacterial speck symptoms on the second and third youngest leaves of tomato cultivars a) cv. H5108, and b) cv. TSH33 treated with zero, one (15 days after seeding (DAS)), two (10 and 15 DAS), or three (10, 12, and 15 DAS) applications of para-aminobenzoic acid (PABA). Foliar PABA treatments (18mM) were applied by coating a fine mist on plants. Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *P. syringae* pv. *tomato* five days after the last PABA application. Disease incidence five days post inoculation is shown. Errors bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Data for cv. H5108 was log transformed to meet assumptions of ANOVA.
Table 2.3 Foliar, root, and total dry weight of tomato cv. H5108 and cv. TSH33 treated with zero, one, two, or three applications of para-aminobenzoic acid (PABA). One, two, or three foliar PABA treatments (18mM) were applied by coating a fine mist on plants 10, 12, and/or 15 days after seeding (DAS). Plants were coated with a fine mist of \(2 \times 10^7\) CFU/ml of \(P.\ syringae\ pv.\ tomato\) five days after the last PABA application. Dry weight of plants five days post inoculation is shown.

<table>
<thead>
<tr>
<th>Application Timing</th>
<th>Foliage Dry weight (mg/plant)</th>
<th>Root Dry weight (mg/plant)</th>
<th>Total Dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H5108</td>
<td>TSH33 (^a)</td>
<td>H5108</td>
</tr>
<tr>
<td>No PABA</td>
<td>125.4 a (^b)</td>
<td>87.0 a</td>
<td>51.1 a</td>
</tr>
<tr>
<td>15 DAS</td>
<td>89.3 b</td>
<td>84.8 a</td>
<td>35.5 b</td>
</tr>
<tr>
<td>10 and 15 DAS</td>
<td>94.4 ab</td>
<td>72.4 a</td>
<td>37.8 b</td>
</tr>
<tr>
<td>10, 12, and 15 DAS</td>
<td>88.1 b</td>
<td>72.3 a</td>
<td>38.8 b</td>
</tr>
</tbody>
</table>

\(^a\) One outlier was removed from the cv. TSH33 foliar dry weight data and total dry weight data. There was a significant trial*trt interaction for cv. TSH33 pooled foliar and total dry weight, however, data was pooled because treatment effects within trials were weak and inconsistent.

\(^b\) Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD. Data from three independent trials for with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

\(^c\) sem = standard error of the mean for all ls means in the same column.
Figure 2.6 Incidence of bacterial speck symptoms on the second and third youngest leaves of tomato cv. H5108 inoculated with *P. syringae* pv. *tomato* (*Pst*) five, seven, or 10 days after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 DAS). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* five, seven, or 10 days after the second PABA application. Disease incidence five days post inoculation is shown for the nontreated control (–) and PABA (–). Errors bars represent standard error of the mean. Data points with the same letter for the same inoculation timing are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. One outlier was removed from the analysis for the data from seven days after the last PABA application.
Table 2.4 Foliar, root, and total dry weight of tomato cv. H5108 inoculated with *P. syringae* pv. *tomato* (*Pst*) five, seven, or 10 days after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 days after seeding (DAS)). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* five, seven, or 10 days after the second PABA application. Dry weight of plants five days post inoculation is shown.

| Inoculation timing (# days after last PABA application) | Foliage | Dry weight (mg/plant) | | | | Roots | | | | Total | | | |
| | No PABA | PABA | sem | No PABA | PABA | sem | No PABA | PABA | sem | | | | |
| 5 | 131.9 a | 124.2 a | 11.8 | 54.8 a | 51.9 a | 6.0 | 186.7 a | 176.1 a | 17.6 | | | |
| 7 | 156.3 a | 138.8 a | 11.9 | 68.9 a | 58.6 a | 5.6 | 225.2 a | 197.4 a | 17.2 | | | |
| 10 | 323.4 a | 284.4 a | 27.1 | 175.7 a | 148.3 a | 17.3 | 499.1 a | 432.7 a | 43.2 | | | |

* a sem = standard error of the mean for all ls means in the same column.

b Means in the same row and group followed by the same letter are not significantly different at $P \leq 0.05$, Tukey's HSD. Data from three independent trials for with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Table 2.5 Incidence of bacterial speck symptoms, population of *P. syringae pv. tomato* (*Pst*) per cm$^2$, and per lesion on the third youngest terminal leaflet of tomato cv. H5108 after para-aminobenzoic acid (PABA) treatment. Two foliar PABA treatments (18 mM) were applied by coating a fine mist on plants beginning at the cotyledon stage (10 and 15 days after seeding (DAS)). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* five days after the second PABA application.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease incidence (lesions/cm$^2$) $^a$</th>
<th>Population (CFU/cm$^2$)</th>
<th>Population (CFU/lesion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No PABA</td>
<td>4.7 a</td>
<td>$1.05 \times 10^6$ a</td>
<td>$3.16 \times 10^1$ b</td>
</tr>
<tr>
<td>PABA</td>
<td>3.2 b</td>
<td>$1.55 \times 10^6$ a</td>
<td>$1.17 \times 10^2$ a</td>
</tr>
</tbody>
</table>

$^a$ Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. There were two missing plots for the control due to poor germination and a sampling error.

$^b$ sem = standard error of the mean (LOG10) for all ls means in the same column, except for No PABA, which was 0.37, 0.12, and 0.16 for disease incidence, population per cm$^2$, and population per lesion.
Figure 2.7 The effect foliar applications of para-aminobenzoic acid (PABA) on a) the incidence of bacterial speck symptoms, b) mean lesion size, c) mean lesion circumference, and d) percent leaf area with lesions on the third youngest terminal leaflet of tomato cv. H5108. Foliar PABA treatments (18mM) were applied 10 and 15 days after seeding (DAS) by coating a fine mist on plants. Plants were coated with a fine mist of 2 x 10⁷ CFU/ml of *P. syringae* pv. *tomato* five days after the last PABA application. Disease incidence and mean lesion size of 10 to 15 lesions is
shown for the nontreated control (■) and PABA (▲). Error bars represent standard error of the mean. Bars with the same letter for the same cultivar are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from two independent trials with 10 (trial 1) and seven (trial 2) replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Figure 2.8 Early season disease progress of bacterial speck symptoms in tomato cv. H5108 treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae* pv. *tomato*, Ridgetown, ON. The percentage of leaves with disease symptoms in the nontreated control ( ), eight applications of CuOH at 7-day intervals ( ), eight applications of PABA at 7-day intervals ( ), two applications of PABA at 5-day intervals ( ), nine applications of PABA at 5-day intervals ( ), and seedlings soaked in PABA for one hour before transplanting ( ) in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Table 2.6. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Figure 2.9 Late season disease progress of bacterial speck symptoms in tomato cv. H5108 treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato*, Ridgetown, ON. Defoliation in the nontreated control (→), eight applications of CuOH at 7-day intervals (←), eight applications of PABA at 7-day intervals (→), two applications of PABA at 5-day intervals (←), nine applications of PABA at 5-day intervals (←), and seedlings soaked in PABA for one hour before transplanting (→) in whole plots is shown. The corresponding area under the disease progress curve is shown in Table 2.6. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Table 2.6 Area under the disease progress curve (AUDPC) for early and late disease in tomato cv. H5108. Early season disease was measured by calculating the percentage of leaves with disease symptoms and late season disease was measured by estimating defoliation in plants treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato*, Ridgetown, ON, in 2014.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of applications</th>
<th>Application interval (days)</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Early season</td>
</tr>
<tr>
<td>Nontreated control</td>
<td>-</td>
<td>-</td>
<td>319 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>8</td>
<td>7</td>
<td>263 a</td>
</tr>
<tr>
<td>PABA</td>
<td>8</td>
<td>7</td>
<td>330 a</td>
</tr>
<tr>
<td>PABA</td>
<td>2</td>
<td>5</td>
<td>352 a</td>
</tr>
<tr>
<td>PABA</td>
<td>9</td>
<td>5</td>
<td>349 a</td>
</tr>
<tr>
<td>PABA</td>
<td>1</td>
<td>seedling soak</td>
<td>316 a</td>
</tr>
</tbody>
</table>

| sem c              | 66.0                | 223.2                      |

*a* All treatments were applied to tomato foliage except for the seedling soak. Tomato seedlings in the soak treatment were soaked in a PABA solution for one hour just prior to transplanting. CuOH was applied at 69 mM for the first four applications and 46 mM for the final four applications because of increasing water volume. Foliar PABA was applied using 18 mM and the seedling soak using 1 mM.

*b* Means in the same column followed by the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD.

*c* sem = standard error of the mean for all ls means in the same column.
Table 2.7 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. H5108, harvested from plots treated with CuOH or para-aminobenzoic acid (PABA) and inoculated with *P. syringae pv. tomato*, Ridgetown, ON, 2014. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of applications</th>
<th>Application interval (days) a</th>
<th>Incidence (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Speck</td>
<td>Spot</td>
</tr>
<tr>
<td>Nontreated control</td>
<td>-</td>
<td>-</td>
<td>23.5 a b</td>
<td>18.0 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>8</td>
<td>7</td>
<td>18.5 a</td>
<td>19.0 a</td>
</tr>
<tr>
<td>PABA</td>
<td>8</td>
<td>7</td>
<td>21.5 a</td>
<td>13.0 a</td>
</tr>
<tr>
<td>PABA</td>
<td>2</td>
<td>5</td>
<td>22.5 a</td>
<td>16.0 a</td>
</tr>
<tr>
<td>PABA</td>
<td>9</td>
<td>5</td>
<td>17.0 a</td>
<td>10.0 a</td>
</tr>
<tr>
<td>PABA</td>
<td>1</td>
<td>seedling soak</td>
<td>19.5 a</td>
<td>17.0 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>7.39</td>
<td>4.72</td>
</tr>
</tbody>
</table>

a All treatments were applied to tomato foliage except for the seedling soak. Tomato seedlings in the soak treatment were soaked in a PABA solution for one hour just prior to transplanting.

b Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.

c sem = standard error of the mean for all ls means in the same column.
Figure 2.10 Relative chlorophyll measured 24, 30, and 36 days after transplanting in tomato cv. H5108 treated with CuOH and para-aminobenzoic acid (PABA) and inoculated with *P. syringae* pv. *tomato* in Ridgetown, ON, 2014. SPAD readings in the nontreated control ( ), eight applications of CuOH at 7-day intervals ( ), eight applications of PABA at 7-day intervals ( ), two applications of PABA at 5-day intervals ( ), nine applications of PABA at 5-day intervals ( ), and seedlings soaked in PABA for one hour before transplanting ( ) in whole plots is shown. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Table 2.8 Total, red, green, and rotten fruit yield in a 2m section of tomato cv. H5108 treated with CuOH and para-aminobenzoic acid (PABA) and inoculated with *P. syringae* pv. *tomato*, Ridgetown, ON, in 2014.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of applications</th>
<th>Application interval (days)</th>
<th>Yield (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Nontreated control</td>
<td>-</td>
<td>-</td>
<td>33.06 a b</td>
</tr>
<tr>
<td>CuOH</td>
<td>8</td>
<td>7</td>
<td>34.46 a</td>
</tr>
<tr>
<td>PABA</td>
<td>8</td>
<td>7</td>
<td>34.85 a</td>
</tr>
<tr>
<td>PABA</td>
<td>2</td>
<td>5</td>
<td>31.12 a</td>
</tr>
<tr>
<td>PABA</td>
<td>9</td>
<td>5</td>
<td>31.33 a</td>
</tr>
<tr>
<td>PABA</td>
<td>1</td>
<td>seedling soak</td>
<td>30.16 a</td>
</tr>
</tbody>
</table>

\( a \) All treatments were applied to tomato foliage except for the seedling soak. Tomato seedlings in the soak treatment were soaked in a PABA solution for one hour just prior to transplanting.

\( b \) Means in the same column followed by the same letter are not significantly different at \( P \leq 0.05 \), Tukey’s HSD.

\( c \) sem = standard error of the mean for all ls means in the same column.
2.4 Discussion

This study demonstrated that foliar applications of PABA can be effective in reducing disease incidence of bacterial speck when tomatoes were grown in growth room conditions. This confirms the effectiveness of PABA under controlled conditions previously reported for a variety of diseases of tomato (Tazhoor, 2014), tobacco (Yang et al., 2011b) and pepper (Song et al., 2013). However, PABA was ineffective in controlling bacterial speck under field conditions, unlike the field control reported with PABA against *P. striiformis* in wheat (Kelman & Cook, 1977) and *Xe/Xp* in pepper (Song et al., 2013). This study also demonstrated that the ability of PABA to suppress bacterial speck of tomato was greatly affected by a number of factors, such as PABA concentration, application method, host genotype, plant age and inoculation timing. Thus, comparison among studies is difficult to make unless such factors are analyzed and then the conditions optimized for PABA efficacy.

2.4.1 Direct antimicrobial effects of PABA

The solubility of PABA in water is 43 mM at 25°C versus 911 mM in alcohol (Anonymous, 2013). Thus, 18.0 mM should be soluble in water, whereas 72.0 mM should not. In water, no toxicity to *Pst* was observed with up to 27 mM PABA, but in ethanol, 18 and 72 mM PABA produced zones of inhibition greater than the control, which also inhibited the pathogen. This provides the first evidence that PABA directly affects *Pst* growth probably in a dose dependent manner. However, the extent of inhibition, which was 23% for 18 mM and 33% for 72 mM was slight. There are reports in the literature that PABA has antimicrobial effects against other bacteria. For example, growth of *Listeria monocytogenes*, *Salmonella* sp., and *E. coli* was inhibited at 9 to 24 mM PABA dissolved in brain heart infusion broth (Richards et al., 1995) and 37 to 111 mM PABA dissolved in edible films (Cagri et al., 2001). Direct antimicrobial effects against *Xe/Xp* were not observed at 0.1 to 10 mM, but the solvent was not specified (Song et al., 2013). It is likely that the mode of action observed *in vivo* in this study is a result of induced resistance because the dose of 18 mM PABA in water caused no direct growth inhibition of *Pst* in vitro. This is also supported by the application of PABA to different leaves than were assessed for bacterial speck incidence, and so there was no direct contact between PABA and the *Pst* populations in leaf tissue examined.

2.4.2 PABA concentrations and application methods

PABA applied as a soil drench was toxic to tomato seedlings at 18 mM, but not at 1 to 9 mM. However, there was no effect on disease incidence using 1 to 9 mM PABA as a soil drench, which is equivalent to 1.4 and 49.4 mg PABA per seedling. This contradicts Song et al. (2013), who showed that
0.1 to 13.7 mg PABA per seedling applied to agar growth medium reduced the severity of disease caused by \textit{Xe/Xp} in peppers, and Yang et al. (2011b), who found that 0.0014 to 0.0247 mg PABA per seedling applied to agar growth medium was effective against \textit{P. carotovorotum} subsp. \textit{carotovorotum} in tobacco. Both Song et al. (2013) and Yang et al. (2011b) did not report any phytotoxicity due to PABA. Thus, similar or even higher amount of PABA applied per seedling in the current study was ineffective against \textit{Pst} in tomatoes, unlike \textit{Xe/Xp} in pepper and \textit{P. carotovorotum} subsp. \textit{carotovorotum} in tobacco. Although host response to plant activators can vary among plants species (Azami-Sardooei et al., 2010), this result is surprising since activators are generally effective across a range of plant species (Boubakri et al., 2016, Durrant & Dong, 2004). One difference is that the tomatoes were grown in a peat-based soilless potting mix in growth rooms in this work, whereas Song et al. (2013) and Yang et al. (2011b) grew plants on MS agar under aseptic conditions. Agar plate growing systems provide a low cost means of producing sterile culture of plants (Conn et al., 2013), and are chemically inert (Best et al., 2014). However, plant growth can be altered, such as with light exposure to roots.

Light exposure can increase root biomass, root length and root hair density and reduce primary root length (Xu et al., 2013) and induce a plant stress response (Yokawa et al., 2014). Also, aseptic conditions alter the expression of SA and JA marker genes in \textit{A. thaliana} compared to non-aseptically grown plants (Carvalhais et al., 2013). Although Song et al. (2013) observe a pepper response to PABA under field conditions, the seedlings were also reared in aseptic conditions. The peat-based soilless potting mix used in the current study is similar to that used by tomato growers to produce seedlings. Peat-based soilless mixes are used because they have good drainage and aeration and are low in soluble salts (Maynard & Hochmuth, 2007), although there can be high variability among batches (Conn et al., 2013). The efficacy of PABA could have been reduced by interactions with components of these mixes as that has been reported for phytohormones and phytohormone inhibitors applied to soil (Best et al., 2014). However, using a higher PABA concentration resulted in phytotoxicity, and thus, it was not possible to find a concentration that provided disease control without plant death using soil drench applications of PABA.

In contrast, two foliar applications of either 9 or 18 mM PABA were effective against \textit{Pst} although they did cause some phytotoxicity. PABA concentration was inversely related to disease incidence demonstrating that the PABA response was dose dependent. One foliar application of 18 mM PABA was also effective against \textit{Pst} in one tomato breeding line (Tazhoor, 2014), but there are no other reports for induced resistance by PABA for foliar applications. For pepper seedlings, growth media applications less than 0.1 mM were not effective, whereas applications of 0.1, 1, and 10 mM PABA effectively reduced the severity of \textit{Xe/Xp}; however, it was not clear if the effect was dose dependent (Song et al., 2013). In tobacco seedlings, growth media applications of 1.0 and 18.0 mM PABA reduced
the severity of *P. carotovotrum* subsp. *carotovotrum* with 18 mM being more effective (Yang et al., 2011b). Dose-dependent responses are also reported for other activators. For example, there was a dose-dependent inverse response to ASM for *X. perforans* bacterial spot of tomato (Huang et al., 2012), and riboflavin for the TMV disease of tobacco, *A. alternata* leaf spot of tobacco and *Peronospora parasitica* downy mildew of *Arabidopsis* (Dong & Beer, 2000). Although the PABA response was dose dependent, one, two, or three applications of 18 mM PABA was readily soluble in water, effective, reproducible and caused limited phytotoxicity to the leaves.

### 2.4.3 Host genotype effect

There are numerous reports of the effectiveness of plant defense activators being affected by plant genotype (Tazhoor, 2014, Dann et al., 1998, Hijwegen & Verhaar, 1994, Ishikawa et al., 2007). Among six commercial processing tomato cultivars in this study, three cultivars, H5108, H9553 and H9909, were responsive to PABA, while three cultivars, H2401, TSH33 and TSH4, were not responsive to PABA. All the responsive cultivars are from Heinz Seed (www.heinzseed.com/). Although they may share some common ancestry, full information about their backgrounds is not publically available (G. Collier, personal communication). One of the non-responsive cultivars is also from Heinz Seed. Cultivar H2401 carries the *Pto* gene for resistance to *Pst* race 0, indicating the isolate used to inoculate tomatoes in this study is race 1. The other two non-responsive cultivars are from Tomato Solutions (www.tomatosolutions.ca), and thus likely share limited ancestry with cvs. H5108, H9553 and H9909. Cultivars TSH33 and TSH4 have different parents, but both reportedly carry tolerance to *Pst* by limiting reproduction of the pathogen on foliage in a non-race specific manner (J. Dick, personal communication). However, only non-treated cv. TSH4 appeared tolerant in this study, perhaps because of the growing conditions and *Pst* population at the time of inoculation. PABA responsiveness was also observed among tomato breeding lines, which had various wild tomatoes in the pedigrees (Tazhoor, 2014). Among eight breeding lines, only one line, which had a lineage from *Solanum lycopersicoides* showed significant induced resistance against *Pst* following a single foliar PABA application. In contrast, Song et al. (2013) and Yang et al. (2011b) only examined single cultivars of pepper and tobacco, respectively, following PABA applications.

Other examples of a genotype effect on induced disease resistance include INA and ASM that were effective in the soybean cultivars Elgin 87 and Williams 82, but less effective in cultivars Corsoy 79 and NKS19-90 against *S. sclerotiorum* (Dann et al. 1998), and INA that was effective in cucumber cultivar Flamingo but not cultivars Corona and Lange Groene Gigant against *Sphaerotheca fuliginea* (Hijwegen and Verhaar 1994). In tomato, SAR against *F. oxysporum* f. sp. *lycopersici* activated by validamycin A varied from over 80% to less than 30% effectiveness among 20 tomato cultivars (Ishikawa
et al., 2007). One explanation for this variation is differences in the response of defense genes to defense activators. The expression pattern of acidic and basic \( PR1 \) (\( PR1b \)) varied among three tomato cultivars after application of ASM (Herman et al., 2007). The differential response to PABA in tomato breeding lines reported by Tazhoor (2014) was associated with a faster and stronger increase in \( SliPR1a \) expression after \( Pst \) inoculation in the responsive breeding line, which suggests that the lines differ in how defense genes are affected by PABA application. Thus, the pedigree of the tomato plays a critical role in determining the effectiveness of PABA. Future studies are needed to determine if the different response of the commercial tomato cultivars in this study may be related to greater increases in defense gene expression.

Phytotoxicity symptoms were observed on tomato leaves treated with the effective concentrations of PABA in the current study, and so one possibility is that the wounding and not PABA itself induced resistance against \( Pst \). Although SAR has been associated with tissue damage in response to necrotizing or HR-inducing pathogens, plant tissue necrosis is not required for SAR (Mishina & Zeier, 2007). Tazhoor (2014) observed a similar response to \( Pst \) in tomato without phytotoxicity symptoms using a single PABA application. Furthermore, phytotoxicity was observed on all tomato cultivars tested, including cultivars that were non-responsive to PABA applications, and was observed with 4.0 mM PABA in a PABA responsive cultivar that did not result in a significant reduction in bacterial speck symptoms. This suggests the response was not solely because of necrosis induced by PABA.

This study and that of Tazhoor (2014) shows that first screening cultivars for their response to PABA is necessary for any practical application of PABA as a disease management tool, since a significant number of cultivars may not respond to PABA as a defense activator. There are many steps from application of a defense activator until disease resistance is expressed (Pieterse et al., 2009a). Further work is needed to determine which step(s) vary between tomato genotypes to PABA, such as differences in PABA uptake, ROS production, SA defense signaling pathways, specific transcription factors and/or other factors.

### 2.4.4 Parameters affecting protection and the duration of protection by PABA

The effect of altering the number of PABA applications in a responsive and non-responsive cultivar was evaluated to determine if this affected the PABA response. Increasing the number of PABA applications from one to three did not induce resistance in the non-responsive cultivar TSH33, and it also did not alter the induced resistance in the responsive cv. H5108. This is the first report on the effect of application number for the level of induced resistance caused by PABA or the other B vitamins thiamine and riboflavin. One reason that multiple applications of PABA may not have improved its effectiveness is because of its mode of action. For the two applications in this study, PABA use was separated by five
days (10 and 15 DAS), and for three applications, PABA use was separated by two and three days (10, 12, and 15 DAS), and all treatments were applied prior to *Pst* inoculation. In previous research in tomato the *PR1a* expression pattern after a single PABA application in a responsive tomato breeding line was not immediately greater than expression in control plants (Tazhoor, 2014). Rather, expression was greater than the control only eight DPT, which was three days after inoculation with *Pst* (Goodwin et al., 2017a). Song et al. (2013) also observed an increase in SA-related gene expression in pepper only after pathogen challenge. This suggests that PABA primes host defenses via SA-dependent signaling, but unlike ASM, it does not result in the immediate activation of defense genes within a few days of application in the absence of a pathogen (Herman et al., 2007).

Priming is a physiological state whereby plant cells respond faster and stronger than non-primed cells to a stimulus (Conrath, 2011). In SAR, priming is associated with the accumulation of mRNA and dormant MPK3 and MPK6 for ASM and *A. thaliana* (Beckers et al., 2009), and ROS- and Ca2+-signaling dependent pathways for MPK3 and MPK6 activation for riboflavin and *A. thaliana* (Nie & Xu, 2016). The priming mechanisms for PABA are unknown, but since previous research associates induced resistance by PABA with SA-signaling, it may also be MPK3 and MPK6 dependent. In this study, one to three applications of PABA were made prior to pathogen challenge with no effect on disease severity, thus it is possible that a priming threshold was reached with a single PABA application, at least within the time period PABA was applied. Preliminary experiments using a single application of PABA in this study were inconsistent, which is why further tests were completed using two PABA applications; however, these experiments were completed using different cultivars and the disease assessment was completed seven DPI instead of five DPI, and later experiments showed efficacy with one to three applications were equivalent. For ASM, applications every eight to 10 days are superior to four or 14 day intervals and re-application of ASM improves induced resistance against *Xanthomonas* spp. in tomato (Huang et al., 2012, Pontes et al., 2016). In a field study of gene expression in tomato cultivars after induction with multiple applications of ASM to tomato in the field resulted in 3.6 fold increases in acidic *PRI* expression in the cultivar Supersonic after the first ASM application, while after the second application the increase was 9.2 fold (Herman et al., 2007). The difference in the ASM and PABA response may be because ASM both primes and activates defense gene expression, or other differences in the molecular mechanisms of induced resistance of ASM compared to PABA. For example, ASM, which is an analogue of SA, functions by inhibiting the enzymes catalase, ascorbate peroxidase, and a mitochondrial oxidase, which results in the production of ROS and increased phenolic compounds (van der Merwe & Dubery, 2006, Wendehenne et al., 1998), but the molecular mechanisms for induced resistance by PABA remain largely unknown.
Song et al. (2013) reported that a single PABA application to roots of seedlings resulted in induced resistance to \( Xe/Xp \) and CMV in peppers for 77 days, indicating that PABA provides very long-lasting protection. PABA was effective when tomato plants were inoculated with \( Pst \) five and seven days, but not 10 days, after the last PABA application. Thus, PABA remained effective for at least seven days after the last application. However, this was clearly less than that described by Song et al. (2013) and this could be due to differences in host and pathogen biology. However, a confounding factor is that the susceptibility of tomato to \( Pst \) may be dependent on developmental stage, also known as age-related resistance (Develey-Rivièrè & Galiana, 2007) as the disease incidence in the control plants dropped from approximately 5 to 2 lesions/cm\(^2\) of leaf tissue from seven to 10 DPT. Susceptibility of Arabidopsis to \( Pst \) decreases 10- to 100-fold as the plant becomes older, which was associated with the accumulation of SA (Kus et al., 2002). In tomato, age-related resistance has been described for \( P. infestans \) (Shah et al., 2015), \( C. michiganensis \) subsp. \( michiganensis \) (Sharabani et al., 2013), and \( Cladosporium fulvum \) (Panter et al., 2002).

2.4.5 Effect of PABA on bacterial speck lesion incidence versus \( Pst \) population

The \( Pst \) population in leaf tissue is normally related to bacterial speck severity as measured by the percentage of leaves with symptoms in \( A. thaliana \) (Vallad et al., 2003) or a severity disease index based on the number of lesions per leaflet in tomato (Baysal et al., 2007, Scarponi et al., 2001). Unexpectedly, the \( Pst \) population per cm\(^2\) of leaf tissue did not differ among treatments, despite reductions in disease incidence. Reductions in bacterial pathogen populations in plant tissues are common for plant defence activators. For example, leaf discs from PABA treated plants contained one order of magnitude fewer \( Xe/Xp \) CFUs than leaf discs from control plants (Song et al., 2013). Similarly, resistance induced by thiamine resulted in \( Pst \) population per gram of fresh weight in \( A. thaliana \) that were lower than control plants (Ahn 2007), and ASM applications reduced \( Xe/Xp \) populations in leaf tissue by almost two orders of magnitude (Louws et al., 2001). However, not all cases of induced resistance involve a reduction in pathogen population (Hammerschmidt, 2009, Summermatter et al., 1995). For example, resistance induced by \( P. s. pv. syringae \) through an incompatible interaction on lower leaves resulted in reduced necrosis in upper leaves after a later challenge in \( A. thaliana \), but the populations of \( P. syringae \) pv. \( syringae \) remained unchanged (Summermatter et al., 1995). Similarly, inoculation with \( Xcv \), avirulent \( Xcv \), or \( Pst \) resulted in induced resistance against subsequent challenge with \( Xcv \) or \( Pst \) in healthy leaves on the same plant based on lower levels of necrosis, increases in the expression of \( PR1a \) and \( PR1b \), and reductions in ion leakage as an indicator of tissue damage, but populations of \( Xcv \) and \( Pst \) in those leaves were not affected (Block et al., 2005). For the latter example, the term systemic acquired tolerance was used to describe a reduction in symptoms but no reduction in pathogen population. The host response to
PABA differs from systemic acquired tolerance in that the number of lesions but not the total amount of necrosis observed was reduced by PABA.

The lack of a correlation between disease incidence and \textit{Pst} population in this study could be due to more bacteria outside of the lesion or more bacteria per lesion in PABA treated plants. To address the first possibility, the \textit{Pst} population in different leaf areas was examined, and populations outside of the lesion in the asymptomatic areas on the same leaflets were more than three orders of magnitude lower than areas of the lesions. Thus, by far the greatest contributor to population per leaf area was lesion area. To address the second possibility that the \textit{Pst} population per lesion in PABA treated plants was higher than lesions in control plants, \textit{Pst} population per lesion was examined. Lesion size and circumference in PABA treated plants were larger than the control, and thus although PABA is suppressing initial infections so that fewer lesions develop, after initial infection, the lesions of PABA-treated plants enlarge faster. This is in contrast to the effect of ASM on bacterial speck, which reduced lesion diameter with ASM increasing concentration in tomato (Scarponi et al., 2001).

The effect of PABA on reducing lesion incidence but increasing their size may be explained by its mode of action. Induced resistance by PABA is dependent on SA-signaling pathways (Song et al., 2013, Yang et al., 2011b, Tazhoor, 2014), which are associated with control of biotrophs as opposed to JA/ET signaling pathways, which are associated with control of necrotrophs (Ton et al., 2006, Pieterse et al., 2009a). For the hemibiotrophic pathogen \textit{Pst}, the first opportunity for induced resistance by PABA to affect infection is during penetration through wounds or natural openings such as stomatal closure. Stomatal closure is a resistance mechanism due to PTI (Melotto et al., 2006, Melotto et al., 2008). However, \textit{Pst} can suppress stomatal closure using the T3SS effector AvrRpt2 and the phytotoxin coronatine (Melotto et al., 2006). If one effect of PABA was to induce resistance by increasing stomatal closure or some other aspect of penetration, then the ability of \textit{Pst} to enter the apoplast of the substomatal cavity would be reduced resulting in a fewer number of infection sites. After successful entry, \textit{Pst} initially multiplies in the apoplastic fluid as a biotroph (Rico & Preston, 2008), where a key element is the release of T3SS effectors to suppress PTI allowing for prolonged feeding on the host cells (Preston, 2000, Cunnac et al., 2009, Munkvold et al., 2009). At this stage, the effect of PABA could be to increase resistance mechanisms so that the ability of these T3SS effectors to suppress PTI in the apoplast is limited, thus resulting in much less biotrophic growth and fewer infection sites where the \textit{Pst} population could increase in size to transition to necrotrophic growth resulting in lesion development. However, the induction of SA-related defenses by PABA may benefit necrotrophic growth of \textit{Pst} resulting in larger lesions, since SA can suppress JA/ET defense signaling (Gimenez-Ibanez & Solano, 2013). As a result, \textit{Pst} could possibly create larger lesions with less JA/ET-mediated defenses. There is evidence for this with PABA as expression of the JA signaling marker gene \textit{CaPIN2} and the ET signaling marker gene.
*CaTIN1* was down-regulated 6 hours after *Xe/Xp* inoculation in PABA-treated peppers (Song et al., 2013). Goodwin et al. (2017a) also observed the down-regulation of the JA-dependent gene, *SlPin2*, and the ET-dependent gene, *SlPR2b*, compared to the water control at one and seven days after *Pst* inoculation, respectively. Evidence that suppression of JA/ET defense signaling by SA can increase disease severity comes from an increase in symptoms for the necrotrophic fungus, *Alternaria brassiccola*, which occurred when resistance was induced in *A. thaliana* by prior inoculation with *Pst*. This inoculation induced SA-related defense genes, such as *PR1* and suppressed JA/ET-related defense genes, such as *PDF1.2*, *HEL*, and *CHI-B* (Spoel et al., 2007). Suppression of the JA signaling pathway was also observed when a combination of the defense activators, ASM, BABA and cis-jasmone was applied to barley resulting in the downregulation of the JA biosynthesis gene *LOX2* and an increase in necrotic symptoms of *Ramularia collo-cygni* (Walters et al., 2011a).

A second possibility is that the leaf damage observed on leaves sprayed directly with PABA created a sub-lethal stress that interfered with the ability of plants to defend themselves against a necrotroph. Abiotic stresses increase ROS concentration in plant cells, which is associated with changes in the expression of detoxification enzymes (Hernández et al., 2001, Alschcer et al., 2002) and hormone signaling pathways (Ben Rejeb et al., 2014). Walters et al. (2011b) observed that prior biotic stress due to infection of barley with *R. secalis* resulted in a failure of the combination of ASM, BABA, and cis-jasmone to induce resistance in new leaves as well as reduce expression of *PR1* and defense-related enzymes. This example shows how a prior stress resulting in necrosis can affect the ability of a defense activator to subsequently induce resistance.

### 2.4.6 Effects of PABA on bacterial speck incidence in the field

A field trial in 2014 was done using a concentration of PABA (18 mM) and a tomato cultivar (cv. H5108) that had resulted in significant reductions in disease incidence in the growth room. Although foliar applications were made from 2 to 9 times as well as a soil soak application, no significant effects of PABA were observed for any *Pst* disease variables, unlike in the growth room. This is in contrast to Song et al. (2013), who found that PABA worked similarly in the growth room and the field for pepper against *Xe/Xp*. One possible explanation for the results in this study is that PABA reduced bacterial speck lesion incidence but not *Pst* populations in growth room studies, and thus a reservoir of the pathogen remained in the leaf. Since bacterial speck is a polycyclic disease, it is possible that even if PABA initially reduced lesion numbers, the lesions would expand more rapidly releasing the pathogen for future infections thus compromising the effect of PABA for sustained disease suppression over the season. Another possibility is that the PABA was rapidly degraded or washed off the plants in the field. PABA is an organic compound and has been found to be degraded by bacteria, such as *Alcaligenes* species isolated from soil.
(Huang et al., 1981). PABA is also known to undergo photodegradation particularly when exposed with dissolved organic matter that acts as a photosensitizer (Zhou et al., 2013). Another factor is that compounds applied to leaves in the field, such as conventional pesticides, can be lost due to being washed off by rain (Oliver & Hewitt, 2014). However, one might have expected some degree of disease control when nine applications were distributed over the growing season. There may have been several reasons for a loss of PABA applied to the foliage in the field, and future tests may require the addition of UV stabilizers to prevent photodegradation or adhesives to stick onto the foliage (Oliver & Hewitt, 2014), such as those used for conventional pesticides. Finally, a notable difference between the growth room and field was that phytotoxicity was not observed in the field, which indicates that PABA did not induce an abiotic stress response, and thus an increase in ROS or induction of hormone signaling pathways may not have occurred or was greatly reduced, which possibly contributed to the resistance observed in the growth room experiments. However, Song et al. (2013) obtained resistance for an entire field season in peppers with only one PABA application to roots of seedlings, in which case, it seems unlikely that increases in ROS or hormones could have persisted for several months.

2.4.7 Effects of PABA on plant growth and development

In addition to inducing disease resistance, there are several reports of PABA altering plant growth. PABA at 7 to 14 mM applied by seed soaking stimulated seed germination in winter wheat and winter barley (Bekusarova et al., 2013). In *A. thaliana* grown on MS agar, the addition of 0.02 to 2 mM PABA reduced root length with increasing concentration and increased lateral root length at low concentrations (Crisan et al., 2014). PABA at 1 mM applied to soil of pepper seedlings increased yield (Song et al., 2013). In this study, 18 mM PABA generally had no effect on tomato root or shoot biomass in the growth room, except for reducing foliar and root weight for the non-responsive cv. TSH33, and reducing root weight in the responsive cv. H9909 in one set of experiments. The effect of plant defense activators on plant growth has been most studied for ASM. Reductions in plant growth and development by ASM is believed to be due to resources being diverted from plant growth to sustained defense responses (Walters & Heil, 2007, Durrant & Dong, 2004). This could be similar for PABA, however, Tazhoor (2014) detected significantly higher levels of PAL5 expression just prior to *Pst* infection in PABA treated tomatoes but not non-treated controls in a PABA responsive breeding line, which indicates induction of gene expression in the absence of the pathogen and is associated with fitness costs, like with ASM. Also, expression of *PR1a* increased in PABA treated tomatoes only after *Pst* inoculation, which is indicative of priming, and priming is associated with fewer fitness costs than immediate induction of resistance (Conrath et al., 2006).

In the field, PABA had no effects on tomato yield, which contradicts the results of Song et al.
(2013) in pepper, where PABA application resulted in a yield increase. However, that may have been due to disease control, and there may have been no effect on yield in this study because PABA did not reduce bacterial speck and bacterial spot severity. Relative chlorophyll was measured as an indicator of N status (Muñoz-Huerta et al., 2013), since plant fitness costs area associated with a deficiency in energy and nutrients (Walters & Heil, 2007). There was no difference in relative chlorophyll in PABA treated and control tomatoes, indicating that chlorophyll levels and N status were not affected by PABA. Thus, there is no evidence for improved plant growth parameters from the field applications of PABA in this study, and the only detection of growth impacts were negative effects in the growth room, which were not consistently observed.

2.4.8 Conclusions

This study demonstrates that PABA can decrease disease incidence of bacterial speck of tomatoes in the growth room based on lesion numbers, although the population of the pathogen and the overall plant surface area affected by bacterial speck were not affected by PABA. Unlike other studies, application of PABA to roots had no effect on disease incidence, possibly because of differences in methods of plant culture. However, foliar applications were effective in the growth room, which was dependent on PABA concentration and host genotype. The timing of Pst inoculation after the last PABA application may influence effectiveness, but this requires further investigation due to confounding effects of plant age and possible developmental resistance to Pst. Surprisingly, other factors, such as the number of PABA applications prior to Pst inoculation did not affect efficacy, indicating that a single PABA application may have a sustained effect that maximizes defense gene expression over at least seven days. Despite selecting factors that provided disease resistance in the growth room, there was no evidence that PABA was effective in one field experiment. The usefulness of PABA as a disease management tool in tomato is limited unless the factors causing PABA to be ineffective under field conditions are discovered. However, even if PABA can be made effective in the field, there are concerns with its use as PABA did not reduce Pst populations or necrotic leaf surface area in treated plants. There could be large reservoirs of the pathogen in treated plants, and thus even more severe disease outbreaks could occur if PABA treatments are stopped or become ineffective during the growing season. Further study on the molecular mechanisms of the effect of PABA on lesion number and size are needed to determine if PABA may actually be counterproductive for disease control in crops such as tomato, which are attacked by a range of different pathogens that are biotrophic, hemibiotrophic and necrotrophic.
Chapter 3: Effects of the plant growth regulator uniconazole with the plant defense activator acibenzolar-S-methyl on incidence and severity of bacterial speck (\textit{P. syringae pv. tomato}) and bacterial spot (\textit{X. gardneri}) in tomato

3.1 Introduction

Bacterial spot, caused by one or more members of the BSX (\textit{Xe}, \textit{Xv}, \textit{Xp} and \textit{Xg}) and bacterial speck caused by \textit{Pst} are the two important foliar diseases of tomato. When established shortly after transplanting, they have caused losses as high as 75\% for speck during the cool and rainy season in Israel (Yunis et al., 1980) and 100\% for spot during the warm and rainy season in Florida (Ritchie, 2000). In the warm and humid growing season in Ontario, losses up to 60\% have been reported as a result of speck and spot epidemics (LeBoeuf et al., 2009). The pathogens infect foliage resulting in the development of small (<5 mm), dark brown, irregular lesions that reduce photosynthetic capacity, accelerate defoliation, and cause premature fruit ripening that reduce yield and disrupt harvest scheduling. Spot and speck lesions on fruit enlarge to 5 to 8 mm and 1 to 2 mm in diameter, and when severe, reduce the ability of processors to effectively peel tomatoes for whole pack and diced markets (LeBoeuf et al., 2009, Jones, 1991a, Jones, 1991b, Koike et al., 2007).

Copper bactericides alone or in combination with the EBDC fungicide mancozeb are the primary chemical option for controlling bacterial spot and speck. However, copper has often proven to be ineffective, possibly because of the appearance of copper tolerant strains of \textit{Pst} and BSX in many parts of the world (Griffin et al., 2017), including Florida (Marco & Stall, 1983), North Carolina (Ritchie & Dittapongpitch, 1991), Arizona and Mexico (Adaskaveg & Hine, 1985), California (Bender & Cooksey, 1986, Bender & Cooksey, 1985, Cooksey et al., 1990, Cooksey & Graham, 1989), Ohio, Taiwan and Argentina (Cooksey, 1990) and Ontario (Cuppels & Elmhirst, 1999, Abbasi et al., 2015). Alternative products, such as kasugamycin (Kasumin), \textit{B. subtilis} QST 713 (Serenade Max), and extract of \textit{R. sachalinensis} (Regalia Maxx), are registered for suppression of bacterial spot in Canada and the USA, but their performance has been inconsistent (Roberts et al., 2008, Trueman, 2015, Miller et al., 2005, Miller & Mera, 2008, Vallad & Huang, 2011).

Another option for chemical control is the plant defence activator ASM, which is marketed as Actigard in North America and Bion in Europe. ASM activates the plant defense response known as SAR resulting in increased resistance for many crops against a variety of pathogens (Kunz et al., 1997). In tomato, for example, ASM increased disease resistance to bacterial spot (Abo-Elyousr & El-Hendawy, 2008, Louws et al., 2001), bacterial speck (Byrne et al., 2005, Herman et al., 2007), grey mould (Achuo et al., 2006), corky root rot (Bubici et al., 2006) and bacterial canker (Baysal et al., 2003), as well as increased
insect pest resistance to two-spotted spider mite (Choh et al., 2004), silverleaf whitefly (Nombela et al., 2005) and green peach aphid (Boughton et al., 2006). However, the level of control by ASM can be highly variable in the field. Louws et al. (2001) reported that an undefined number of ASM applications on a 7 or 10-day interval reduced foliar severity of bacterial spot ranging from 17 to 85% in 13 of 14 experiments and bacterial speck from 23 to 100% in all seven experiments compared to the nontreated controls showing high variability. Similarly, Roberts et al. (2008) reported no effect of 11 ASM application compared to a nontreated control in one experiment but reductions in foliar bacterial spot disease severity of 66% in another experiment, and no effect of six ASM applications in one experiment but a 67% reduction in disease in one other experiment. Wilson et al. (2002) reported foliar bacterial speck reductions of 35 to 70% in three experiments with 8 to 10 ASM applications, and Obradovic et al. (2004) reported foliar bacterial spot disease reductions of 18 to 37% in three experiments with 6 ASM applications. Although Huang et al. (2012) reported that weekly applications of ASM effectively reduced XP severity with an inverse relationship between ASM rates and disease severity, the inverse relationship accounted for only 37 and 35% of the variation observed, suggesting that other factors also influenced the effectiveness of ASM in the field.

One explanation for the variability of ASM effectiveness could be related to it having host fitness costs (Durrant & Dong, 2004, Walters & Heil, 2007). During the SAR response, ASM, which is a functional analog of SA, inhibits mitochondrial NADH:ubiquinone oxidoreductase of complex I of the electron transport chain (van der Merwe & Dubery, 2006) as well as the H₂O₂ scavenging enzymes catalase and ascorbate peroxidase (Wendehenne et al., 1998), resulting in increased levels of ROS and thus possibly plant stress. The accumulation of ROS and SA triggers a systemic response that upregulates the expression of genes encoding defense compounds, like PR proteins, that require plant nutrients and energy that would otherwise be allocated to plant growth and development and stress resistance (Pieterse et al., 2009a, Spoel & Dong, 2012). In some cases, yield losses have been associated with ASM application to tomato under field conditions (Damicone & Trent, 2003, Lange & Smart, 2005, Louws et al., 2001, Pontes et al., 2016). However, the concentrations used in those studies were either more than two times higher than the Canadian label rate or included more than the recommended number of applications per season (PMRA, 2011), whereas no yield reductions were observed in tomatoes at concentrations closer to the Canadian label rate (Alexander & Waldenmaier, 2003, Graves & Alexander, 2002, Lange et al., 2007, Lewis Ivey et al., 2004, Miller et al., 2002, Roberts et al., 2008). Foliar ASM applications to grafted field tomato also resulted in a reduction in yield compared to grafted control plants growing in R. solanacearum infested soils using a concentration that was 25% higher than the Canadian label rate (Kunwar et al., 2017). Nevertheless, the observation that ASM-treated plants can occasionally lower yields indicates plant stress.
One possible way to minimize the fitness costs of ASM is to provide the plant with other treatments that alter the plant’s physiology for increased stress tolerance. Application of the triazole-type PGR UNI, which is marketed as Sumagic in North America, inhibits the synthesis of GA in plants by blocking the cytochrome P-450-dependent monoxygenases which prevents the oxidation of ent-kaurene into ent-kaurenoic acid in the GA biosynthesis pathway (Rademacher, 2000). It is used in commercial tomato seedling production to prevent stem elongation and limit the production of over-sized plants that are difficult to transplant into the field (Zandstra et al., 2006). In addition, UNI application increases stress tolerance. UNI reduced symptoms of chilling injury in tomato, salt stress in Datura spp., and drought stress in soybean and wheat, which was linked to increased antioxidant levels, protein contents, photosynthetic rates, and chlorophyll content, as well as changes in other phytohormone levels (Al-Rumaih & Al-Rumaih, 2007, Duan et al., 2008, Senaratna et al., 1988, Zhang et al., 2007). Combining ASM with paclobutrazol, another triazole-type PGR structurally similar to UNI, reduced symptoms of Pst on tomato leaves under greenhouse conditions to a greater extent than ASM alone, but the effect on subsequent disease development and plant growth in the field was not evaluated (Mahesaniya, 2002). The combination of ASM and paclobutrazol applied to tomato seedlings was evaluated for effects on disease severity in field trials completed between 2002 and 2004 at the University of Guelph, Ridgetown Campus, but results were inconsistent and the BSX species used to inoculate in the field is unknown (Dr. Ron Pitblado unpublished).

As previous results using the combination of ASM and a triazole-type PGR have been inconsistent and/or not well described, the potential benefits of applying ASM to triazole-type PGR-treated tomato seedlings were explored in this chapter. UNI was chosen as the triazole-type PGR instead of paclobutrazol because paclobutrazol was not approved for use on tomato seedlings in Canada by the PMRA, whereas UNI was approved for use in Canada in 2011 (J. LeBoeuf, pers. communication). The objective of this research was to determine if an ASM and UNI combination might result in reducing ASM induced fitness costs and improve the consistency of bacterial spot and bacterial speck suppression in processing tomatoes under Ontario field conditions. This was tested by evaluating season-long effects on bacterial speck and spot development and tomato growth with one greenhouse application of UNI followed by six field applications of ASM or one greenhouse application of UNI followed by three greenhouse applications of ASM and no field applications of ASM.

3.2 Materials & Methods

3.2.1 Weather conditions

Outdoor minimum and maximum daily temperature and rainfall during the study period each year was obtained from the Environment Canada (http://climate.weather.gc.ca/) weather station located at
Ridgetown Campus. Ten year averages for daily minimum and maximum temperature and monthly precipitation were calculated for 2004-2013. For periods with missing values, data from the New Glasgow weather station locate 20 km northeast was used instead.

3.2.2 Greenhouse treatment with UNI followed by field treatment with ASM

3.2.2.1 Experimental design and treatments

Tomato cv. TSH4 was seeded in a commercial transplant production greenhouse located approximately 25 km from Ridgetown, Ontario, Canada on 5 April 2011, 4 April 2012, and 8 April 2013, and the seedlings were produced using normal grower practices. The treatments were a non-treated control, 0.02 mM UNI applied as Sumagic (Valent Canada, Guelph, ON; 0.055% UNI), 0.14 mM ASM applied as Actigard (Syngenta Canada, Guelph, ON, CA; 50% ASM), and 0.14 mM ASM + 0.02 mM UNI (Table 3.1). Treatment with UNI occurred once by spraying tomato foliage in the greenhouse on 27 April 2011 (22 DAS), 23 April 2012 (19 DAS), and 6 May (32 DAS) at the two-leaf stage. UNI applications were made using a hand mist sprayer with 100 mL of solution applied per 288-cell tray (Table 3.1). ASM treatments were performed in field plots established at the Ridgetown Campus (42.4406° N, 81.8842° W). The tomato seedlings were transplanted into twin-rows in the field using a mechanical transplanter on 31 May 2011 (56 DAS), 18 May 2012 (44 DAS) and 22 May 2013 (44 DAS). Individual rows within each set of twin rows were 50 cm apart with plants within rows on a 33 cm spacing. Each set of twin-rows was spaced 1.5 m apart. Each plot consisted of one 7 m twin-row. Treatments were arranged in a randomized complete block design with four replications per treatment. ASM treatment was done using six applications beginning 2 or 3 DAT to the field at 7-day intervals, except in 2011 when poor weather delayed some treatment applications (Table 3.1). ASM was applied at 280 L/Ha for the first two applications one and two, 467 L/Ha for third and fourth applications, and 655 L/Ha for the fifth and sixth applications in accordance with the Canadian product label (Syngenta Canada 2012). ASM was applied using a hand-held 2 m CO₂ boom sprayer with ULD 120-02 nozzles (Pentair Ltd., New Brighton, MN, USA) at a pressure of 241 kPa.

Preventative fungicide applications of Quadris (azoxystrobin, Syngenta Canada, Guelph, ON, CA) were made for early blight (Alternaria solani) on 10 July (96 DAS) and Bravo (chlorothalonil, Syngenta Canada, Guelph, ON, CA) on 24 July and 2 Aug 2012 (110 and 123 DAS). Applications of Bravo were also applied for prevention of early blight and late blight on 19 June, 4, 17, and 30 July 2013 (73, 89, 102, and 115 DAS). All insecticide and fungicide applications were applied using labelled Canadian product rates.

3.2.2.2 Pathogen inoculations
Pst isolate 06T2 and Xg isolate DC00T7A were obtained from Dr. Diane Cuppels, Agriculture and Agri-Food Canada, London, Ontario, Canada. These were originally isolated from tomatoes in southwestern Ontario in 2006 and 2000, respectively. Isolates were grown separately in tryptic soy broth overnight with shaking at 150 rpm, and the concentration of each adjusted to approximately 1 x 10^6 CFU/mL in distilled water with a surfactant, 0.025% Sylgard 309 (siloxylated polyether; Dow Corning, Georgetown, ON, CA). Pst isolate 06T2 and Xg isolate DC00T7A were mixed to obtain equal populations and applied using a hand-held 2 m CO2 boom sprayer with ULD 120-02 nozzles at a pressure of 241 kPa at a rate of 200 L water/Ha on 16 and 30 June 2011 (72 and 86 DAS), 30 May 2012 (55 DAS), and 29 May and 5 June 2013 (51 and 58 DAS) to tomatoes post-transplanting. Only one inoculation was made in 2012 because symptoms were clearly visible within seven days after the first inoculation date.

3.2.2.3 Disease and plant growth assessments

Disease incidence was determined by counting the number of compound leaves with symptoms of small necrotic dark brown lesions indicating bacterial spot and speck in a 1.24 m^2 area (five plants) in each plot on 22, 30 June, 8, 19 and 27 July 2011 (78, 86, 94, 105, and 113 DAS), 7, 15, 21 June, and 4 July 2012 (63, 71, 77, and 90 DAS), and 11, 20 and 29 June 2013 (57, 66, and 75 DAS). In 2013, the total number of leaves in the area was also counted and the percentage of leaves with symptoms was calculated. Leaf counts did not continue beyond these dates as the amount of foliage present made it impossible to accurately assess all leaves present in the area. Disease incidence was also determined by estimating the percent defoliation for the entire area of each plot at the times when leaves with lesions were counted, as well as 8, 18 and 23 Aug 2011 (125, 135, and 140 DAS), 11, 23 July, 7 Aug 2012 (97, 109, and 124 DAS), and 29 June, 7, 16, 24 July, 1, 13, and 19 Aug 2013 (75, 83, 92, 100, 108, 120, and 126 DAS). The diseased leaf number and percent defoliation values were used to calculate an early and late season AUDPC using the following equation: AUDPC = \( \sum \left[ \left( Y_i + Y_{i+1} \right) \left( X_i - X_{i-1} \right) / 2 \right] \). Y_i is number of infected leaves or percent defoliation at day X_i, and Y_{i+1} is number of infected leaves or percent defoliation at day X_{i+1}.

Plant growth stages for the five plants used for leaf counts were also recorded on each disease assessment date. Growth stages were: seedling (no branches), vegetative growth (branched plant with no flowers), flowering (minimum first inflorescence developed), or ripening (one or more fruit with breaker colour). Relative plant chlorophyll levels on one newly emerged leaflet from each of the 5 plants used for disease assessment was measured using a Minolta SPAD-502 Chlorophyll Meter (Konica Minolta, Osaka, Japan) at 18, 36 and 56 DAT each year (74, 92 and 112 DAS in 2011, and 62, 80, and 100 DAS in 2012 and 2013).

Tomatoes were harvested from all four replicated blocks on 24 and 25 Aug 2011 (141 and 142
DAS) and 13 Aug 2012 (130 DAS). In 2013, tomatoes from three replicated blocks were harvested on 23 Aug (130 DAS), but the fourth replicated block was not harvested because of poor plant stand due to frost damage in May. A 2 m section of twin-row was harvested in each plot, and the red, green and rotted fruit were weighed. Fifty green fruit were randomly selected and evaluated for incidence of bacterial spot (dark brown lesions 3 to 6 mm in diameter) and bacterial speck (dark brown lesions 1 to 2 mm in diameter). Fifty red fruit were also randomly selected and evaluated similarly for the incidence of bacterial spot and speck, and the red fruit were then stored at room temperature for three days and assessed a second time for the severity of anthracnose (sunken lesions with concentric rings usually at least 1 cm in diameter) using the following scale: 0 = no anthracnose, 1 = one lesion, 2 = two lesions, 3 = three lesions, 4 = four or more lesions. A disease severity index for anthracnose was calculated using the following equation (Kobriger and Hagedorn 1983):

\[
DSI = \frac{\sum [(\text{class no.})(\text{no. of fruit in each class})]}{\text{(total no. fruit per sample)}(\text{no. classes -1})} \times 100
\]

Fifty additional red fruit were washed and submitted for processing tomato quality assessment by the Ridgetown Campus tomato breeding lab of S. Loewen. Soluble solids, pulp pH, and tomato colour were measured as described by Van Eerd & Loewen (2009).

3.2.2.4 Statistical analysis

Statistical analysis was completed using SAS v9.4 (SAS Institute, Cary, NC, USA). Data were tested for normality using the Shapiro-Wilk statistic. Outliers were identified using Lund’s test of standardized residuals (Lund 1975). Analysis of variance was completed using Proc Mixed with treatment as a fixed effect and replication as a random effect. Data were analyzed as a randomized complete block design. Means comparisons were performed when \( P \leq 0.05 \), and means were separated using Tukey’s HSD. A preliminary analysis combining data from all years revealed treatment x year interactions, and therefore it was decided to analyse data separately from each year.

3.2.3 Greenhouse treatment with UNI followed by greenhouse treatment with ASM or CuOH

3.2.3.1 Experimental design and treatments

Tomato cv. TSH4 and cv. H9909 were seeded in a commercial transplant production greenhouse located approximately 25 km from Ridgetown, Ontario, Canada on 5 April 2011, 4 April 2012, and 8 April 2013, and the seedlings were produced using normal grower practices. Greenhouse plots consisted of one-half of a 288-cell tray arranged in a randomized split-plot design with four replications, with cultivar as the split-plot and chemical treatment as the main plot. The treatments were a non-treated control, 17.6 mM CuOH applied as Kocide 2000 (E.I. du Pont Canada Co, Missisauga, ON; 53.8%
CuOH), 17.6 mM CuOH + 0.02 mM UNI applied as Sumagic, 0.14 mM ASM applied as Actigard, and 0.14 mM ASM + 0.02 mM UNI (Table 3.2). An additional treatment, UNI (0.02 mM) alone, was included in 2013. UNI treatments were applied using registered label application timing and rates to tomato foliage in the greenhouse at the two-leaf stage on 27 April 2011 (22 DAS), 23 April 2012 (19 DAS), and 6 May 2013 (32 DAS). The applications were made using the same methods as described for greenhouse applications in section 3.2.1.1. CuOH treatment was done by spraying foliage six times in 2011 and 2012 and five times in 2013 on a 5 to 8 day interval beginning 20 April 2011 (15 DAS), 21 April 2012 (17 DAS), and 26 April 2013 (18 DAS). ASM treatment was done spraying foliage on 28 April 6 and 17 May 2011 (23, 31, and 42 DAS), 25 April, 4 and 14 May 2012 (21, 30, and 40 DAS), 30 April and 1 and 20 May 1 2013 (22, 33, and 42 DAS). CuOH and ASM were applied using a hand mist sprayer using a water volume of 1000 L/Ha.

The seedlings were then transplanted to a field located at the Ridgetown Campus, University of Guelph on 31 May 2011 (56 DAS) and 21 May 2012 (47 DAS). For 2013, the transplanting occurred on 5 June 2013 (57 DAS), as an earlier transplanting on 22 May (44 DAS) was discarded due to a serious frost event on 25 and 26 May causing approximately 80% plant loss. The field plots were established using the methods described in section 3.2.1.1. Fungicide and insecticide applications were made on the same dates as those listed in section 3.2.1.1, except an additional application of Bravo was applied 22 Aug 2013 (135 DAS).

3.2.3.2 Pathogen inoculations

Tomato plants were inoculated with Pst isolate 06T2 and Xg isolate DC00T7A using the methods described in section 3.2.1.2 on 16, 30 June, and 9 July 2011 (72, 86, and 95 DAS), 30 May and 12 June 2012 (55 and 67 DAS), and 11 and 19 June 2013 (63 and 71 DAS). However, the inoculation of 9 July 2011 (95 DAS) was done by soaking a collection of infected leaves from a grower's field located approximately 150 km from Ridgetown in water, filtering through cheesecloth, and applying the solution at a rate of 100 mL slurry/L. This was done because disease symptoms were slow to develop.

3.2.3.3 Disease and plant growth assessments

Transplant canopy height was measured at 14, 22, 28, 35, 41 and 52 DAS (19, 25 April, 1, 7 and 18 May 2011, 18, 24, 30 April, 6 and 17 May 2012, and 22, 28 April, 4, 10 and 21 May 2013) at five locations in each plot from the soil line to the top of the canopy. Stem diameter at the base of five seedlings was measured using digital calipers at 35 and 41 DAS in each year as well as at 52 DAS in 2011 and 2013. Root and foliar dry weight was measured at 56 DAS in 2011 (22 May) and 44 DAS in 2012 and 2013 (9 and 13 May), which was just prior to transplanting. Dry weights were obtained from roots and foliage from 5 plants per replicate that were rinsed to remove media and then dried in a
greenhouse for 1 to 2 weeks.

Disease, plant growth, and yield evaluations were completed using the methods described in Section 3.2.1.3. Plant leaf counts were performed 22, 30 June, 8, 19, and 27 July 2011 (78, 86, 94, 105 and 113 DAS), 8, 15, 21 June, and 5 July 2012 (63, 70, 76 and 90 DAS), and 22 June, 1 and 8 July 2013 (74, 83, and 90 DAS). Percent defoliation was estimated on all dates where lesions were counted, as well as 8, 18 and 29 Aug 2011 (125, 135 and 146 DAS), 11, 23 July, 7 and 17 Aug 2012 (102, 114, 129, and 139 DAS), and 16, 23 July, 1, 13 and 23 Aug 2013 (98, 105, 114, 126 and 135 DAS). All four replicate blocks of tomatoes were harvested on 30 Aug 2011 (147 DAS), 21 Aug 2012 (143 DAS), and 30 Aug 2013 (TSH4) (142 DAS), but only three out of four replicate blocks were harvested on 3 Sept 2013 (cv. H9909) (146 DAS). The fourth replicate block of cv. H9909 was harvested on 11 Sept (153 DAS) as there was a data entry error for this block on 3 Sept. TSH4 was harvested before cv. H9909 in 2013 because they matured earlier.

3.2.3.4 Statistical analysis

Statistical analysis was completed using the same methods described in section 3.2.1.4. A preliminary analysis combining data from all years and cultivars revealed numerous treatment x year, treatment x cultivar, and year x cultivar interactions, therefore it was decided to analyse data separately from each year and cultivar for each variable.
Table 3.1 Treatment descriptions and application timings for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. Further details on applications methods are described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application Timing (# days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DAS</td>
</tr>
<tr>
<td>1. Control</td>
<td>-</td>
</tr>
<tr>
<td>2. UNI (0.02 mM) (^b)</td>
<td>22</td>
</tr>
<tr>
<td>3. ASM (0.14 mM)</td>
<td>-</td>
</tr>
<tr>
<td>4. UNI (0.02 mM) ASM (0.14 mM)</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^a\) DAS = days after seeding; DAT = days after transplanting.

\(^b\) UNI applications were made when seedlings were at the two-leaf stage.
Table 3.2 Treatment descriptions and application timings for tomato cv. TSH4 and H9909 treated with uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. Further details on applications methods are described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Application Timing (DAS) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2011</td>
</tr>
<tr>
<td>1. Control</td>
<td>-</td>
</tr>
<tr>
<td>2. CuOH (17.6 mM)</td>
<td>15, 23, 27, 32, 36, 43</td>
</tr>
<tr>
<td>3. UNI (0.02 mM) (^b)</td>
<td>22</td>
</tr>
<tr>
<td>CuOH (17.6 mM)</td>
<td>15, 23, 27, 32, 36, 43</td>
</tr>
<tr>
<td>4. ASM (0.14 mM)</td>
<td>23, 31, 42</td>
</tr>
<tr>
<td>5. UNI (0.02 mM)</td>
<td>22</td>
</tr>
<tr>
<td>ASM (0.14 mM)</td>
<td>23, 31, 42</td>
</tr>
<tr>
<td>6. UNI (0.02 mM)</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) DAS = days after seeding.  
\(^b\) UNI applications were made when seedlings were at the two-leaf stage.
3.3 Results

3.3.1 Weather Conditions

Seasonal variation in maximum and minimum daily temperatures in 2011, 2012 and 2013 tended to follow the 10-year historical trend with temperatures peaking in late July. However, there were days above 30°C in early June 2011 and multiple days over 30°C from late May to late July in 2012 (Figure 3.1a-d). Frost events on 25 and 26 May damaged tomato plants in 2013 (Figure 3.1c). Average monthly maximum and minimum temperatures in 2011 were similar to the average, except for the maximum daily average being 2.2°C above the 10-year average in July (Figure 3.2a), and minimum daily average being 1.1 and 2.2°C above the 10-year average in May and July (Figure 3.2b). Monthly maximum and minimum daily temperatures in 2012 ranged from 0.5 to 2.5°C and 0.7 to 1.5°C degrees higher than the 10-year average in May, June, and July. Average maximum daily temperatures in June, July, and Aug 2013 were 1.6, 1.3, and 0.9°C below the 10-year average. Thus, temperatures were generally above average in the early part of the season in 2011 and most of the season in 2012, and below average most of the season in 2013.

Daily rainfall records showed that heavy rain events (>25 mm) occurred twice in May and once in June in 2011, once in June, four times in July, and once in Aug in 2012, and once in May, twice in June, and once in July and Aug in 2013 (Figure 3.1a-c). Two of the July rain events in 2012 exceeded 50 mm of precipitation. Total rainfall in 2011 was similar to the 10 year monthly average, except that the heavy rainfall events in May resulted in a total monthly rainfall 50 mm greater than the 10-year average (Figure 3.2c). Rainfall in 2012 was 60% below the 10-year average in May, but was 179% above the 10-year average in July, mainly due to two rainfall events that exceeded 50 mm of precipitation. Rainfall in 2013 was below the 10-year average in all months except June. Thus, monthly rainfall went from above average to similar to the 10-year average in 2011, below average to above the 10-year average in 2012, and generally below the 10-year average in 2013.

3.3.2 Greenhouse treatment with UNI followed by field treatment with ASM

In this section of the study, UNI was applied once in the greenhouse before transplanting to the field using the recommended application timing for plant height control, and ASM was applied six times after transplanting in the field in order to evaluate the effect of ASM on disease development during the growing season following the currently registered use pattern of ASM in Canada.

3.3.2.1 Effect of greenhouse UNI followed by field ASM on bacterial speck and bacterial spot

Early season bacterial speck and spot incidence on cv. TSH4 was measured as the number of
symptomatic leaves in a 1.24 m² area in each plot, which was assessed from late June to late July in 2011, early June to early July in 2012 and mid-June to early July in 2013 (Figure 3.3). In 2011, applications of ASM first reduced disease incidence (74% fewer symptomatic leaves) compared to the nontreated control on 19 July (105 DAS) and then by 68 and 51% compared to the nontreated control and UNI on 27 July (113 DAS) in 2011 (Figure 3.3a). UNI and ASM+UNI treatments reduced early season disease compared to the nontreated control on 27 July (113 DAS). Early season AUDPC in 2011 (22 June to 27 July) was 65% lower in the ASM treatment than the nontreated control (Figure 3.4a). In 2012, applications of ASM reduced early season disease incidence compared to the nontreated control on 4 July (90 DAS) (Figure 3.3b). Early season disease for the UNI treatment was never significantly lower than the nontreated control, but ASM+UNI treatment was significantly lower than the nontreated control starting on 15 June (71 DAS) (86% fewer symptomatic leaves) and then was 32 and 28% lower than the nontreated control and UNI, respectively, on 4 July (90 DAS). Early season AUDPC in 2012 (7 June to 4 July) was 38 and 42% lower in treatments with ASM and ASM+UNI than the nontreated control (Figure 3.4b). In 2013, early season disease incidence for all the treatments (ASM, UNI and ASM+UNI) was significantly lower than the nontreated control on 20 June 2013 (66 DAS) (Figure 3.3c). Early season AUDPC in 2013 (11 June to 29 June) was 31 and 50% lower in the ASM and ASM+UNI treatments than the nontreated control (Figure 3.4c). Thus, ASM applications resulted in a reduction in early season AUDPC compared to the nontreated control in all years but UNI+ASM only in 2012 and 2013.

Late season bacterial speck and spot incidence was measured as the percent defoliation per plot, which was assessed from late July to late Aug in 2011 and late June to mid-Aug in 2012 and 2013 (Figure 3.5). In 2011, ASM, UNI and UNI+ASM treatments all had less defoliation than the nontreated control on 27 July, but by 23 Aug (140 DAS), only defoliation in the ASM treatment was lower than the nontreated control (Figure 3.5a). Late season AUDPC in 2011 (27 July to 23 Aug) with ASM was the only treatment that was significantly lower than the nontreated control (by 58%) (Figure 3.6a). In 2012 and 2013, there was no difference among treatments for defoliation (Figure 3.5b & Figure 3.5c, Figure 3.6b & Figure 3.6c). Thus, ASM provided some reduction in defoliation in one of three years compared to the nontreated control and there was no effect of UNI on defoliation, indicating limited consistent benefits of either treatment in preventing premature tomato defoliation.

The incidence of bacterial speck on red fruit ranged from 2.0 to 10.5% from 2011 to 2013, while the incidence of bacterial spot on red fruit ranged from 9.0 to 24.2% from 2011 to 2013 (Table 3.3). Xg was the predominate red fruit pathogen in 2012 and 2013, but was similar in incidence to Pst in 2011. There was no significant reduction in either bacterial speck or spot symptoms among treatments on red (Table 3.3) or green fruit (Table A.3). There was no effect on the incidence or severity of anthracnose on tomato fruit in any year (Table A.4).
3.3.2.2 Effect of greenhouse UNI followed by field ASM on tomato growth, yield, and quality

In 2011, relative chlorophyll content of the nontreated control at 18 DAT (74 DAS) was equivalent to ASM but was 8.6 and 10.2 SPAD units lower than UNI and UNI+ASM, respectively (Figure 3.7a). In 2012, relative chlorophyll content among treatments at 18, 36 and 56 DAT was not different (Figure 3.7b). In 2013, relative chlorophyll content of the nontreated control at 18 DAT (62 DAS) was equivalent to ASM but was 5.7 and 7.6 SPAD units lower than UNI and UNI+ASM, respectively (Figure 3.7c). There were no differences among treatments at 36 DAT in 2013 but UNI was higher than the control and other treatments at 56 DAT. Thus, ASM did not alter chlorophyll content compared to the nontreated control, whereas UNI or UNI+ASM applications increased chlorophyll content at 18 DAT in two of three years.

The number of days to reach first inflorescence, fruit set, and ripening was not affected by treatment (Table A.5). Total red, green and rotten fruit tomato yield was 22% higher in the ASM+UNI treatment than the nontreated control in 2011; however, ASM and UNI alone did not significantly affect red, green or rotten tomato yield in 2011 (Table 3.4). There were no significant differences among treatments in 2012 and 2013. There was no difference among treatments for tomato fruit colour, pH and soluble solids, except in 2011 when treatment UNI had 0.05 higher pH units than the control (Table 3.5).

3.3.3 Greenhouse treatment with UNI followed by greenhouse treatment with ASM

In this section of the study, UNI was applied once in the greenhouse before transplanting to the field using the recommended application timing for plant height control, and ASM was applied three times in the greenhouse before transplanting in order to evaluate long term effects of ASM on disease development and plant growth post-transplanting. Greenhouse treatment with ASM was examined to determine if the effects of ASM persisted for long periods of time, since three applications from late April to mid-May were done for the greenhouse ASM treatment versus six applications from late May to early July was done for the field ASM treatments. Assessments were completed using similar methods and similar timings as those described in the previous section for greenhouse treatment with UNI followed by field treatment with ASM. However, cv. H9909 was included as well as cv. TSH4.

3.3.3.1 Effect of greenhouse UNI followed by greenhouse ASM on bacterial speck and bacterial spot

For cv. TSH4, there was no difference among treatments for early season foliar disease incidence in 2011 (22 June to 27 July; Figure 3.8a) or the early season AUDPC (Figure 3.9a). In 2012, ASM and CuOH+UNI had no effect on early season incidence compared to the nontreated control at any time point
(8 June to 4 July), but ASM+UNI treatment was 55% lower than the control on 5 July (91 DAS) (Figure 3.8b). The ASM+UNI treatment also had 52, 57, and 51% lower total early season AUDPC than the control, CuOH and CuOH+UNI (Figure 3.9b). In 2013, there was significant differences between treatments only on 22 June with ASM+UNI having lower incidence than the nontreated control and CuOH, and UNI and CuOH+UNI having lower incidence than the nontreated control (Figure 3.8c). However, there was no difference among treatments for total early season AUDPC in 2013 (Figure 3.9c). Thus, compared to the nontreated control, CuOH+UNI and UNI (2013 only) provided additional benefits, but only on specific assessment dates. The only treatment with lower early season AUDPC than the control was ASM+UNI which reduced early season AUDPC compared to the nontreated control in one of three years.

For cv. H9909 in 2011, ASM treatment was equivalent to the nontreated control on all assessment dates (22 June to 27 July; Figure 3.8d) and for total early season AUDPC (Figure 3.8b). The CuOH+UNI and ASM+UNI treatments had fewer early season symptomatic leaves than the control and an equivalent number to CuOH treatment on 30 June, 8 and 19 July (86, 94 and 105 DAS). Total early season AUDPC for 2011 was lowest for CuOH and CuOH+UNI treatments, which were both significantly lower than the control but equivalent to the ASM+UNI treatment. In 2012, applications of ASM, CuOH+UNI, and ASM+UNI were equivalent to the nontreated control on all assessment dates (8 June to 4 July; Figure 3.8e), and for early season AUDPC (Figure 3.9b). Similarly, there was no effect in 2013 of ASM, UNI, CuOH+UNI or ASM+UNI treatments on early season disease incidence or total early season AUDPC (Figure 3.8f and Figure 3.9c). Thus, ASM or ASM+UNI did not reduce total early season disease compared to the nontreated control in any year in cv. H9909, which differed from cv. TSH4 where applications of ASM+UNI provided benefits based on AUDPC in one year.

Late season defoliation was assessed in the same manner as the section on field applications of ASM. In 2011 for cv. TSH4, applications of ASM and ASM+UNI resulted in defoliation levels that were equivalent to the control and CuOH on all assessment dates (27 July to 29 Aug; Figure 3.10a) and for total late season AUDPC (Figure 3.11a). Applications of CuOH+UNI resulted in a reduction in defoliation compared to the control on 8 and 18 Aug. Late season AUDPC was lower than the nontreated control for CuOH+UNI. In 2012, ASM and ASM+UNI had similar effects with the treatments resulting in a significant reduction in defoliation compared to the control on 23 July, and compared to the control and CuOH on 7 and 18 Aug (5 July to 18 Aug; Figure 3.10c). Applications of CuOH+UNI did not reduce defoliation compared to the control or CuOH on any assessment date. Late season AUDPC was lower for ASM and ASM+UNI than the control, CuOH and CuOH+UNI treatments (Figure 3.11b). In 2013, applications of ASM, UNI, CuOH+UNI and ASM+UNI had no effect on defoliation or total late season AUDPC (1 July to 23 Aug; Figure 3.10c and Figure 3.11c). Thus, treatments with ASM had lower late
season AUDPC compared to the nontreated control in only one of three years with cv. TSH4.

For cv. H9909, there was no difference among treatments for late season defoliation in 2011 (27 July to 29 Aug; Figure 3.10b and Figure 3.11a), including AUDPC. In 2012, ASM treatment had higher defoliation than the nontreated control and CuOH+UNI treatment on Aug 18 (Figure 3.10d). The ASM+UNI treatment also had higher defoliation than these treatments on Aug 18, and had higher total late season AUDPC than CuOH+UNI (Figure 3.11b). In 2013, applications of ASM, UNI, CuOH+UNI, and ASM+UNI had defoliation levels that were equivalent to the nontreated control and CuOH (Figure 3.10f and Figure 3.10c). Thus, treatments including ASM increased defoliation in one of three years and had no effects on defoliation in the other two years. UNI had little effect on defoliation, except when UNI was combined with ASM, where increased defoliation was also observed in one of three years. This differed from the response in cv. TSH4, where applications of ASM and ASM+UNI provided marginal benefits in the same year that ASM increased defoliation in cv. H9909.

None of the treatments reduced the incidence of bacterial speck and spot on red or green fruit for either cultivar in any year, except ASM+UNI in cv. TSH4 in 2012 (Table 3.6, Table A.6). The incidence of bacterial spot and bacterial speck on fruit followed a similar pattern to that observed in the ASM field applications discussed earlier and suggests that Xg was also the predominant pathogen with the greenhouse ASM applications in 2012 and 2013, but that both Xg and Pst caused similar levels of damage to fruit in 2011. There was no treatment effect on the incidence or severity of anthracnose (Table A.7).

3.3.3.2 Effect of greenhouse UNI followed by greenhouse ASM on tomato growth, yield and quality

Treatment effects on transplant height were measured at weekly intervals beginning two weeks after seeding up until the time of transplanting. For cv. TSH4 in 2011, plant height in the ASM treatment was equivalent to the control and CuOH on all assessment dates (Figure 3.12a). For CuOH+UNI treatment, plants were shorter than the control and CuOH at 35 and 41 DAS (13 and 19 days after UNI application). ASM+UNI plant height was less than CuOH at 28 DAS and less than CuOH and the control at 35 and 41 DAS. In 2012, applications of ASM reduced plant height compared to the control at 28 and 35 DAS, but were equivalent to CuOH on all dates (Figure 3.12b). CuOH+UNI and ASM+UNI treated plants had lower height than the control and CuOH at 28, 35 and 41 DAS (nine, 16 and 22 days after UNI application). In 2013, plant height with ASM treatment was lower than the control and CuOH at 35 DAS (Figure 3.12c). Applications of UNI, CuOH+UNI and ASM+UNI resulted in shorter plant height than the control at 35, 41 and 52 DAS (three, nine and 20 days after UNI application). Thus, treatments including UNI consistently reduced plant height in cv. TSH4 in all years within at least 16 days after application.

For cv. H9909, plant height in the ASM treatment was equivalent to the control and CuOH
treatments on all assessment dates in all years (Figure 3.12d). Plant heights with CuOH+UNI and ASM+UNI treatments were shorter than the control and CuOH treatments at 28, 35 and 41 DAS (six, 13 and 19 days after UNI application) in 2011. In 2012, CuOH+UNI and ASM+UNI applications resulted in lower plant height at 22, 28, 35 and 41 DAS (three, nine, 16, and 22 days after UNI application; Figure 3.12e). In 2013, the UNI, CuOH+UNI, and ASM+UNI had lower plant height than the control and CuOH treatments at 35, 41 and 52 DAS (three, nine, and 20 days after UNI application; Figure 3.12f). UNI applications to cv. H9909 resulted in shorter plants in all three years within at least 10 days after UNI application, regardless of whether plants were also treated with ASM or CuOH during the study period. UNI applications reduced plant height by 32 to 34% in cv. TSH4 and 26 to 32% in cv. H9909, suggesting a slightly greater effect of UNI on cv. TSH4.

Stem diameter was measured on tomato seedlings at 35 and 41 DAS. In cv. TSH4 in 2011, stem diameters in plants treated with ASM was equivalent to the control and CuOH treatments (Table 3.7). However, stem diameters with the CuOH+UNI and ASM+UNI treatments was lower than the control and CuOH at 35 DAS, and 35 and 41 DAS, respectively. In 2012, stem diameter with ASM treatment was equivalent to the control and CuOH on both assessment dates. CuOH+UNI treatment had lower stem diameters than the control, and ASM+UNI treatment had lower stem diameter than the control and CuOH 35 DAS, but there were no differences among these treatments by 41 DAS. In 2013, stem diameter with ASM treatment was also equivalent to the control and CuOH at 35 and 41 DAS. However, UNI treated plants had lower stem diameter than CuOH treated plants, and applications of CuOH+UNI and ASM+UNI resulted in lower stem diameters than with nontreated control and CuOH application at 41 DAS. Thus, applications of ASM or CuOH alone did not affect stem diameter, whereas UNI sometimes but not always resulted in lower stem diameter than treatments that did not receive UNI.

Stem diameter in cv. H9909 in 2011 was equivalent for the nontreated control, CuOH and ASM treated plants, which were all higher than CuOH+UNI and ASM+UNI treated plants, except for ASM at 41 DAS (Table 3.7). In 2012, there were no differences among treatments for stem diameter. In 2013, there were no differences among treatments for stem diameter at 35 DAS, but by 41 DAS, application of UNI and ASM+UNI had the lowest stem diameters followed by ASM and CuOH+UNI application. The nontreated control had the largest stem diameter, equivalent to CuOH treatment. Unlike cv. TSH4, cv. H9909 had reduced stem diameters in one year with ASM alone but CuOH alone was never significantly different from the control. The effect of UNI on reducing stem diameter in cv. H9909 was not as consistent from year to year as in cv. TSH4, but also generally resulted in a decreased stem diameter compared to treatments where no UNI was applied.

Relative chlorophyll was measured at 18, 37 and 56 DAT in each year (74, 93 and 112 DAS in 2011 and 62, 81 and 100 DAS in 2012 and 2013) (Figure 3.13). In cv. TSH4 in 2011, there was no
treatment effect on relative chlorophyll content (Figure 3.13a), but in 2012, ASM treatment had chlorophyll content equivalent to the nontreated control and CuOH at 18 and 37 DAT (Figure 3.13b). At 56 DAT, relative chlorophyll was also equivalent to the control but was lower than the CuOH treatment. Both CuOH+UNI and ASM+UNI had higher relative chlorophyll than the control and CuOH at 18 DAT, but levels were equivalent to these treatments at 37 and 56 DAT. In 2013, the ASM treatment had chlorophyll content equivalent to the nontreated control and CuOH at 18 and 37 DAT, but by 56 DAT, the relative chlorophyll was lower than the CuOH treatment (Figure 3.13c). The CuOH+UNI treatment had higher relative chlorophyll than the control and CuOH only at 18 DAT. For ASM+UNI, relative chlorophyll was higher than the control and CuOH at 18 DAT, and higher than CuOH at 56 DAT. The UNI treatment, which was only included in 2013, had higher relative chlorophyll than the nontreated control and CuOH at 18 DAT but was equivalent to these treatments at 37 DAT and lower than CuOH treatment, but not the control, at 56 DAT. Thus, in two of three years, applications of UNI resulted in higher relative chlorophyll content at least at 18 DAT and sometimes ASM treated plants had lower relative chlorophyll than CuOH, CuOH+UNI and ASM+UNI treated plants.

For cv. H9909 in 2011, ASM treatment had relative chlorophyll content equivalent to the control and CuOH at 18, 37 and 56 DAT (Figure 3.13d). Both CuOH+UNI and ASM+UNI treated plants had higher relative chlorophyll content than the control and CuOH treated plants at 18 DAT, but these treatments were equivalent at 37 and 56 DAT. In 2012, the ASM and ASM+UNI treatments were equivalent to the control and CuOH treatments on all assessment dates (Figure 3.13e). Applications of CuOH+UNI resulted in higher relative chlorophyll content than the control and CuOH treated plants at 18 DAT, but had no effect on chlorophyll content at 37 and 56 DAT. In 2013, relative chlorophyll in the ASM treatment was equivalent to the control and CuOH treatments on all assessment dates (Figure 3.13f). Applications of UNI and CuOH+UNI resulted in higher relative chlorophyll content than the CuOH treatment at 18 DAT, whereas the ASM+UNI treatment was equivalent to both CuOH and the control treatments on the same assessment date. For cv. H9909, treatments that included UNI frequently but not always resulted in higher relative chlorophyll content 18 DAT in all three years, but there was no effect of UNI on relative chlorophyll content at 37 or 56 DAT, indicating that the effects of UNI were no longer apparent by 37 DAT. ASM or CuOH alone treatments had relative chlorophyll contents equivalent to the nontreated control in all three years. These results were similar to those observed for cv. TSH4.

There were no effects on the number of days to reach first inflorescence, fruit set, and fruit ripening for either cultivar, except for cv. H9909 in 2013, where the number of days to reach fruit set was five days fewer for the UNI than the ASM treatment, but neither was different than the nontreated control (Table A.8).

For cv. TSH4 in 2011, there were no differences among treatments for total, red, green or rotten
tomato yield (Figure 3.14a). In 2012, red tomato yield was 29% higher with ASM and ASM+UNI treatments compared to the nontreated control and CuOH+UNI, but were equivalent to the CuOH treatment (Figure 3.14b). In 2013, there were again no differences among treatments for tomato yield (Figure 3.14c). Thus, in one of three years ASM application had a positive effect on tomato yield, but this effect was not augmented with the inclusion of UNI into the ASM application.

For cv. H9909 in 2011, tomato yield in the ASM treatment was equivalent to the control and CuOH treatments, but total and red tomato yield was 31 and 26% lower than the yield obtained in the CuOH+UNI treatment (Figure 3.14d). Yields in the CuOH+UNI and ASM+UNI treatment were equivalent to all other treatments. In 2012, ASM and ASM+UNI reduced total yield by 24% compared to the control, CuOH, and CuOH+UNI treatments (Figure 3.14e). Red tomato yield in treatment ASM was also 24 and 22% lower than CuOH and CuOH+UNI treatments. In 2013, there were no differences among treatments for yield (Figure 3.14f). Thus, in two of three years, negative effects of ASM on yield were observed at some level compared to the best treatment, CuOH+UNI. This even included less yield than the nontreated control in 2012. This result is in contrast to the results in cv. TSH4 in the same year, where ASM treatment increased yield.

There were no treatment effects on tomato quality parameters including colour, pH, and soluble solids in cv. TSH4 in any year (Table 3.8). Treatments with ASM and ASM+UNI had lower soluble solids than the control in cv. H9909 in 2012, but not in 2011 and 2013. However, soluble solids for all treatments were relatively high for Ontario growing conditions in 2012, possibly because of hot and dry growing conditions. Juice pH in treatment ASM+UNI was also higher than the control for cv. H9909 in 2012. Overall, treatments only had effects on cv. H9909, which would be considered negative, but the effects on the quality parameters tested were slight.
Figure 3.1 Daily maximum (—) and minimum (———) temperatures, and total daily rainfall (■) for a) 2011, b) 2012, c) 2013, and d) 10-year average (2004-2013) at Ridgetown Campus, University of Guelph.
Figure 3.2 Mean monthly a) maximum temperature, b) minimum temperature, and c) monthly rainfall in 2011 ( ), 2012 ( ), 2013 ( ) and 10-year average ( ) (2004-2013) at the Ridgetown Campus, University of Guelph.
Figure 3.3 Early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \( P.\ syringae \) \textit{pv. tomato} and \( X.\ gardneri \), Ridgetown, ON, in a) 2011, b) 2012, c) 2013. The number of leaves with disease symptoms in the nontreated control (–), UNI (\(-\rightarrow\)), ASM (\(-\rightarrow\)), and ASM + UNI (\(-\rightarrow\rightarrow\)) treatments in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Figure 3.2. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at \( P \leq 0.05 \), Tukey’s HSD. NS = no significant difference.
Figure 3.4 Area under the disease progress curve (AUDPC) for early season disease. Early season disease was measured by the number of leaves with disease symptoms (shown in fig. 2) for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (22 June-27 July), b) 2012 (7 June-4 July), c) 2013 (11 June-29 June). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.
Figure 3.5 Late season progress of bacterial spot and speck on tomato cv. TSH4 treated with uniconazole (UNI, in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011, b) 2012, c) 2013. Defoliation in the nontreated control ( ), UNI ( ), ASM ( ), and ASM + UNI ( ) treatments in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Figure 3.4. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Figure 3.6 Area under the disease progress curve (AUDPC) for late season disease. Late season disease was measured by the percent defoliation (shown in fig. 4) for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae pv. tomato} and \textit{X. gardneri}, Ridgetown, ON, in a) 2011 (27 July-23 Aug), b) 2012 (4 July-27 Aug), c) 2013 (29 June-19 Aug). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.
Table 3.3 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae} pv. \textit{tomato} and \textit{X. gardneri}, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial speck on fruit (%)</th>
<th>2013</th>
<th>2012</th>
<th>2011</th>
<th>2013</th>
<th>2012</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacterial spot on fruit (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.0 a</td>
<td>8.8 a</td>
<td>10.5 a</td>
<td>14.7 a</td>
<td>24.4 a</td>
<td>9.0 a</td>
<td></td>
</tr>
<tr>
<td>UNI</td>
<td>5.3 a</td>
<td>9.5 a</td>
<td>4.5 a</td>
<td>18.0 a</td>
<td>35.5 a</td>
<td>10.5 a</td>
<td></td>
</tr>
<tr>
<td>ASM</td>
<td>2.7 a</td>
<td>10.5 a</td>
<td>8.5 a</td>
<td>11.3 a</td>
<td>31.6 a</td>
<td>5.0 a</td>
<td></td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>5.3 a</td>
<td>11.0 a</td>
<td>7.5 a</td>
<td>18.7 a</td>
<td>30.0 a</td>
<td>5.5 a</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD.

\textsuperscript{b} sem = standard error of the mean for all ls means in the same column.
Figure 3.7 SPAD chlorophyll readings measured 18, 36, and 56 days after transplanting in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in the nontreated control (−−), UNI (−→), ASM (→−), and ASM + UNI (−−→) treatments a) 2011, b) 2012, c) 2013. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference.
Table 3.4 Total, red, green, and rotten fruit yield in a 2m section of tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.69 a</td>
<td>14.59 a</td>
<td>18.00 a</td>
<td>13.07 a</td>
<td>14.67 a</td>
<td>6.65 a</td>
<td>1.44 a</td>
<td>3.36 a</td>
<td>0.05 a</td>
<td>0.08 a</td>
<td>0.15 a</td>
<td></td>
</tr>
<tr>
<td>UNI</td>
<td>27.23 a</td>
<td>14.56 a</td>
<td>20.51 ab</td>
<td>23.10 a</td>
<td>12.90 a</td>
<td>18.29 a</td>
<td>4.09 a</td>
<td>1.41 a</td>
<td>2.17 a</td>
<td>0.04 a</td>
<td>0.25 a</td>
<td>0.05 a</td>
</tr>
<tr>
<td>ASM</td>
<td>25.72 a</td>
<td>15.24 a</td>
<td>19.70 ab</td>
<td>21.09 a</td>
<td>13.73 a</td>
<td>16.51 a</td>
<td>4.63 a</td>
<td>1.41 a</td>
<td>3.07 a</td>
<td>0.00 a</td>
<td>0.10 a</td>
<td>0.12 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>26.27 a</td>
<td>15.38 a</td>
<td>22.12 a</td>
<td>22.05 a</td>
<td>13.81 a</td>
<td>19.03 a</td>
<td>3.95 a</td>
<td>1.44 a</td>
<td>2.81 a</td>
<td>0.07 a</td>
<td>0.13 a</td>
<td>0.28 a</td>
</tr>
</tbody>
</table>

sem $^b$ 1.878 0.882 0.907 1.177 0.0954 1.234 1.384 0.324 0.509 0.029 0.075 0.082

$^a$ Means in the same column grouping followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.

$^b$ sem = standard error of the mean for all ls means in the same column.
Table 3.5 Soluble solids, Agtron colour readings, and juice pH of ripe tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was processed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble solids (%)</th>
<th>Agtron colour</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2012</td>
<td>2011</td>
<td>2012</td>
</tr>
<tr>
<td>Control</td>
<td>4.8 a</td>
<td>5.1 a</td>
<td>21 a</td>
</tr>
<tr>
<td>UNI</td>
<td>4.8 a</td>
<td>4.8 a</td>
<td>20 a</td>
</tr>
<tr>
<td>ASM</td>
<td>4.9 a</td>
<td>5.1 a</td>
<td>19 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>4.8 a</td>
<td>4.9 a</td>
<td>20 a</td>
</tr>
</tbody>
</table>

|                | 2011               | 2011          | 2011   |
|                | 4.34 a             | 4.20 b        |        |
|                | 4.35 a             | 4.25 a        |        |
|                | 4.36 a             | 4.21 ab       |        |
|                | 4.37 a             | 4.24 ab       |        |

|                | 0.34               | 0.18          | 1.5    | 2.4    | 0.021  | 0.011  |

\(^a\) Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD.
\(^b\) sem = standard error of the mean for all ls means in the same column.
Figure 3.8 Early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with P. syringae pv. tomato and X. gardneri, Ridgetown, ON, in a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. The number of leaves with disease symptoms in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments in a 1.24 m² area is shown. The corresponding area under the disease progress curve is shown in Figure 3.9. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference. An ‘**’ indicates differences among treatments which are discussed in the text.
**Figure 3.9** Area under the disease progress curve (AUDPC) for early season disease. Early season disease was measured by the number of leaves with disease symptoms (Figure 3.8) for tomato cv. TSH4 (black bars) and cv. H9909 (grey bars) treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (22 June-27 July), b) 2012 (8 June-5 July), c) 2013 (22 June-8 July). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.
Figure 3.10 Late season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Defoliation in the nontreated control (---), CuOH (– – –), CuOH + UNI (– – –), ASM (– – –), ASM + UNI (– – –), and UNI (– – –) treatments in whole plots is shown. The corresponding area under the disease progress curve is shown in Figure 3.11. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. NS = no significant difference.
Figure 3.11 Area under the disease progress curve (AUDPC) for late season disease. Late season disease was measured by the percent defoliation (shown in fig. 8) for tomato cv. TSH4 (black bars) and cv. H9909 (grey bars) treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011 (27 July-29 Aug), b) 2012 (5 July-8 Aug), c) 2013 (1 July-23 Aug). Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.
Table 3.6 The incidence of bacterial speck and bacterial spot on red tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit harvested in a 2m section of each plot was evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TSH4</th>
<th>H9909</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacterial speck</td>
<td>Bacterial spot</td>
</tr>
<tr>
<td>Control</td>
<td>0.0 a 7.0 a 1.0 a 7.0 a 30.0 a 16.0 a 2.5 a 5.2 ab 1.0 a 25.5 a 30.0 a 25.5 a</td>
<td></td>
</tr>
<tr>
<td>CuOH</td>
<td>0.5 a 6.0 a 1.0 a 14.0 a 24.5 a 12.0 a 3.0 a 10.5 a 2.1 a 28.5 a 34.5 a 24.5 a</td>
<td></td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>1.5 a 3.0 a 1.0 a 10.5 a 29.5 a 13.0 a 1.0 a 9.5 ab 1.5 a 23.0 a 27.5 a 28.0 a</td>
<td></td>
</tr>
<tr>
<td>ASM</td>
<td>0.5 a 7.5 a 2.5 a 12.0 a 27.0 a 15.5 a 2.0 a 2.5 b 1.5 a 20.5 a 24.0 a 30.0 a</td>
<td></td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>1.0 a 8.0 a 0.5 a 8.5 a 14.0 b 16.0 a 3.0 a 7.5 ab 3.0 a 25.5 a 24.5 a 24.5 a</td>
<td></td>
</tr>
<tr>
<td>UNI</td>
<td>0.5 a NT NT 19.0 a NT NT 3.0 a NT NT 25.0 a NT NT</td>
<td></td>
</tr>
<tr>
<td>sem b</td>
<td>0.47 1.69 0.66 3.54 2.74 5.80 1.65 1.95 0.97 3.29 4.65 7.77</td>
<td></td>
</tr>
</tbody>
</table>

* Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NT = not tested.

b sem = standard error of the mean for all ls means in the same column, except for the control treatment, cv. H9909, 2012 the sem is 2.21 and the CuOH treatment, cv. H9909, 2011 the sem is 1.08.
Figure 3.12 Height of tomato seedlings cv. TSH4 and H9909 treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse, Ridgetown, ON, in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Table 3.7 Stem diameter and foliar and root dry weight of tomato seedlings, cvs. TSH4 and H9909, treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse, Ridgetown, ON, 2011-2013.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Stem diameter (35 DAS) a</td>
<td>Stem diameter (35 DAS) a</td>
<td>Stem diameter (35 DAS) a</td>
<td>Stem diameter (35 DAS) a</td>
<td>Stem diameter (35 DAS) a</td>
<td>Stem diameter (35 DAS) a</td>
</tr>
<tr>
<td>Control</td>
<td>1.83 a</td>
<td>1.97 a</td>
<td>1.83 a</td>
<td>1.95 a</td>
<td>2.01 a</td>
<td>1.87 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>1.79 a</td>
<td>1.94 a</td>
<td>1.70 a</td>
<td>1.97 a</td>
<td>2.06 a</td>
<td>1.86 a</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>1.61 b</td>
<td>1.79 b</td>
<td>1.64 a</td>
<td>1.77 b</td>
<td>1.99 a</td>
<td>1.82 a</td>
</tr>
<tr>
<td>ASM</td>
<td>1.83 a</td>
<td>1.97 a</td>
<td>1.75 a</td>
<td>1.94 a</td>
<td>2.09 a</td>
<td>1.85 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>1.71 ab</td>
<td>1.71 b</td>
<td>1.74 a</td>
<td>1.76 b</td>
<td>1.88 a</td>
<td>1.81 a</td>
</tr>
<tr>
<td>UNI</td>
<td>NT</td>
<td>NT</td>
<td>1.73 a</td>
<td>NT</td>
<td>NT</td>
<td>1.85 a</td>
</tr>
</tbody>
</table>

| sem c          | 0.043    | 0.037    | 0.069    | 0.040      | 0.054      | 0.044      |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Foliar dry weight (g/plant) d</th>
<th>Foliar dry weight (g/plant) d</th>
<th>Foliar dry weight (g/plant) d</th>
<th>Foliar dry weight (g/plant) d</th>
<th>Foliar dry weight (g/plant) d</th>
<th>Foliar dry weight (g/plant) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.143 a</td>
<td>0.108 a</td>
<td>0.126 a</td>
<td>0.188 a</td>
<td>0.116 a</td>
<td>0.154 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>0.130 ab</td>
<td>0.102 a</td>
<td>0.121 ab</td>
<td>0.146 a</td>
<td>0.112 ab</td>
<td>0.135 ab</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>0.112 bc</td>
<td>0.104 a</td>
<td>0.099 bcd</td>
<td>0.172 a</td>
<td>0.110 ab</td>
<td>0.112 bc</td>
</tr>
<tr>
<td>ASM</td>
<td>0.123 ab</td>
<td>0.080 b</td>
<td>0.112 abc</td>
<td>0.144 a</td>
<td>0.085 b</td>
<td>0.126 ab</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>0.098 c</td>
<td>0.080 b</td>
<td>0.085 d</td>
<td>0.111 a</td>
<td>0.096 ab</td>
<td>0.085 c</td>
</tr>
<tr>
<td>UNI</td>
<td>NT</td>
<td>NT</td>
<td>0.096 cd</td>
<td>NT</td>
<td>NT</td>
<td>0.123 ab</td>
</tr>
</tbody>
</table>

| sem            | 0.0052                         | 0.0053                         | 0.0061                         | 0.0226                        | 0.0062                        | 0.0071                        |

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Root dry weight (g/plant) d</th>
<th>Root dry weight (g/plant) d</th>
<th>Root dry weight (g/plant) d</th>
<th>Root dry weight (g/plant) d</th>
<th>Root dry weight (g/plant) d</th>
<th>Root dry weight (g/plant) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.053 a</td>
<td>0.054 a</td>
<td>0.052 a</td>
<td>0.052 a</td>
<td>0.052 a</td>
<td>0.035 a e</td>
</tr>
<tr>
<td>CuOH</td>
<td>0.055 a</td>
<td>0.065 a</td>
<td>0.058 a</td>
<td>0.055 a</td>
<td>0.046 b</td>
<td>0.042 a</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>0.058 a</td>
<td>0.075 a</td>
<td>0.060 a</td>
<td>0.058 a</td>
<td>0.050 ab</td>
<td>0.038 a</td>
</tr>
<tr>
<td>ASM</td>
<td>0.063 a</td>
<td>0.053 a</td>
<td>0.051 a</td>
<td>0.045 a</td>
<td>0.047 b</td>
<td>0.035 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>0.059 a</td>
<td>0.076 a</td>
<td>0.057 a</td>
<td>0.053 a</td>
<td>0.067 a</td>
<td>0.036 a</td>
</tr>
<tr>
<td>UNI</td>
<td>NT</td>
<td>NT</td>
<td>0.056 a</td>
<td>NT</td>
<td>NT</td>
<td>0.038 a</td>
</tr>
</tbody>
</table>

| sem            | 0.0050                         | 0.0070                         | 0.0044                         | 0.006                         | 0.004                         | 0.069                         |

a DAS = days after seeding.
b Means in the same row followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NT = not tested.
b sem = standard error of the mean for all ls means in the same row.
c Dry weight of 5 seedlings per plot was measured 56 DAS in 2011, and 44 DAS in 2012 and 2013.
d Means in this row were transformed for ANOVA using a log transformation. The back-transformed means are shown here. The sem is for the transformed means.
Figure 3.13 SPAD chlorophyll readings measured 18, 37, and 56 days after transplanting in tomato cv. TSH4 and cv. H9909 treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in the nontreated control ( ), CuOH ( ), CuOH + UNI ( ), ASM ( ), ASM + UNI ( ), and UNI ( ) treatments a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD. NS = no significant difference.
Figure 3.14 Total ( ), red ( ), green ( ), and rotten ( ) fruit yield in a 2m section of tomato cv. TSH4 and cv. H9909 treated with CuOH, uniconazole (UNI), and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, in a) 2011-TSH4, b) 2012-TSH4, c) 2013-TSH4, d) 2011-H9909, e) 2012-H9909, and f) 2013-H9909. Error bars represent standard error of the mean. Bars with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.
Table 3.8 Soluble solids, Agtron colour readings, and juice pH of ripe tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2012. A subsample of 50 red fruit harvested in a 2m section of each plot was processed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble solids (%)</th>
<th>Agtron colour</th>
<th>pH</th>
<th>Soluble solids (%)</th>
<th>Agtron colour</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.9 a</td>
<td>5.2 a</td>
<td>18 a</td>
<td>18 a</td>
<td>4.36 a</td>
<td>4.31 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>4.7 a</td>
<td>5.2 a</td>
<td>18 a</td>
<td>18 a</td>
<td>4.35 a</td>
<td>4.32 a</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>4.9 a</td>
<td>5.4 a</td>
<td>18 a</td>
<td>17 a</td>
<td>4.35 a</td>
<td>4.32 a</td>
</tr>
<tr>
<td>ASM</td>
<td>5.0 a</td>
<td>5.5 a</td>
<td>18 a</td>
<td>17 a</td>
<td>4.31 a</td>
<td>4.28 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>5.1 a</td>
<td>5.3 a</td>
<td>19 a</td>
<td>17 a</td>
<td>4.31 a</td>
<td>4.30 a</td>
</tr>
</tbody>
</table>

sem $^b$ 0.17 0.23 0.1 1.1 0.017 0.021 0.14 0.22 0.8 1.0 0.010 0.027

$^a$ Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD.

$^b$ sem = standard error of the mean for all ls means in the same column.
3.4 Discussion

For these experiments, the combination of ASM and UNI for bacterial speck and spot control was assessed by applying them together in two different sequences over three years. In the first sequence of the UNI+ASM combination, UNI was applied in the greenhouse and ASM was applied in the field, while in the second sequence of the combination, both UNI and ASM were applied in the greenhouse. For the first ASM+UNI sequence, treatments with ASM alone, UNI alone or a nontreated control were included over the three years. Experiments with the second ASM+UNI sequence included treatments each year with ASM alone, CuOH and CuOH+UNI as well as a nontreated control. In the final year for the second ASM+UNI sequence, a UNI alone treatment was also included. ASM+UNI was compared to the nontreated control to determine if the combination controlled disease, and compared to ASM alone and UNI alone to see if the addition of UNI to ASM affected its level of disease control. Treatment with CuOH was included to compare ASM+UNI to a standard control, and CuOH+UNI was also examined to compare ASM+UNI to a UNI modified standard control.

Typically, field trials evaluating bacterial speck and spot management only involve measurements of late season disease severity (i.e. defoliation or percent leaf area affected) and yield (Louws et al., 2001, Huang et al., 2012, Roberts et al., 2008). However, in this work, early season disease incidence was also assessed to better capture differences in disease development during early phases of the field epidemic each year, since many of the treatment applications occurred during the greenhouse seedling stage and it was unknown how long potential effects would be detectable. Those results could then be compared to the standard late season disease severity assessments for defoliation. In addition to yield measurements, this work also measured tomato fruit quality as both UNI and ASM are known to impact tomato physiology.

3.4.1 Greenhouse UNI with field ASM application

Application of UNI to greenhouse tomato seedlings followed by ASM in the field suppressed early season AUDPC compared to the nontreated control in two of three years, but had no effect on late season AUDPC, fruit yield or quality in any year, except for yield in 2011. However, ASM without UNI suppressed early season AUDPC in all three years, and late season AUDPC in one of three years. Thus, the addition of UNI to ASM reduced the period of ASM effectiveness in one year, making ASM effectiveness more variable. UNI application alone did not reduce early or late season AUDPC, yield or fruit quality compared to the control in any year. Based on these results, UNI did not appear to have any disease control effectiveness alone; however, UNI did provide lower disease incidence and percent defoliation than the control on 27 July in 2011, and lower disease incidence on 20 June in 2013.
Therefore, application of UNI alone did affect disease, but this was slight and inconsistent. This is the first report of any effect of UNI alone on bacterial spot or speck severity in tomato and suggests that there are long-term effects of UNI on tomato growth and development. This is consistent with reported long-term effects of UNI on plant growth and development in bedding plants (Blanchard & Runkle, 2007). Taken together, the results indicate that the addition of greenhouse applications of UNI with field applications of ASM may reduce the relative effectiveness of ASM in some years, and that UNI alone has little effect on bacterial speck and spot.

It was expected that reductions in disease would be reflected in higher yield and greater tomato quality, since bacterial speck and spot are considered economically important field diseases that affect fruit in Ontario (LeBoeuf et al., 2009, Jones, 1991a, Jones, 1991b, Koike et al., 2007). The ASM+UNI combination had an effect on yield compared to the control in only one of the three years, 2011, when a significant increase was observed. However, this did not correspond to reductions in early or late season AUDPC in the same year, and so may have been due to a factor not related to bacterial speck or spot. Applications of ASM alone or UNI alone did not result in any yield benefit compared to the control in any year. This suggests that applications of UNI with ASM may have a synergistic effect on yield in some years. Since both early and late season AUDPC with ASM and ASM+UNI were equivalent in 2011, this effect appears to be unrelated to disease intensity. ASM+UNI resulted in a higher relative chlorophyll content on 18 DAT than ASM alone in 2011, suggesting higher plant N status may have played a role in the yield increase, but the same chlorophyll content response was observed in 2013 with ASM+UNI and no yield benefit was observed, not supporting this hypothesis. Thus, the mechanism of the yield benefit by ASM+UNI in 2011 is unknown. Future studies examining the effect of the greenhouse applications of UNI and field applications of ASM combination on yield in the absence of disease may provide additional clues with regards to the mechanism of this response. UNI is associated with stress tolerance in other plant systems (Al-Rumaih & Al-Rumaih, 2007, Duan et al., 2008, Senaratna et al., 1988, Zhang et al., 2007), but it is not clear if similar mechanisms were at play here.

Applications of UNI alone did not affect yield compared to the nontreated control. A previous report from Ontario associated UNI application with earlier bloom, advanced fruit maturity, and increased biomass (Zandstra et al., 2006). Few details on experimental methods are provided by Zandstra et al. (2006), but the authors reported that higher rates of fertilizer were used during seedling production than in the nontreated control plants. Fertilizer regimes were not altered for UNI treated plants in this study to avoid confounding factors in comparisons among treatments.

Relative chlorophyll content in the ASM+UNI treatment was higher than the nontreated control and ASM alone at 18 DAT in 2011 and 2013, and at 56 DAT in 2013, but were equivalent to UNI alone on the same dates. Applications of ASM had no effect on chlorophyll levels. These results suggest that
while UNI application can increase relative chlorophyll content, field applications of ASM alone or in combination with UNI have no effect on relative chlorophyll content.

The combination of greenhouse applications of UNI and field applications of ASM did not affect tomato colour, pH, or soluble. Applications of UNI alone or ASM alone also did not affect these results with the notable exception of UNI alone resulting in higher tomato juice pH than the control in 2011. However, the value of pH 4.25 measured in the UNI treatment was not of concern because it is still within an acceptable range for processing tomatoes (Monti, 1980).

3.4.2 Greenhouse UNI and greenhouse ASM application

In the second series of trials, UNI and ASM were applied to seedlings in the greenhouse, and cv. H9909 was included as well as cv. TSH4. Combining UNI and ASM applications reduced early and late season AUDPC and fruit with bacterial spot in cv. TSH4 in 2012, but not in 2011 or 2013. ASM and ASM+UNI applications alone did not reduce early season AUDPC, but ASM+UNI did reduce late season AUDPC compared to the nontreated control in 2012. There was no effect of ASM alone on fruit disease incidence in any year, except that it resulted in an increase in the incidence of bacterial speck on green fruit in 2012 compared to all other treatments. Thus, the addition of UNI to ASM increased ASM effectiveness for cv. TSH4 against the nontreated control compared to ASM alone, but only in 2012.

Applications of the ASM+UNI combination to cv. H9909 did not result in any reductions in early and late season AUDPC or diseased fruit, except for a reduction in the incidence of green fruit with bacterial speck in 2012. The same was true for application of ASM alone including the reduction in the incidence of green fruit with bacterial speck in 2012. In fact, the late season AUDPC in 2012 for ASM+UNI was higher than CuOH+UNI, suggesting some negative impact of ASM+UNI on disease control. Thus, cv. H9909 was largely unresponsive to the combination of ASM+UNI applied in the greenhouse.

Greenhouse ASM+UNI applications increased red tomato yield compared to the nontreated control for cv. TSH4 in 2012, but not in 2011 or 2013. ASM alone also had higher red tomato yield only than the nontreated control in 2012, and so the addition of UNI did not further increase yield compared to ASM alone. In contrast, greenhouse applications of ASM+UNI with cv. H9909 resulted in lower red tomato yield in 2012. The yield response for ASM+UNI and ASM were equivalent, with ASM alone also having lower total yield than the control and standard treatments. Similarly, total yield in the ASM-only treatment for cv. H9909 in 2011 was lower than the modified standard CuOH+UNI treatment, suggesting ASM and not UNI as the main driver of this yield effect. The increase in disease with greenhouse applications of ASM in cv. H9909 is consistent with observations of higher disease levels in the same
treatments in 2012, whereas in cv. TSH4 there was lower disease and higher yields with the same greenhouse ASM treatments, suggesting a genotype dependent effect.

Relative chlorophyll readings in the greenhouse ASM+UNI treatment in cv. TSH4 were equivalent to the nontreated control in 2011, but higher than the nontreated control and ASM only treatments 18 DAT in 2012 and 2013. This indicates that UNI increases relative chlorophyll for several weeks after application. Relative chlorophyll in the ASM-only treatment had lower relative chlorophyll content than the CuOH treatment in cv. TSH4 in 2012 and 2013 56 DAT. For cv. H9909, the impact of ASM+UNI on relative chlorophyll was less apparent. This treatment was equivalent to the control 18 DAT in all three years but had higher relative chlorophyll 18 DAT than ASM alone in 2011 and 2012. Similar to the previous discussion on the results of field applications of ASM, yield differences did not appear to be associated with differences in relative chlorophyll content, which tended to be higher across both cultivars when UNI was applied regardless of the negative yield effects in cv. H9909 in 2011 and 2012 or positive yield effects in cv. TSH4 in 2012. These results support the observation that ASM can have long term impacts on tomato physiology.

In most cases, greenhouse ASM combined with UNI did not have a negative impact on tomato quality. Treatments including ASM had lower soluble solids than the control in cv. H9909 in 2012. However, levels of soluble solids greater than 4.5% are desirable in processing tomato production soluble solids, and all treatments were higher than this value in all years (Monti, 1980). Juice pH in treatment ASM+UNI was higher than the control for cv. H9909 in 2012 when ASM was applied to seedlings, but juice pH for all treatments except the control was higher than the acceptable limit of pH 4.30.

Overall, plant response to ASM and UNI when both products were applied at the greenhouse seedling stage varied from the results of the first application sequence when ASM was applied in the field. Although the effect was marginal, greenhouse applications of ASM+UNI to cv. TSH4 resulted in more reductions in disease severity compared to the nontreated control than ASM alone, which is in contrast to the result from field only applications, where ASM combined with UNI reduced the effectiveness of ASM in some years. However, this effect was not noted in cv. H9909. The effect of ASM on yield also varied in 2012 with no yield response to field only applications but a positive yield response to greenhouse ASM application with cv. TSH4 and a negative response to greenhouse ASM application with cv. H9909.

3.4.3 Greenhouse UNI with greenhouse CuOH application

A standard treatment, CuOH, and modified standard, CuOH+UNI were also applied in the greenhouse as a control. The greenhouse treatment of CuOH was chosen instead of field applications of CuOH so that the greenhouse treatments could be directly compared to each other. CuOH+UNI was
included to observe if it may have an effect on CuOH since at the time of the experiments CuOH was widely used and it was expected that UNI may be widely adopted in greenhouse production in Ontario. The greenhouse UNI treatment was added in 2013 as a control to compare the response of UNI treated plants to that of CuOH+UNI treated plants.

Greenhouse applications of CuOH+UNI was the only treatment to have lower late season AUDPC than the nontreated control for cv. TSH4 in 2011, but was equivalent to the nontreated control in all other years. Furthermore, this treatment was equivalent to the nontreated control for early season AUDPC in all years, and had the equivalent early season AUDPC as ASM+UNI in 2012. Similarly, applications of CuOH resulted in early and late season AUDPC equivalent to the nontreated control in all years, and in 2012 early season AUDPC was higher with CuOH than ASM alone. For cv. H9909, CuOH+UNI was the only treatment to have lower late season AUDPC than ASM+UNI in 2012. Early season AUDPC was lower with CuOH+UNI and CuOH alone than the nontreated control and ASM alone in 2011, but in all other years, early and late season AUDPC for these treatments was equivalent to the nontreated control, ASM alone or ASM+UNI. Thus, the effect of the addition of UNI to CuOH is similar to that of the addition of UNI to ASM, which indicated occasional slight benefits of UNI to tomato health, except under conditions of drought stress for cv. H9909 in 2012. When greenhouse UNI was included in 2013, AUDPC for early and late season disease was equivalent to CuOH+UNI in both cultivars, indicating that the addition of CuOH to UNI provided no benefits. Greenhouse applications of CuOH rarely resulted in any benefit in field control of bacterial spot and speck.

Greenhouse applications of CuOH and CuOH+UNI resulted in yields equivalent to the nontreated control in all years. It is not surprising that applications of CuOH provided few advantages for disease management or tomato growth, since the product was only applied to tomato seedlings, and CuOH is a contact bactericide. Other studies show limited benefit of CuOH applications for long-term, consistent management of bacterial speck and spot, even when applications are made in the field (Griffin et al., 2017). This may be due to copper tolerance, as reported by Griffin et al. (2017). However, this is unlikely as the Xg isolate used in this study is not copper tolerant (Abbasi et al., 2015), and thus demonstrates limited long term benefits of greenhouse CuOH. As previously discussed, Zandstra et al. (2006) reported benefits in earliness and tomato yield with the use of UNI, but these effects were not observed here, possibly because fertilizer programs were not increased as Zandstra et al. (2006) had done.

Greenhouse applications of UNI frequently increased relative chlorophyll content compared to plants that were not treated with UNI in both cultivars, which was a response similar to that observed for ASM+UNI. Higher relative chlorophyll suggests higher N status in UNI-treated tomatoes, however as discussed above this did not result in a consistent increase in yield. Higher N status could be one factor that explains the slight benefit in bacterial disease tolerance observed in this trial, however, relative
chlorophyll content 18 DAT, when the response to UNI was most consistent among site years, was not consistently correlated with tomato yield or disease intensity (*data not shown*). Tomato quality was not affected by treatments of CuOH alone, UNI alone or CuOH+UNI.

3.4.4 Improvement of ASM effectiveness

It was hypothesized that the combination of greenhouse UNI with field or greenhouse ASM applications would improve the consistency of disease suppression with ASM both in foliage and fruit. Previous reports have shown inconsistency of field ASM applications for management of late season disease severity and fruit yield losses in the field due to bacterial spot and speck in tomato. Late season disease severity (Obradovic et al. 2004) and season-long AUDPC (Roberts et al. 2008) in ASM treated plots were not consistently lower than the nontreated control and only occasionally lower than standard copper + EBDC treatments in field tomatoes. ASM also did not reduce the severity of bacterial spot on tomato foliage in one of 14 and 10 of 14 trials based on season-long AUDPC and final disease severity ratings (Louws et al. 2001). However, Louws et al. (2001) found that seven out of seven trials had reduced foliar disease severity of bacterial speck with ASM compared to the nontreated control. For fruit disease incidence, only five out of eight trials against spot and one out of three trials against speck showed significant reductions (Louws et al., 2001). Similarly, there were few effects on fruit disease incidence when ASM was tested in Ontario from 2010 to 2012 (Trueman, 2015). Roberts et al. (2008), Lewis Ivey et al. (2004), and Graves and Alexander (2002) all reported percent marketable yield after ASM application as opposed to fruit disease incidence. However, it is difficult to determine if the results were only due to control of bacterial spot and speck because they include evaluations of other diseases, such as anthracnose, or were completed using fresh market tomatoes, where tolerance for fruit lesions is zero. Mahesaniya (2002) reported improved control of bacterial speck on tomato seedlings treatments with ASM and paclobutrazol, however, disease assessments were completed at the tomato seedlings stage and disease intensity in the field was not evaluated.

This study was undertaken under the assumption that applications of ASM can induce fitness costs in the host plant due to the re-allocation of resources from growth and development to plant defence pathways. However, relative chlorophyll readings in ASM-only treatments were not significantly lower than the nontreated control when ASM was applied in the field, suggesting no fitness costs associated with ASM application and plant nitrogen status. In two of three years UNI treated plants had higher relative chlorophyll than ASM and the control, but this was not correlated with higher tomato yield or disease tolerance (*data not shown*). Gianquinto et al. (2006) found relative chlorophyll 29 to 92 DAT was positively correlated with tomato yield, but there is a lack of additional information in the literature on the relationship between relative chlorophyll and yield earlier in the growing season. In addition, no negative
effects of ASM on tomato yield were observed when it was applied in the field and according to current label directions, despite conditions that included limited precipitation and irrigation for several weeks in 2012 and frost damage in 2013. In the cases where ASM-induced yield loss in tomato is reported by Louws et al. (2001), Damicone and Trent (2003), and Lange et al. (2007) application rates were 26.3 to 35.0 g ASM/Ha, which are more than two times higher than the rate of 12.5 g/Ha used in this study, included application on grafted plants (Kunwar et al., 2017), or included more than eight applications in a single season (Pontes et al., 2016). Thus, at the application rates and timings used, fitness cost potential of ASM may have been limited.

3.4.5 Environmental impacts on disease and control measures

Tomato response to ASM and UNI application varied among years and may be related to environmental conditions, since effects of ASM on greenhouse tomato and *Pst* are generally reported as being very consistent (Herman et al., 2008, Lanna-Filho et al., 2017). Several abiotic stress factors could have affected tomato response to ASM and UNI applications including transplanting delays in 2011 due to wet field conditions, unusually hot and dry field conditions in 2012, and low temperature and frost events in 2013 that occurred within days of transplanting. The ASM differing response among genotypes observed in 2012 may be related to differences in SAR induction, ability to compensate for growth changes induced by ASM, or ability to tolerate heat and water stress. This is consistent with Dietrich et al. (2005) who reports that the ability of *A. thaliana* to compensate for reductions in growth immediately after ASM application is influenced by environmental conditions. Plant response to abiotic stress is complex, particularly when multiple abiotic stresses occur simultaneously or in combination with a biotic stress such as pathogen infection (Suzuki et al., 2014). For example, water stress in tomatoes involves 19 different metabolic pathways, including those related to production of plant secondary metabolites, *PR* genes, hormones such as GA and SA (Gong et al., 2010). Water stress increased susceptibility to *B. cinerea* and reduced efficacy of ASM in one greenhouse study in tomato (Maymoune et al., 2015), but reduced susceptibility to the same pathogen in another greenhouse study (Achuo et al., 2006). In *A. thaliana*, a number of hormones including ABA, JA, GA, and ET affected water stress-related gene expression (Huang et al., 2008). Furthermore, ABA is reported to alter JA/ET-dependent gene expression and JA biosynthesis in an *A. thaliana*-Pythium *irregular* system (Adie et al., 2007), and suppress SAR induction in an *A. thaliana*-Pst system (Mohr & Cahill, 2007). Huang et al. (2012) reported that ASM concentration explained only 35 and 37% of the variation in AUDPC for bacterial spot in two Florida field trials. UNI applications are associated with reductions in chilling injury in tomato when UNI was applied four days prior the chilling stress (Senaratna et al., 1988), salt stress in *Datura* spp. (Al-Rumaih & Al-Rumaih, 2007), and drought stress in soybean (Zhang et al., 2007) and wheat (Duan et al., 2008), but
UNI appeared ineffective at reducing plant stress in the present study for cv. H9909. These findings are important as positive or negative yield effects have important implications for industry, even if the risk varies from year to year. Differences in yield response in cv. TSH4 and cv. H9909 support the hypothesis that ASM applications to tomato seedlings can induce fitness costs in tomato, but this effect is dependent on the environment and host genotype. In other field trials completed at the same location, applications of ASM to cv. H9909 in the field did not negatively affect tomato yield (Trueman, 2015), suggesting fitness costs may also be related to plant age at the time of ASM application.

3.4.6 Duration of ASM effectiveness

In 2012, greenhouse applications of ASM alone or in combination with greenhouse applications of UNI for cv. TSH4 had lower late season AUDPC than the nontreated control CuOH and CuOH+UNI. The final greenhouse ASM application in 2012 occurred 15 days prior to the first inoculation with \( \text{Pst} \) and \( \text{Xg} \), suggesting that ASM applied within the first 6 weeks of emergence can have long-term effects on host immunity and other components of host physiology under certain conditions. Previous research has demonstrated that ASM is translocated and degraded in tomato leaves within 72h of application in tomatoes at the 5 to 6 true leaf stage (Scarponi et al., 2001), and increases in \( \text{PRIa} \) gene expression in field tomatoes are apparent within 1 day of application (Herman et al., 2007), but less is known about factors affecting the duration of the response. Application of ASM to approximately 22-week old tomato plants induced \( \text{PRIa} \) gene expression in all three cultivars tested in a New York field trial, and although the pattern of expression varied among the three cultivars tested, expression returned to baseline levels within seven days of activation (Herman et al., 2007). That result was supported by observations that 14-day interval field applications of ASM were ineffective at reducing bacterial spot severity in Florida (Huang et al. 2012) and that the optimal levels of disease control was achieved with a 8 to 10 interval in Brazil (Pontes et al., 2016). Tomato seedlings treated with ASM at 15 DAS with and without \( \text{Xv} \) challenge had higher levels of peroxidase and catalase compared to controls with and without pathogen challenge 12 days after ASM application (Cavalcani et al., 2006). Similarly, peroxidase and chitinase activity in tomato seedlings treated with ASM with and without challenge by \( \text{Clavibacter michiganensis} \) ssp. \( \text{michiganensis} \) remained higher than the water and water and pathogen control for 10 days after ASM application (Baysal et al., 2003), and tomato seedlings grown from ASM-treated seeds showed differential gene expression at 12 to 22 DPT compared to a non-treated control (Goodwin et al., 2017b). In tobacco, ASM induced SAR genes including \( \text{PRI} \) were induced up to 20 days after application in 8-week old greenhouse grown plants (Friedrich et al., 1996), and ASM application to canola 21 days prior to inoculation with \( \text{P. syringae pv. maculicola} \) still resulted in a reduction in pathogen growth and
increased *PRI* and *PR2* gene expression (Potlakayala et al., 2007). This indicates there are long term effects of ASM on host defense in tomato and other plant species.

### 3.4.7 Comparison of disease assessment methods

Disease incidence in the early part of the season was completed by counting the number of leaves with any bacterial speck or spot symptoms in an area of 1.24 m², which represents the approximate area occupied by five tomato plants. The incidence values therefore represent the density of symptomatic leaves in an area. No visual differences in plant size were observed among treatments in the trials in any year. An alternative method for calculating incidence was also implemented in both trials in 2013, where the total number of leaves in 1.24 m² and the number of symptomatic leaves was counted, so that the percentage of leaves with symptoms could be calculated. A comparison of total leaf counts in the trials when ASM was applied in the field showed no difference in total leaf counts among treatments when early season disease assessments were completed (Figure A.2). Means separation differed slightly from the symptomatic leaf counts per 1.24 m², but the conclusion that ASM and ASM+UNI provided equivalent levels of bacterial disease suppression remains the same. For the trial when ASM was applied to seedlings, there was also no difference among treatments for the number of leaves in 1.24 m² for cv. H9909, and no change in means separation when the percent symptomatic leaves was analysed compared to the number of symptomatic leaves per 1.24 m² (Figure A.3). For TSH4, the control had fewer total leaves than ASM+UNI on the final early season assessment date (8 July, 90 DAS), and there were some slight differences in means separation on the first two assessment dates (22 June/74 DAS and 1 July/83 DAS). Percent symptomatic leaf analysis showed that the ASM and ASM+UNI treatments were equivalent on 22 June (74 DAS), whereas the analysis using the number of symptomatic leaves per 1.24 m² showed the ASM+UNI treatment had lower disease incidence than ASM. The analysis of percent symptomatic leaves also showed that on 1 July (83 DAS) the ASM+UNI treatment had fewer infected leaves than the control, whereas the analysis using the number of symptomatic leaves per 1.24 m² showed no difference among treatments. Overall, there was little difference in the two assessment methods and the overall conclusions of the study were not affected.

Disease intensity was evaluated using different methods in the early compared to the late part of the growing season. Although this could be considered a disadvantage because disease progress over the course of the season could not be directly compared or summarized, the early season assessment method allowed for assessment of treatment effects at the beginning of disease onset, but prior to the beginning of defoliation symptoms, so that any early treatment effects could be detected. It was not possible to continue using the early season disease assessment throughout the entire season due to the quantity of plant biomass produced by the tomato plants and difficulty in counting leaves without damaging plants.
Defoliation was assessed on a percent scale using 5% increments which is demonstrated to be equivalent to the commonly used Horsfall-Barratt when disease severity is greater than 1% (Chiang et al., 2014).

Symptoms of other foliar plant diseases such as early blight, Septoria leaf spot, and late blight were not observed in the trials in any year, and preventative fungicide applications were applied to all treatments in 2012 and 2013, confirming that any treatment differences observed were a result of bacterial speck and spot.

3.4.8 Conclusions

This research explored the possibility that tomatoes treated with UNI at the seedling stage combined with ASM at either the greenhouse seedling or field stage will have higher tolerance to bacterial speck and spot under field conditions in Ontario, Canada. The results indicate that under the best case scenario, applications of UNI may occasionally provide marginal benefits for suppression of these diseases when combined with or without ASM. Due to the limited benefits and inconsistent response to UNI for bacterial disease management, use of UNI should be for transplant height management only with the understanding that, in some years, its use combined with ASM in the field may be beneficial. ASM applied to tomato seedlings can result in a reduction in bacterial speck and spot intensity several weeks after treatment in some years; however, in certain genotypes and under certain environmental conditions there is the potential for increased disease susceptibility and lower yield. Commercial application of crop protection tools requires a predictable response under field conditions so that products can be used efficiently and effectively to limit losses to plant disease. Plant defense inducers have the potential to be part of an integrated solution for management of bacterial speck and spot in Ontario, but additional research on the long term effects of plant defense inducers on host immunity, physiology, growth, and host interactions with environmental stresses is required to realize the full potential of these tools in commercial tomato production.
Chapter 4: Use of bacterial endophytes to control the incidence of bacterial speck disease 
(Pseudomonas syringae pv. tomato) and alter plant growth in processing tomato seedlings

4.1 Introduction

Bacterial endophytes of plants are bacteria that reside in surface sterilized plant tissue with neutral or beneficial effects on their host (Hallmann et al. 1997). Many are culturable, but there are also viable but not culturable bacterial endophytes that are identified as the set of microbial genomes located inside plant organs (Gaiero et al., 2013, Bulgarelli et al., 2013). The major sources of endophytes are seeds, propagative material, the phylloplane and soil, but the latter is considered the most important inoculum source at least for root endophytes (Hallmann et al., 1997, Hardoim et al., 2008, Rosenblueth & Martinez-Romero, 2006). Taxonomically, bacterial endophytes are mainly members of the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, which is less diverse than rhizosphere bacteria (Berg et al., 2005, Bulgarelli et al., 2013). Based on their adaptations for endophytism, Hardoim et al. (2008) divided bacterial endophytes into passenger endophytes, which have few adaptations and are present in the outer root cortex tissue only by chance, opportunistic endophytes, which have more adaptations but are still confined to root cortex tissues, and competent endophytes, which are the most adapted to the plant environment allowing for higher populations in tissues, vascular colonization and spread inside the plant for near complete plant colonization. Competent endophytes are believed to have been under selection pressure because they provide benefits to the host in the interaction.

For tomato, bacterial endophyte species appear to be relatively diverse. Cultures of endophytic Pseudomonas oleovorans, Pseudomonas plecoglossicida, P. ananatis, Citrobacter freundii, Staphylococcus hominis, Sphingobacterium multivorum, Enterobacter cloacae, Arthrobacter globiformis and Rhizobium radiobacter were obtained from tomato roots (Upreti & Thomas, 2015). In another example, Feng et al. (2013) used a non-culture-dependent method (PCR- restriction fragment length polymorphism and 16S rDNA sequencing) to identify Sphingomonas yanoikuya, Pseudomonas pseudoalcaligenes, S. marcescens, B. megaterium, Paenibacillus polymyxa, B. pumilus, B. cereus, P. fluorescens and A. globiformis as endophytes from roots and stems of two tomato cultivars. However, mass sequencing of 16S-ribosomal RNAs demonstrated that the endophyte population in tomato is even more diverse as 80 operational taxonomic units were found for tomato leaf bacterial endophytes, which were dominated by members of the Proteobacteria with relatively small numbers in the Actinobacteria, Planctomycetes, Verrucomicrobia and Acidobacteria (Romero et al., 2014). In another study, the diversity of 41 bacterial endophyte isolates cultured from tomato stems was less clear, as only two of them, Staphylococcus epidermidis and B. amyloliquefaciens, were identified (Nawangsih et al., 2011).

Two important benefits that bacterial endophytes provide plants are induced disease resistance
and plant growth promotion (Hardoim et al., 2015). Endophytes mostly induce resistance via ISR, which involves priming of ET and JA dependent signalling pathways and typically induces resistance against hemibiotrophs and necrotrophs, but some endophytes could be considered to induce SAR, which involves SA dependent signaling pathways and typically induces resistance against biotrophs and hemibiotrophs (Pieterse et al., 2009a, Ton et al., 2006). Bacterial endophytes can promote plant growth by increasing the availability of nitrogen and phosphorous, and modulating levels of phytohormones including IAA, cytokinins and ET (Hardoim et al., 2015). Part of the plant growth promotion is also due to competition with plant pathogens through production of antimicrobial compounds, nutrient competition and siderophores, which can act in addition to induced resistance (Hallmann et al. 1997; Lugtenberg and Kamilova 2009; Rosenblueth and Martinez-Romero 2006).

For tomato, a number of bacterial endophytes have been shown to be beneficial. For example, endophytic Bacillus and Arthrobacter species from tomato that produced IAA and siderophores and solubilized inorganic phosphate, caused tomato growth promotion (Amaresan et al., 2012). A. johnsonii, S. marcescens, Sinorhizobium sp. and B. megaterium induced resistance against early blight (A. solani) and bacterial speck, and also increased plant height (Barretti et al., 2009). Since the endophytes were applied to cut stems and the pathogens were inoculated on leaves, it is likely that induced resistance was involved. Isolates of B. pumilus and B. amyloliquefaciens from tomato induced resistance against bacterial speck in tomato, which was associated with induction of peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase associated with resistance (Lanna-Filho et al., 2017). P. oleovorans and Agrobacterium tumefaciens, which were also isolated from tomato, increased seedling vigour and reduced tomato bacterial wilt, although induced resistance was not established (Thomas & Upreti, 2016, Upreti & Thomas, 2015). B. pumilus SE34 induced resistance against F. oxysporum f. sp. radicis-lycopersici by inducing ultrastructural changes in tomato, such as the production of root wall appositions and electron-dense substances that are associated with resistance (Benhamou et al., 1998). Thus, there is ample evidence for tomato bacterial endophytes as providing both plant growth promotion and induced resistance.

Bacterial endophyte populations in plants can be influenced by a number of factors including host species (McInroy & Kloepper, 1995), cultivar (Hardoim et al., 2011, Rasche et al., 2006) and plant development stage (Andreote et al., 2010, Monteiro et al., 2011). The effect of plant development, for example, was observed with diazotrophic bacterial populations of vetiver that were more common after transplanting at 3, 6 and 12 months than 1 month (Monteiro et al. 2011), and populations of phosphate solubilising bacteria in soybean were higher in roots at the vegetative than the flowering and senescence stages (Kuklinsky-Sobral et al., 2004). The effect of plant genotype, for example, was observed with more IAA-producing bacterial endophytes in an early ripening than a late-ripening soybean cultivar.
(Kuklinsky-Sobral et al., 2004), and the diversity of tomato root endophytes was greater in a bacterial wilt resistant than a wilt susceptible cultivar, which was related to there being more bacteria with antagonistic activity against *R. solanacearum* in the resistant cultivar (Upreti & Thomas 2015).

Developing new bacterial endophytes for tomato disease protection is appealing because endophytes are already adapted to colonizing the plant environment and may also promote plant growth (Mercado-Blanco & Lugtenberg, 2014). However, their effectiveness when applied to plants is determined by many factors, such as the inoculum density, environment and plant genotype (Pillay & Nowak, 1997). Thus, developing bacterial endophytes for disease control and plant growth promotion in tomato requires not just obtaining a promising endophyte but also examining many aspects of the system. The objective of this study was to screen bacterial endophytes from cultivated tomato (*S. lycopersicum*), wild tomato (*S. arcanum, S. chmielewskii* and *S. cheesmaniae*) and *N. benthamianum* for their ability to induce resistance against *Pst* and then optimize conditions for inducing resistance against bacterial speck through an examination of endophyte inoculation timing, inoculation method and concentration, and the impact of host genotype.

4.2 Materials & Methods

4.2.1 Isolation of endophytes from tomato and wild tomato species

Seeds of tomato cv. Tiny Tim and TSH4 were obtained from the A.E. McKenzie Co, Brandon, MB and Tomato Solutions, Chatham, ON, respectively, and seeds of the wild tomato species, *S. arcanum* LA2152, *S. chmielewskii* LA1327 and LA1306, *S. cheesmaniae* LA1040, and *S. galapagense* LA1508 were obtained from the C.M. Rick Tomato Genetics Resource Centre, University of California at Davis, CA, USA. Soil was collected from range A7 (sandy loam with more than 30 years of vegetable production) or a grass buffer strip located near the edge of a wood lot at the Ridgetown Campus, University of Guelph (42.4406° N, 81.8842° W) and pasteurized in a drying oven for 11 min per L of soil at 60°C to obtain an internal temperature of 60°C for 30 minutes. The pasteurized soil was mixed with Fafard germination mix (Fafard et Frères, St. Bonaventure, PQ) and fine granulated washed sand (Alltreat Farms, Arthur, ON) at a ratio of 40:40:20 and placed in 4-inch pots. Tomato cv. Tiny Tim or TSH4 were seeded in the range A7 soil and *S. arcanum* LA2152, *S. chmielewskii* LA1327 and LA1306, *S. cheesesmaniae* LA1040, and *S. galapagense* LA1508 were seeded in the grass buffer strip soil. The second soil source was included in later experiments to try and increase the diversity of endophytes isolated from tissues, as there was no history of crop production in this soil. Plants were grown in a growth chamber at 22°C with a 16 h photoperiod with light intensity of approximately 95 μmol/m²/s for four to six weeks to obtain roots for endophyte isolation. Tomato cv. TSH4 was also grown in the field for approximately three months after transplanting in range A7 using standard practices for processing tomatoes in Ontario.
Roots were separated from the soil and gently but thoroughly washed in tap water, surface sterilized for 60 sec in 70% ethanol, 120 sec in 10% bleach, rinsed three times in sterile distilled water, and then blot dried on sterile filter paper. The fresh weight of each root mass was recorded, and then roots were macerated using a mortar and pestle until no root pieces were visible. Approximately 2 mL sterile distilled water with 0.02% Tween 80 was added to the macerated roots, which was then serially diluted using sterile distilled water with 0.02% Tween 80. 100 uL of the macerated solution of each dilution and the wash water from the third root rinse was plated on TSA in duplicate and incubated at room temperature (22°C). The number and type of colonies was recorded after two days, and representative single colonies of common phenotypes were streaked on TSA to obtain pure cultures. Long term storage cultures of each isolate were prepared by inoculating LB broth from a single colony, growing overnight, and mixing with sterile glycerol to reach a final concentration of 15% glycerol. Cultures were stored at -80°C.

4.2.2 Screening of endophytes for induced resistance and plant growth effects

Twenty bacterial endophytes from tomato roots isolated at Ridgetown and seven from Nicotiana benthamiana roots isolated in the laboratory of P. H. Goodwin, University of Guelph were screened for their ability to induce resistance against Pst using an overnight seedling root soak + soil drench assay (Valenzuela-Soto et al. 2010). Seeds of tomato cv. TSH4 were planted in Fafard germination mix in a 288-cell plug tray (approximately 14 mL per cell). Endophytes were grown for 72 hrs on TSA, transferred to 10 mM MgCl2 using a sterile swab, vortexed, adjusted to absorbance 1.0 (+/- 0.050; OD = 600) and then diluted 10-fold. Nine days after planting, seedlings were carefully removed from the soil, and the roots were placed in the endophyte solution and shaken for 30 min at 50 rpm. The seedlings were then transplanted into 4-inch (500 mL) round pots filled with a 50:50 mix of pasteurized soil from the same buffer strip described previously and Fafard germination mix. 10 mL of the endophyte solution used to soak the seedling roots onto the soil surface of each pot. Control treatments included equal volumes of 10 mM MgCl2 in place of all endophyte solutions.

For this study, the tomato, bacterial root endophyte and foliar bacterial speck (Pst) system was chosen as application of bacterial endophytes can induce resistance in tomato (Benhamou et al., 1998, Lanna-Filho et al., 2017), and Pst can be controlled by both SAR and ISR (Herman et al., 2008). Pst causes bacterial speck, which causes foliar lesions, defoliation, early fruit ripening and sunscald in commercial tomatoes (Preston 2000; Young et al. 1978). Plants were inoculated with approximately 2 x 10^7 CFU/mL of Pst DC06T2-4 in distilled water with 0.01% Sylgard 309 at 20 DAS. The upper leaf surface of each plant was sprayed with inoculum until just before runoff using a hand-held mist applicator, and then the plants were covered with a translucent plastic container for 24 hours in a growth
chamber. No fertilizer was applied during the study period. The number of lesions on all compound leaves on each plant was recorded seven DPI with Pst. Leaf area was determined by detaching compound leaves, tracing the circumference of each leaflet on a clear acetate sheet, scanning the image, and determining the number of pixels using Image J v.1.47 (http://imagej.nih.gov/ij/). Pixel number was converted to area (cm²) using the following equation: area = ((0.0171*no. of pixels) + 5.3828) * 0.01. The equation was developed by measuring the numbers of pixels measured for circles with known areas and creating a standard curve of pixel number versus area. At seven DPI, plant height from the soil line to the growing point, foliar fresh weight and root fresh weight were determined, and the relative chlorophyll the first leaflet of each compound leaf was recorded using a Minolta SPAD-502 Chlorophyll Meter (Konica Minolta, Tokyo Japan). Foliar and root dry weights were recorded after fresh samples were placed in a greenhouse for 1 to 2 weeks until dry.

After one experiment using 27 endophytes, endophytes R9, R17, R19, R20, R21 and Serenade Max (B. subtilis QST713) (Bayer CropScience, Calgary, AB) were applied using the seedling soak assay as described above. The purpose of these preliminary experiments was to further evaluate the selected endophytes as candidates for future experiments, and to refine the experimental method, thus experimental methods varied. Serenade Max was meant to serve as a possible positive control for disease suppression. The seedlings were grown in 50:50 Promix PGX (Premier Tech Horticulture, Rivière-du-Loup, PQ) with pasteurized range A7 soil in experiment one and Promix PGX only in experiment two. Also, Promix PGX Biofungicide (B. subtilis MBI600) (Premier Tech Horticulture, Rivièr du-Loup, PQ) was applied by growing the seedlings in the Promix PGX potting media mixed 50:50 with pasteurized range A7 soil in experiment one and non-pasteurized range A7 soil in experiment two. The purpose of using the Promix PGX Biofungicide was to determine its utility as a positive control. Inoculation with Pst was the same as described for the initial screening, except inoculum was applied to both the upper and lower leaf surfaces. The experiment was a randomized complete block design with four replications per treatment and one pot per replicate.

In addition, a seed soak + seed drench + seedling drench inoculation method was developed and tested for endophytes R9, R19 and R20 in three experiments, and R17 and R21 in four experiments, each in a randomized complete block design with 4 replications per treatment. Seeds of tomato cv. TSH4 were surface sterilized in 2% sodium hypochlorite for 10 min and rinsed three times in distilled water for five minutes (Tyburski & Tretyn, 2004). Bacterial endophyte solutions were prepared as previously described. For the seed soak portion of the application, tomato seeds were added to the endophyte solution and incubated overnight at 150 rpm. Seeds for the control were placed in 10 mM MgCl₂ only. After 14 to 16 h the seeds were carefully removed from the solution, placed on paper towels, sown in 3.5-inch square pots (530 mL) filled with Fafard germination mix, covered with vermiculite, and placed in a growth chamber.
at 24°C and 16 h photoperiod. For the seed drench portion of the application, a pipette was used to apply 4.95 mL of the endophyte solution that had been used to soak the seeds overnight to the surface of the germination mix. For the seedling drench portion of the application, a fresh solution of each endophyte solution was prepared as above and applied to the surface of the germination mix at 10 DAS using the same method as the seed drench. No fertilizer was applied during the study period. *Pst* inoculation and plant growth and disease assessments were completed as previously described.

### 4.2.3 Standard curve of CFU versus A600

A standard growth curve was developed for R17 and R21 by plating dilutions of the bacteria grown on TSA for 72 h and then diluting in sterile distilled water to 0.2, 0.4, 0.6, 0.8, and 1.0 absorbance (OD = 600). Serial dilutions at each absorbance level were plated onto TSA and counted at 48 hours after incubation at approximately 22°C. From this, a formula was developed for R17: CFU/mL = [(9x10⁷) (absorbance)] - 1x10⁷, and a formula was developed for R21: CFU/mL = [(2x10⁹) (absorbance)] - 2x10⁸. Based on populations obtained from the serial dilutions at 1.0 absorbance, the approximate population of R17 and R21 used in the previous trials was calculated to be approx. 8 x 10⁶ and 3 x 10⁸ CFU/mL. The formulas were used to calculate OD = 600 required for the R17 and R21 cells to be adjusted to 1 x 10⁷ and 1 x 10⁸ CFU/mL for R17 and R21 for all additional experiments. Only absorbance measurements at 600 nm were used at < 0.500 to avoid erratic measurements at higher absorbance levels.

### 4.2.4 Direct antimicrobial effects of R17 and R21

The direct antimicrobial effects of R17 and R21 were evaluated using an agar overlay assay and a modified cross-streaking assay (Jinhua et al., 2002). For the agar overlay assay, TSA was prepared using standard methods, and a 2 cm diameter circle traced on the bottom of each plate. The area inside each circle was then streaked with endophyte solution, and the plates were incubated at room temperature for two days. TSA was prepared (1.50% agar), and 5 mL added to sterile test tubes and incubated in a 51°C water bath. The molten agar was then inoculated with 0.1 mL of 2 x 10⁷ CFU/mL *Pst* 06T2-4, inverted three times to mix, and then slowly poured over the endophyte inoculated TSA plate. The plates were incubated at room temperature and assessed for a zone of inhibition around the endophyte inoculated area two days after *Pst* inoculation. The overlay assay was repeated three times with eight replications per treatment.

A cross streaking assay was completed by streaking a single endophyte on TSA to form a single linear streak across the centre of each plate. Plates were incubated for two days at room temperature, and then a single colony of *Pst* 06T2-4 was streaked at a 90° angle from the edge of the plate to the edge of
the endophyte streak. Care was taken to finish the streak as close as possible to the endophyte without touching it. Plates were incubated for two days at which time the streak of *Pst* was well grown and plates could be assessed for a zone of inhibition. The *Pst* streak width at the rim end of the plate and the center of the plate where the streak stopped was also measured and used to calculate the percent reduction in streak width. Each assay was repeated three times with five replications per treatment.

4.2.5 **Optimization of bacterial endophyte treatment**

To compare the effects of bacterial culture media, R17 and R21 were grown on TSA, 0.1 TSA, and LB agar for three days and then prepared as previously described for seed soak + seed drench + seedling drench inoculation in one experiment. To compare the effects of soil fertility, the seed soak + seed drench + seedling drench inoculation was done with R17 and R21 after growth on 0.1 TSA and TSA as previously described, but plants were grown with no added fertilizer versus addition of 20 mL (three experiments) or 80 mL (one experiment) of Plant-Prod Ultimate fertilizer (Plant Products Co Ltd, Brantford, ON, CA) at 11, 16 and 21 DAS. The fertilizer was a 20-20-20+micronutrients fertilizer solution mixed at 1.26 g/L. The incidence of bacterial speck lesions, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined as previously described, except assessments were completed at five DPI and the number of lesions per cm\(^2\) on only the second and third youngest mature leaves on each plant was calculated. The second youngest leaf was identified using the definition of the youngest developing leaf as the first leaf from the apical meristem with a terminal leaflet midrib length of 1.5 cm or longer.

To evaluate the effects of the endophyte application method, R17 and R21 were applied as described above for seed soak, seed drench, seedling drench, seed soak + seed drench or seed soak + seed drench + seedling drench. Applications of 10 mM MgCl\(_2\) at the time of the different endophyte treatments were used as controls. Plants were grown as above with 20 mL of fertilizer in the first trial, and 80 mL fertilizer in the second and third trials, and disease and plant growth assessments were taken at five DPI. Lesion counts and leaf area were evaluated for the second and third youngest leaves of each plant. To determine the effect of raw and pelleted seed with different application methods, the seed soak, seed drench or seed soak + seed drench application methods for endophyte R17 was repeated in two trials with seven replications as above. At five DPI, the incidence of bacterial speck lesions on the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined.

A comparison of five commercial tomato cultivars on the effectiveness of endophyte R17 was done with tomato cv. H2401, cv. H5108, and cv. H9553 (H.J. Heinz, Leamington, ON), and cv. TSH33 and cv. TSH4 (Tomato Solutions, Chatham, ON). The effect of different doses of R17 on induced
resistance in cv. TSH4 was determined by evaluating five concentrations of R17 (1x10^5, 1x10^6, 1x10^7, 1x10^8, and 1x10^9 CFU/mL) and a nontreated control in cv. TSH4 using the seed drench method. The duration of the plant response to R17 was determined by Pst inoculation performed 15, 20 and 25 DAS on cv. TSH4. Except for the dose experiment, R17 was applied at 1 x 10^7 cfu/mL in 10 mM MgCl₂ using the seed drench method, and the plants were fertilized at 10, 15 and 21 DAS with 80 mL fertilizer solution, except that plants inoculated 25 DAS in the timing of induction trials were also fertilized at 26 DAS. Control plants received 10 mM MgCl₂. At five dpi, the incidence of bacterial speck lesions on the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined.

4.2.6 Effect of R17 inoculation on populations in planta

Spontaneous rifampicin resistant strains of R17 were developed using a method similar to Andreote et al. (2008). Briefly, an overnight culture of R17 was grown in tryptic soy broth with shaking. A 4 mL sample was removed from the overnight culture, centrifuged at 6000 rpm for 15 minutes, the supernatant removed and the bacterial pellet resuspended in 1 mL sterile distilled water. The bacteria were then spread on TSA amended with 50 µg/mL rifampicin and incubated at room temperature for four days. Single colonies were picked from the rifampicin-amended plates, grown overnight in TS broth, and stored in 15% glycerol at -80°C.

To confirm the rifampicin resistant R17 strains have the same disease suppression characteristics as the wt R17, three rifampicin resistant strains (R17-RfpA, R17-RfpB, and R17-RfpC) were compared to the wt R17 (R17-Wt) using cv. TSH4. Three trials with five or six replications per treatment were completed using the seed drench application method and effects on disease suppression and plant growth were assessed as previously described.

The ability of R17-RfpC to colonize the rhizosphere and plant tissues was examined at 10 and 25 DAS. R17-RfpC was applied as a seed drench and plants were grown as previously described, except each replicate consisted of five pots with 10 plants per replicate at 10 DAS (2 plants/pot) and five plants per replicate at 25 DAS (1 plant/pot). Cotyledon leaves were used for foliar samples at 10 DAS because there was little or no true leaf tissue present. At 25 DAS, the terminal leaflet on the second and third youngest leaves were collected. Root tissue was collected by gently digging out roots from pots and removing the Fafard germination mix by rinsing in tap water. Prior to rinsing in tap water, the germination mix from around the roots was collected for rhizosphere samples. Root and leaf tissue was wrapped in paper towel, placed in a plastic bag, and stored at ambient temperature until processing, which was within four hours of sample collection. Rhizosphere samples were collected in plastic centrifuge tubes to avoid moisture loss prior to processing. To ensure R17 was not present in the germination mix.
prior to drenching, samples were collected directly from the Fafard germination mix bag and processed using the same method as the rhizosphere samples. No colonies resembling R17 were detected.

Root samples were processed as previously described for endophyte isolation, except only 1.5 mL sterile distilled water with 0.02% Tween 80 was added to macerated roots, which was directly plated onto 10 plates of TSA amended with 50 µg/mL rifampicin. The method for foliar samples was the same as the root tissue except the tissue was surface sterilized for 90 seconds in 2-3% sodium hypochlorite, followed by 60 seconds in 70% ethanol, and rinsing in sterile distilled water. For rhizosphere samples, 1 g of germination mix was mixed with 10 mL sterile distilled water with 0.02% Tween 80, macerated in a mortar and pestle to reduce particle size, and incubated at room temperature for 30 to 40 minutes. The solution was then vortexed and plated as described above for root and foliar samples. Dry weight of the rhizosphere samples was determined by using an equal subsample of the germination mix, and the percent moisture of rhizosphere samples and population of R17-RfpC per g of dry soilless mix calculated.

Colony types matching R17 were counted at six days after plating, and the presence of R17 was confirmed by streaking random single colonies on TSA and submitting samples to Laboratory Services, University of Guelph for identification using 16S and gyrB DNA sequencing described below.

4.2.7 Effect of R17 inoculation on Pst populations and lesion size in planta

Tomato cv. TSH4 was grown and inoculated with R17 with the seed drench method. On the day of disease assessment (five DPI), the third terminal leaflets of control and R17 inoculated plants were excised, and the leaf area of each terminal leaflet was determined as previously described. The fresh weight of each sample was recorded and surface sterilization was completed by immersing each leaflet in 3.1% sodium hypochlorite solution for 90 seconds, 70% alcohol for 60 seconds, and then rinsing in sterile distilled water for 30 seconds. Leaflets were blot dried on filter paper and homogenized in 2 mL of sterile distilled water using a mortar and pestle. A dilution series was completed and 100 µL from dilutions 10⁻² through 10⁻⁶ were plated on VBT, a semi-selective media for Pst (Cuppels & Elmhirst, 1999). The efficacy of the surface sterilization method was confirmed by plating rinse water from each sample on TSA. The number of colonies on each plate was recorded after incubation at room temperature for four days. The number of CFU per cm² of leaf tissue and per lesion was then calculated. The trial was repeated three times with three (experiments 1 and 2) or two (experiment 3) replications per treatment in each experiment.

To determine the effect of R17 inoculation on lesion size and circumference, tomato cv. TSH4 was grown as previously described, and the terminal leaflet on the third youngest tomato leaf collected on the day of assessment (five DPI). A Nikon D300s camera (Nikon Canada Inc., Mississauga, ON) set to JPEG fine quality was used to take a photo (12.2 MB, 4288 x 2824 pixels) of each leaflet. A ruler was
also included within each image. Photos were uploaded to PowerPoint 2010 (Microsoft, Redmond, Washington, USA), increased in size to allow for tracing of the lesion circumference, printed in colour, and the circumference of 12 to 15 lesions per leaflet traced using a fine point permanent marker. Lesions were selected by tracing all lesions within a randomly placed 225 mm² quadrant, and then 12 to 15 lesions were randomly selected. The lesion circumferences were then scanned and the number of pixels determined as previously described for leaf area, except the size of each image was standardized by calibrating each image. This was completed in Image J by using the line tool to draw a straight line representing a distance of 2 cm on the ruler included in the original image, clicking ‘analyze’ and ‘set scale’, setting the distance in pixels to 40 and the known distance to 2 cm. Lesion circumference was calculated using the equation \( C = 2\pi \sqrt{\text{mean lesion area}/\pi} \). The percent leaf area covered in lesions was calculated using the equation \( \text{area} = (\# \text{lesions}/\text{cm}^2 \times \text{mean lesion area} \text{(cm}^2)) \times 100 \). The experiment was repeated twice with seven (experiment 1) and six (experiment 2) replications per treatment.

4.2.8 Identification of endophytes R17 and R21

The 16S DNA partial sequences of R17 and R21 were amplified with primers 27f (5’GCYTAACACATGCAAGTCGA-3’) and 1495r (5’-GTGTGTACAAGNCCCGGGAA-3’) (Laboratory Services, University of Guelph), where Y designates C or T, and N designates any nucleotide, to obtain a 1265 bp amplicon of each endophyte. In addition, the 16S sequence of R17 was also determined using primers 8F (5′-AGAGTTTGATCCTGGCTCAG -3′) and universal primer 515F (5′-GTGCCAGCMGCCGCGGTAA -3′), where M designates A or C (Lane, 1991). For R21, a rpoB gene fragment was also amplified using primers Vic3 (5′-GGCGAAATGGCWGAGAACCA-3’) and Vic2 (5′-GAGTCTTCGAAGTTGTAACC-3’) (Paauw et al., 2008), where W designates A or T to obtain a 951 bp amplicon. For R17, primers gyrB560F (5′-ACTCGTATGCGTGAGTTGGC-3’) and gyrB1840R (5′-CAAGTTTCCCTTCAAGATGCA-3’) (Laboratory Services, University of Guelph) were used to amplify and sequence a 1134 bp product of the gyrB sequence. Primers were synthesized by Laboratory Services, University of Guelph. Pure cultures were submitted to the Pest Diagnostic Clinic, University of Guelph, for DNA extraction and sequencing.

To design the gyrB primers, gene sequences were obtained of CP009692 for B. mycoides and CP009746 for B. weihenstephanensis from the NCBI nr database and aligned with ClustalW. Conserved regions for all species were identified that would result in an amplicon between 1000 and 1400 bp. The primer melting temperatures were determined to ensure both forward and reverse temperatures were similar and greater than 52°C.

In order to determine the phylogenetic relationship of R17 and R21 to other species of bacteria, a BLASTn search of the 16S sequences were done against the GenBank 16S ribosomal RNA gene database.
(http://blast.ncbi.nlm.nih.gov/Blast.cgi), and BLASTn of the \textit{rpoB} and \textit{gyrB} sequences were done using the Genbank nr database. Sequences from different species with e-values of 0.0 were collected. The sequences of the overlapping regions of each endophyte and selected matches were aligned by ClustalW v.1.83 (Kyoto University Bioinformatics Centre, Kyoto, Japan) multiple sequence alignment tool (http://www.genome.jp/tools/clustalw/). Neighbour-joining trees for gene sequences of each endophyte were constructed in ClustalW with 1000 bootstrap replicates and visualized with TreeView v1.6.6 (Glasgow University, Glasgow, UK).

The ability of R17 to grow at cold temperatures was evaluated by streaking a single colony on TSA and incubating at 4°C for six days. Motility was determined in a wet mount from an overnight culture grown in TSB using a light microscope (100x) (Cleary et al., 2002, Chester & Poulos, 1980).

4.2.9 Statistical analysis

Statistical analysis was completed using SAS v9.4 (SAS Institute, Cary, NC, USA). Data were tested for normality using the Shapiro-Wilk statistic. Outliers were identified using Lund’s test of standardized residuals (Lund 1975). Analysis of variance was completed using Proc Mixed with treatment as a fixed effect and replication as a random effect. Data from different trials was pooled together when statistical analysis showed no treatment x trial interaction ($P \leq 0.05$), except where noted in some tables and figures. Trial was treated as a fixed effect. Data were analyzed as a randomized complete block design. Means comparisons were performed when $P \leq 0.05$, and means were separated using Tukey’s HSD. Regression analysis for endophyte concentration (LOG10) was completed using Proc Reg to determine model significance and Proc Glm to obtain parameter estimates.

4.3 Results

4.3.1 Bacterial endophytes isolated from tomato and wild tomato species

A total of 21 phenotypically different bacterial endophytes were collected from roots of tomato and wild tomato species, including seven from cv. Tiny Tim, eight from cv. THS4, two from \textit{S. arcanum} LA2152, three from \textit{S. chmieleskii} LA1327, and one from \textit{S. cheesmaniae} LA1040 (Table 4.1). Most isolates were smooth, translucent, entire, circular, and cream in colour.

4.3.2 Screening of bacterial endophytes for induced resistance and plant growth effects

Initial screening of 20 endophytes from tomato and wild tomato, seven bacterial endophytes isolated from \textit{N. benthamiana}, \textit{B. subtilis} QST 713, and \textit{B. subtilis} MBI600 was completed using the overnight seedling root soak + soil drench assay (Valenzuela-Soto et al. 2010) with the concentrations of
endophytes adjusted to OD600=1.0 at least once for all isolates except R4, which could not be evaluated due to contamination of the long term storage culture. There was no effect of endophyte inoculation on the number of bacterial speck lesions per cm² of leaf tissue at seven DPI, relative chlorophyll, plant height, foliar dry weight, or root dry weight compared to the MgCl₂ control (Table 4.2 and Table 4.3). However, there was a numerical trend toward lower disease incidence for endophytes R9, R17, R19, R20 and R21.

These endophytes were examined again using a modified version of the Valenzuela-Soto et al. (2010) method, where the seed rather than seedling roots were soaked and the soil was drenched at the time of planting and then once the seedling had developed. This was done to reduce damage to the roots, and the method was designated the seed soak + seed drench + seedling drench inoculation method. Inoculation with this method significantly reduced the number of bacterial speck lesions per cm² of leaf tissue at seven DPI for R9, R17 and R21 compared to the MgCl₂ control (Table 4.4). However, there was a significant treatment × trial interaction with R9. While R9 significantly reduced lesion numbers by 41% in one experiment, it had no significant effect in another experiment. Therefore, R9 was considered inconsistent. Lesion numbers were reduced by 27% with both R17 and R21 compared to the control. None of the endophytes affected relative chlorophyll, plant height, or dry root weight (Table 4.5). Foliar dry weight was not affected by any endophyte, except for an increase with R17, which was inconsistent as it was significantly higher than the control in experiment one but equivalent to the control in the final two experiments. Based on these results, only R17 and R21 were further examined.

4.3.3 Direct antimicrobial effects of R17 and R21

The direct antagonistic effects of R17 and R21 against Pst were evaluated in cross-streaking and agar overlay assays. In the cross-streaking assay, R17 produced a zone of inhibition with the Pst streak width near R17 being reduced by 90.2% compared to streak width at the edge of the petri plate distant from R17 (Table 4.6). In the agar overlay assay, the zone of inhibition of Pst by R17 was 8 mm compared to none for the control (Table 4.7). R21 produced a zone of inhibition in the cross-streaking assay with the Pst streak width near R17 being reduced by 46.5% compared that at the opposite end of the petri plate (Table 4.6). However, no zone of inhibition of Pst by R21 was observed in the agar overlay assay (Table 4.7). The results demonstrate both R17 and R21 have antagonistic effects against Pst in vitro, but R21 had only slight effects on Pst growth because it only slightly reduced growth in the cross-streak assay and produced no zones of inhibition in the overlay assay.

4.3.4 Optimization of bacterial endophyte treatment
To compare the effects of bacterial culture media, the seed soak + seed drench + seedling drench inoculation was done with R17 and R21 that were grown on TSA, 0.1 TSA, and LB agar. The level of induced resistance using R17 and R21 grown on 0.1 TSA was numerically better than 1.0 TSA or LB, and so for future experiments, the endophytes were grown on 0.1 TSA (*data not shown*). To compare the effects of soil fertility, the plants either had no plant fertilizer or a 20 mL or 80 mL fertilizer program. Preliminary results suggested a more consistent response using the 80 mL fertilizer program, and so the 80 mL fertilizer program was chosen (*data not shown*). To determine the endophyte concentration for inoculation, a standard curve of R17 population versus absorbance was made (Figure A.4). Based on that, the endophyte concentrations were adjusted to 1 x 10^7 CFU/ml for R17 and 1 x 10^8 CFU/ml for R21 because the concentrations of R17 and R21 used in the previous trials was calculated to be approx. 8 x 10^6 and 3 x 10^8 CFU/mL, respectively. Also, it was decided to assess plants for disease incidence at five DPI instead of seven DPI to make it easier to distinguish individual bacterial speck lesions, and to count bacterial speck lesions on only the second and third youngest leaves instead of all compound leaves to reduce variability associated with abscised or dying leaves.

Using the above conditions, seed soak + seed drench + seedling drench treatment with R17 or R21 reduced the number of bacterial speck lesions per cm² of leaf tissue at five DPI by 33 and 38 %, respectively. There was a non-target effect of both endophyte treatments reducing plant height by 0.5 to 0.7 cm, but neither endophyte treatment had an effect on relative chlorophyll or foliar and root dry weight (Table 4.8). These results suggest that under the conditions tested, both R17 and R21 induced host resistance in tomato cv. TSH4 against *Pst*, reduced plant height, and had no effect on relative chlorophyll and plant biomass accumulation.

Different endophyte application methods of R17 and R21 were compared: seed soak, seed drench, seedling drench, seed soak + seed drench, and seed soak + seed drench + seedling drench. Although all endophyte treatments resulted in a numerically lower number of bacterial speck lesions per cm² of leaf tissue, only the seed soak and seed drench treatments with R17 resulted in a significant reduction in disease incidence (Table 4.9). There were no other effects of endophyte inoculation on relative chlorophyll or plant growth. Based on this, only R17 was further studied, even though R21 had previously been effective (Table 4.8). The seed soak and seed drench application methods for R17 were also evaluated on both raw and pelleted seed. Disease incidence with raw seed was never different from pelleted seed for any of the application methods (Table 4.10). The greatest reduction in the number of bacterial speck lesions per cm² of leaf tissue occurred when R17 was applied as a seed drench or seed soak + seed drench, similar to the previous set of trials that evaluated endophyte application method, there were no significant effect of R17 application on plant height, or foliar and root dry weight. The seed drench application method was selected for future experiments because of its consistency and because it
was less labour intensive than the seed soak treatment, which required an overnight seed soak.

Four commercial processing tomato cultivars were evaluated to determine if the host response to R17 application was consistent among different host genotypes. A reduction in the number of bacterial speck lesions per cm² of leaf tissue was only observed in cv. TSH4 (Figure 4.1a). Another commercial processing tomato cultivar, H2401, was also tested, but it was inconsistent between experiments, with disease incidence being lower than the control in experiment one but not in experiments two and three (Table A.9). Relative chlorophyll was higher in R17 treated plants than the control for cv. TSH4, but there was no effect on cv. H5108, TSH33, or H2401 (Figure 4.1b, Table A.9). Plant height and foliar and root dry weight were not affected by R17 application (Figure 4.1c-e), except for cv. H2401 where the response for foliar and dry root weight was again inconsistent among experiments (Table A.9). These results suggest that the disease suppression response to R17 is genotype dependent. Future experiments were completed using cv. TSH4 because this was the only cultivar where R17 application resulted in a consistent reduction in bacterial speck incidence.

The dose response of R17 seed drench application was determined using concentrations of 1 x 10⁵ to 1 x 10⁹ CFU/mL. There was a significant quadratic response to R17 concentration: disease incidence = 4.85 – 0.22(LOG10 (R17 concentration)) + 0.01(LOG10 (R17 concentration))² (Table 4.11). Peak disease suppression was achieved at approximately 1 x 10⁷ CFU/mL. There was no dose response observed for relative chlorophyll, plant height, or foliar and root dry weight. These results confirmed that applications of R17 at a concentration of 1 x 10⁷ CFU/mL provided the best opportunity for bacterial speck suppression.

To determine the duration of the plant response to R17, tomatoes were inoculated with Pst at 15, 20, and 25 days after R17 seed drench application. A reduction in the number of bacterial speck lesions per cm² of leaf tissue occurred in R17 treated plants when Pst inoculation occurred at 15 and 20 DAS, but not at 25 DAS (Figure 4.2a). Susceptibility of cv. TSH4 to bacterial speck appeared to decline between 15 and 25 DAS, indicating potential age-related host resistance to Pst. The results for plant growth and relative chlorophyll were inconsistent with previous results at 20 DAS (Figure 4.2b).

4.3.5 Effect of R17 inoculation on Pst populations and lesion size in planta

The influence of R17 application on Pst population in symptomatic leaves was assessed by determining disease incidence and Pst population on the third youngest terminal leaflet. R17 application did not reduce total Pst population per cm² of leaf tissue when there was a reduction in the number of bacterial speck lesions per cm² of leaf tissue (Table 4.12). However, the mean Pst population per lesion in R17 treated plants was 75% higher compared to the mean population in control plants. This is an indication that bacterial speck lesions appearing in R17 treated plants contain more bacteria than those in
control plants.

Additional experiments were completed to further examine bacterial speck lesion size among R17 treated and control plants. Lesion area and lesion circumference were 45 and 18% higher in R17 treated plants (Figure 4.3a-c). However, there was no change in the total leaf tissue surface area covered with lesions (Figure 4.3d). These results suggest that R17 is suppressing initial infections so that fewer lesions develop, but after initial infection, the lesions of R17 treated plants reached a larger size by five DPI than those of control plants. For control plants, a comparison of Pst populations in symptomatic (i.e., bacterial speck lesions) and asymptomatic leaf tissue showed that symptomatic tissue had Pst populations three orders of magnitude higher (5.12 x 10^8 CFU per cm^2 leaf tissue) than asymptomatic tissue (1.12 x 10^5 CFU per cm^2 leaf tissue) from the same leaflet. Thus, lesions appear to have made the greatest contribution to total Pst population in a leaflet.

### 4.3.6 Effect of R17 inoculation on populations in planta

Rifampicin resistant mutants R17-RfpA, R17-RfpB, and R17-RfpC were developed in order to determine the colonization pattern of R17 in tomato 10 and 25 DAS. These mutants suppressed the number of bacterial speck lesions per cm^2 of leaf tissue to the same level as the R17 wt (Table 4.13). The R17-wt and the Rfp mutants also did not affect relative chlorophyll, plant height, or foliar and root dry weight. Thus, it was assumed that the Rfp mutants also colonize cv. TSH4 in a similar manner to R17-wt.

R17-RfpC was never observed in leaves or roots of buffer treated plants, but a low population of a colony type matching R17 with rifampicin resistance was observed in the rhizosphere soil (Table 4.14). At 10 DAS, colonies matching R17-RfpC were recovered in relatively large amount from rhizosphere soil, approximately four orders of magnitude less from roots and six orders of magnitude less from leaves. This indicates that the bacteria were able to infect roots and cotyledon leaves. At 25 DAS, the population in rhizosphere had declined by approx. half from 10 DAS. However, the population in root tissue at 25 DAS was two orders of magnitude lower than root tissue at 10 DAS, and the population in true leaves was undetectable. Thus, colonization of plant tissues by R17-RfpC declined over time in all samples. Randomly picked colonies from the rhizosphere and different tissues were confirmed to be R17 based on gyrB sequencing described below. This included the rhizosphere colonies from buffer treated plants.

### 4.3.7 Identification of R17 and R21

The relationship of endophyte R21 to other bacteria was determined by partially sequencing 16S DNA and rpoB. To create the R21 16S neighbour-joining tree, representative sequences of all Enterobacter sp. and all other species with a 98% nt match or higher in a BLASTn search were selected.
for multiple alignment. For the R21 rpoB neighbour-joining tree, representative Enterobacter, Citrobacter and Leclercia sp. in the top 500 matches and all Enterobacter ludwigii in the top 1000 matches were included in the multiple alignment. When multiple sequences of a species were available, preference was given to published sequences. Enterobacter soli was selected as the outgroup because it was the Enterobacter sp. with the lowest match in the 16S query that is not a member of the E. cloacae complex, and sequences for both 16S and rpoB were available. R21 clustered most closely with all 11 sequences of E. ludwigii, three of nine sequences of E. cloacae, and one of three sequences of Pantoea agglomerans in the 16S neighbour-joining tree (Figure 4.4), and all five sequences of E. ludwigii and three of eight sequences of E. cloacae in the rpoB neighbour-joining tree (Figure 4.5). P. agglomerans did not appear within the top 1000 matches of the BLASTn search for rpoB. These results suggest that R21 is a member of the E. cloacae complex and most closely related to all E. ludwigii and some E. cloacae isolates entered in the BLASTn database. To create the R17 16S neighbour-joining tree, selected representative sequences with a 98% nt match or higher in BLASTn were used including all available B. mycoides and B. weihenstephanensis sequences. The outgroup Bacillus thermoamylovorans was selected because it was the best matching Bacillus species that is not a member of the B. cereus group. Attempts to produce a partial sequence of the gyrB region of R17 using primers Up-1S and Up-2Sr (Yamamoto & Harayama, 1995), Up1-F and Up2-R (Yamamoto & Harayama, 1995), gyrB293F and gyrB1494R (Laboratory Services, University of Guelph), and gyrB560F and gyrB1840R (Laboratory Services, University of Guelph) were made. Only primers gyrB560F and gyrB1840R resulted in the successful amplification of gyrB gene sequences. For the R17 gyrB neighbour-joining tree, all available B. mycoides and B. weihenstephanensis were included as well as up to five sequences of species that also appeared in the 16S neighbour-joining tree. Published sequences were given priority when more than five sequences of a given species were available. The outgroup Bacillus bombysepticus was selected because it was the best matching Bacillus species that is not a member of the B. cereus group. R17 clustered most closely with both sequences of B. weihenstephanensis and all three B. mycoides in the 16S neighbour-joining tree (Figure 4.6), and all six sequences of B. weihenstephanensis and two of three sequences of B. mycoides in the gyrB neighbour-joining tree (Figure 4.7). Thus, R17 is most likely a member of the B. cereus group and most closely related to sequences identified as B. mycoides and B. weihenstephanensis. R17 appeared to be psychrotolerant as it grew on TSA at 4°C and motile, as it demonstrated movement in TSB when cells from an overnight culture were observed under the microscope.
Table 4.1 Source and colony description of endophytes isolated from roots of *S. lycopersici, S. arcanum, S. chmielewskii, S. cheesmaniae*, and *N. benthamiana* after growth on tryptic soy agar at room temperature (~22°C) for two days. Endophytes were isolated from macerated roots of plants growing outdoors in the field at Ridgetown Campus, in media containing 40 to 50% pasteurized field soil mixed with sand under controlled conditions (Ridgetown ‘R’ isolates), or in a 1:1 mixture of pasteurized soil from the Guelph Turfgrass Institute (Guelph, ON) and potting mix (Guelph ‘G’ isolates).

<table>
<thead>
<tr>
<th>Isolate Code</th>
<th>Host</th>
<th>Growing location</th>
<th>Soil source</th>
<th>Colour</th>
<th>Surface</th>
<th>Opacity</th>
<th>Margin</th>
<th>Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-LR</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R2-SWR</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R3-Y</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Yellow</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R4-LC</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R5-LR</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R6-LR</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R7-LR</td>
<td><em>S. lycopersici</em> cv. Tiny Tim</td>
<td>Controlled</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R8-SWR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R9-VLR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R10-Y</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Yellow</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R11-Y</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Yellow</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R12-LR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R13-SWR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R14-VLR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R15-LR</td>
<td><em>S. lycopersici</em> cv. TSH4</td>
<td>Field</td>
<td>RCUG-A7</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R16-2152PY</td>
<td><em>S. arcanum</em> LA2152</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Pale yellow</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R17-2152B</td>
<td><em>S. arcanum</em> LA2152</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Cream</td>
<td>Rough</td>
<td>Opaque</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R18-1306PY</td>
<td><em>S. chmielewskii</em> LA1306</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Pale yellow</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R19-1306B</td>
<td><em>S. chmielewskii</em> LA1306</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Cream</td>
<td>Rough</td>
<td>Opaque</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R20-1327LR</td>
<td><em>S. chmielewskii</em> LA1327</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>R21-1040LR</td>
<td><em>S. cheesmaniae</em> LA1040</td>
<td>Controlled</td>
<td>RCUG-B</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
<tr>
<td>G1-LW7</td>
<td><em>N. benthamiana</em></td>
<td>Controlled</td>
<td>GTI</td>
<td>Cream</td>
<td>Smooth</td>
<td>Translucent</td>
<td>Entire</td>
<td>Circular</td>
</tr>
</tbody>
</table>

*(Pseudomonas)*
| G2-LW1(CW1) (Bacillus cereus) | N. benthamiana | Controlled | GTI | Cream | Rough | Opaque | Entire | Circular |
| G3-SW1 (Pseudomonas alcaligenes) | N. benthamiana | Controlled | GTI | Cream | Smooth | Translucent | Entire | Circular |
| G4-LbW(cbW) (Bacillus simplex) | N. benthamiana | Controlled | GTI | Light brown | Smooth | Translucent | Entire | Circular |
| G5-LW(CW2) (Bacillus megaterium) | N. benthamiana | Controlled | GTI | Cream | Smooth | Translucent | Entire | Circular |
| G7-LY (Bacillus marisflavi) | N. benthamiana | Controlled | GTI | Yellow | Smooth | Translucent | Entire | Circular |
| G8-DC (Bacillus pumilis) | N. benthamiana | Controlled | GTI | Cream | Smooth | Very translucent | Entire | Circular |

a RCUG-B = grassed buffer strip on edge of woodlot at Ridgetown Campus, University of Guelph; RCUG-A7 = agricultural research range A7 at Ridgetown Campus, University of Guelph; GTI = Guelph Turfgrass Institute, University of Guelph, Guelph, ON.
Disease incidence of bacterial speck on non-fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes listed in Table 4.1, Serenade Max (\textit{B. subtilis} QST713) or Promix PGX (\textit{B. subtilis} MBI600). Endophytes were inoculated following the Valenzuela-Soto et al. (2010) method using a solution of each endophyte with A600=1.000 then diluted 1:10 in 10 mM MgCl$_2$, except for MBI600 which was included in the potting mix (1 x 10$^7$ CFU/mL) and QST713 which was applied at 1 x 10$^6$ CFU/mL. Plants receiving 10 mM MgCl$_2$ were used as a control. Plants were inoculated with \textit{P. syringae} pv. \textit{tomato} at 20 days after seeding, and disease incidence assessed at seven days post inoculation. Disease was assessed on all leaflets of each plant.

<table>
<thead>
<tr>
<th>Endophyte $^a$</th>
<th>MgCl$_2$</th>
<th>Endophyte treated</th>
<th>sem $^b$</th>
<th>No. Trials</th>
<th>No. Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>2.4 a</td>
<td>1.8 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R2</td>
<td>2.6 a</td>
<td>2.5 a</td>
<td>0.88</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R3</td>
<td>2.4 a</td>
<td>2.3 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R5</td>
<td>1.8 a</td>
<td>2.3 a</td>
<td>0.46</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R6</td>
<td>2.6 a</td>
<td>2.6 a</td>
<td>0.88</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R7</td>
<td>1.8 a</td>
<td>2.3 a</td>
<td>0.46</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R8</td>
<td>2.4 a</td>
<td>1.7 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R9</td>
<td>7.3 a</td>
<td>4.0 a</td>
<td>1.40</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>R10</td>
<td>2.6 a</td>
<td>2.2 a</td>
<td>0.88</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R11</td>
<td>1.8 a</td>
<td>2.7 a</td>
<td>0.46</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R12</td>
<td>2.9 a</td>
<td>2.1 a</td>
<td>0.85</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R13</td>
<td>1.8 a</td>
<td>2.7 a</td>
<td>0.46</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R14</td>
<td>2.6 a</td>
<td>2.8 a</td>
<td>0.88</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R15</td>
<td>1.8 a</td>
<td>2.9 a</td>
<td>0.46</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R16</td>
<td>2.9 a</td>
<td>2.9 a</td>
<td>0.85</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R17</td>
<td>7.3 a</td>
<td>4.0 a</td>
<td>1.40</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>R18</td>
<td>2.9 a</td>
<td>2.4 a</td>
<td>0.85</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R19</td>
<td>7.3 a</td>
<td>3.8 a</td>
<td>1.40</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>R20</td>
<td>7.3 a</td>
<td>4.7 a</td>
<td>1.40</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>R21</td>
<td>7.3 a</td>
<td>4.2 a</td>
<td>1.40</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>G1</td>
<td>2.2 a</td>
<td>3.2 a</td>
<td>1.53</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G2</td>
<td>2.4 a</td>
<td>1.9 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G3</td>
<td>2.2 a</td>
<td>4.4 a</td>
<td>1.53</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G4</td>
<td>2.4 a</td>
<td>3.2 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G5</td>
<td>2.4 a</td>
<td>1.6 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G7</td>
<td>2.2 a</td>
<td>4.1 a</td>
<td>1.53</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>G8</td>
<td>2.4 a</td>
<td>2.0 a</td>
<td>0.66</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>MBI600 $^d$</td>
<td>4.1 a</td>
<td>5.2 a</td>
<td>0.19</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>QST713 $^d$</td>
<td>4.1 a</td>
<td>4.8 a</td>
<td>0.17</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$ R4 was not tested due to problems with contamination. For endophytes R9, R17, R19, R20, R21, MBI600, and QST713 one trial was completed with plants growing in Promix potting mix and not Fafard germination mix.

$^b$ sem = standard error of the mean for all ls means in the same column. The sem for MBI600 and QST713 for the control treatment was 0.20. This value is for the ls means calculated from a log transformation. The sem differs from the inoculated treatments due to missing plots.

$^c$ Means in the same row followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD. Data from different trials was pooled together because ANOVA showed no treatment x trial interaction.

$^d$ Data in these rows was transformed using a log transformation, the back transformed means are shown here.
Table 4.3 Growth parameters (relative chlorophyll, plant height, and root and foliage weight) of non-fertilized tomato cv. TSH4 following inoculation with the endophytes listed in Table 4.1, Serenade Max (B. subtilis QST713) or Promix PGX (B. subtilis MB1600). Endophytes were inoculated following the Valenzuela-Soto et al. (2010) method using a solution of each endophyte with A600=1.000 that was diluted 1:10 in 10 mM MgCl₂, except for MB1600 which was included in the potting mix (1 x 10⁶ CFU/mL) and QST713 which was applied at 1 x 10⁷ CFU/mL. Plants receiving 10 mM MgCl₂ were used as a control. Plants were assessed at 27 days after seeding on the same day as assessment of disease incidence.

<table>
<thead>
<tr>
<th>Endophyte</th>
<th>Relative Chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
<th>No. Trials</th>
<th>No. Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgCl₂</td>
<td>Endophyte treated</td>
<td>sem</td>
<td>MgCl₂</td>
<td>Endophyte treated</td>
</tr>
<tr>
<td>R1</td>
<td>31.0 a</td>
<td>2.00</td>
<td>4.5 a</td>
<td>9 a</td>
<td>4.5 a</td>
</tr>
<tr>
<td>R2</td>
<td>22.7 a</td>
<td>2.25</td>
<td>9.7 a</td>
<td>9 a</td>
<td>9.7 a</td>
</tr>
<tr>
<td>R3</td>
<td>31.0 a</td>
<td>2.00</td>
<td>4.5 a</td>
<td>9 a</td>
<td>4.5 a</td>
</tr>
<tr>
<td>R4</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R5</td>
<td>22.7 a</td>
<td>2.25</td>
<td>9.7 a</td>
<td>9 a</td>
<td>9.7 a</td>
</tr>
<tr>
<td>R6</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R7</td>
<td>31.0 a</td>
<td>2.00</td>
<td>4.5 a</td>
<td>9 a</td>
<td>4.5 a</td>
</tr>
<tr>
<td>R8</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R9</td>
<td>31.0 a</td>
<td>2.00</td>
<td>4.5 a</td>
<td>9 a</td>
<td>4.5 a</td>
</tr>
<tr>
<td>R10</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R11</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R12</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R13</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R14</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R15</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R16</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R17</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R18</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R19</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R20</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>R21</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G1</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G2</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G3</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G4</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G5</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G6</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G7</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>G8</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>MB1600</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
<tr>
<td>QST713</td>
<td>34.6 a</td>
<td>1.60</td>
<td>5.3 a</td>
<td>9 a</td>
<td>5.3 a</td>
</tr>
</tbody>
</table>

*R4 was not tested due to problems with contamination. For endophytes R9, R17, R19, R20, R21, MB1600, and QST713 one trial was completed with plants growing in Promix potting mix and not Fafard germination mix.*
sem = standard error of the mean for all is means in the same column. The sem for MBI600 and QST713 for the control treatment was 1.12 for relative chlorophyll, 0.34 for height, 0.00401 for foliar dry weight, and 0.00103 for root dry weight.

Means in the same row and group followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from different trials was pooled together because ANOVA showed no treatment x trial interaction. There was a significant treatment x trial interaction for foliar dry weight for MBI600 and QST713 but there was no difference among these treatments and the control in either trial and so the pooled results are presented.
Table 4.4 Disease incidence of bacterial speck on non-fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes R9, R17, R19, R20 and R21. Endophytes were inoculated using the seed soak + seed drench + seedling drench inoculation method (A600=1.000 then diluted 1:10 in 10 mM MgCl$_2$). Plants receiving 10 mM MgCl$_2$ were used as a control. Plants were inoculated with *Pst* at 20 days after seeding, and disease incidence assessed at seven days post inoculation on all leaflets of each plant.

<table>
<thead>
<tr>
<th>Endophyte</th>
<th>MgCl$_2$</th>
<th>Endophyte treated</th>
<th>sem $^a$</th>
<th>No. Trials</th>
<th>No. Reps</th>
</tr>
</thead>
<tbody>
<tr>
<td>R9</td>
<td>7.5 a $^b$</td>
<td>4.4 b</td>
<td>0.72</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R9</td>
<td>3.0 a</td>
<td>2.5 a</td>
<td>0.30</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R17</td>
<td>4.4 a</td>
<td>3.2 b</td>
<td>0.24</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>R19</td>
<td>4.9 a</td>
<td>3.9 a</td>
<td>0.32</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>R20</td>
<td>4.9 a</td>
<td>3.8 a</td>
<td>0.34</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>R21</td>
<td>4.4 a</td>
<td>3.2 b</td>
<td>0.24</td>
<td>5</td>
<td>24</td>
</tr>
</tbody>
</table>

$^a$ sem = standard error of the mean for all ls means in the same row. The sem for R9 for the control treatment was 0.39 and for R19 0.34.

$^b$ Means in the same row and group followed by the same letter are not significantly different at P $\leq$ 0.05, Tukey’s HSD. Data from different trials was pooled together because ANOVA showed no treatment x trial interaction. There was a significant treatment x trial interaction for disease incidence for R9 and results are presented separately due to a different treatment response in each trial. In one trial, R17 was grown on 0.1 tryptic soy agar (TSA) instead of TSA.
Table 4.5 Growth parameters (relative chlorophyll, plant height, and root and foliage weight) of non-fertilized tomato cv. TSH4 following inoculation with the endophytes R9, R17, R19, R20 and R21. Endophytes were inoculated using the seed soak + seed drench + seedling drench inoculation method (A600=1.000 diluted by a factor of 10 in 10 mM MgCl₂). Plants receiving 10 mM MgCl₂ were used as a control. Plants were assessed at 27 days after seeding on the same day as assessment of disease incidence.

<table>
<thead>
<tr>
<th>Endophyte</th>
<th>Relative Chlorophyll</th>
<th>Height (cm)</th>
<th>Foliage</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgCl₂ Endophyte</td>
<td>MgCl₂</td>
<td>MgCl₂ Endophyte</td>
<td>MgCl₂</td>
</tr>
<tr>
<td></td>
<td>treated sem</td>
<td>treated sem</td>
<td>treated sem</td>
<td>treated sem</td>
</tr>
<tr>
<td>R9</td>
<td>24.5 a b</td>
<td>25.1 a</td>
<td>7.2 a</td>
<td>7.8 a</td>
</tr>
<tr>
<td>R17</td>
<td>25.0 a</td>
<td>25.5 a</td>
<td>8.7 a</td>
<td>8.7 a</td>
</tr>
<tr>
<td>R19</td>
<td>25.7 a</td>
<td>26.0 a</td>
<td>7.9 a</td>
<td>8.7 a</td>
</tr>
<tr>
<td>R20</td>
<td>25.7 a</td>
<td>24.9 a</td>
<td>7.9 a</td>
<td>8.4 a</td>
</tr>
<tr>
<td>R21</td>
<td>25.0 a</td>
<td>26.4 a</td>
<td>8.7 a</td>
<td>9.3 a</td>
</tr>
</tbody>
</table>

a sem = standard error of the mean for all ls means in the same column. The sem for R9 for the control treatment was 0.15 for relative chlorophyll and 0.51 for plant height, and for R19 for the control treatment was 0.96 for relative chlorophyll, 0.35 for plant height, 0.01871 for foliar dry weight, and 0.02241 for dry root weight.

b Means in the same row and group followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from different trials was pooled together when ANOVA showed no treatment x trial interaction.
Table 4.6 In vitro inhibition of *P. syringae* pv. *tomato* (*Pst*) strain DC06T2-4 by co-incubation with endophytes R17 and R21 using a cross streaking assay. Each endophyte was streaked in a single line across the centre of a plate of tryptic soy agar and incubated for two days. *Pst* was then streaked in a straight line from the edge of the plate to the endophyte streak at a 90° angle. Plates were assessed two days after streaking *Pst*.

<table>
<thead>
<tr>
<th>Endophyte</th>
<th>Zone of Inhibition (mm)</th>
<th>Streak width reduction (%) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 b (^b)</td>
<td>12.0 c</td>
</tr>
<tr>
<td>R17</td>
<td>5.5 a</td>
<td>90.2 a</td>
</tr>
<tr>
<td>R21</td>
<td>0.2 b (^b)</td>
<td>46.5 b</td>
</tr>
<tr>
<td>sem (^c)</td>
<td>0.34</td>
<td>3.43</td>
</tr>
</tbody>
</table>

\(^a\) Percent reduction in *Pst* streak width at the rim end and the centre of the plate where the streak stopped.

\(^b\) Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

\(^c\) sem = standard error of the mean for all ls means in the same column.
Table 4.7 In vitro inhibition of *P. syringae* pv. *tomato* (*Pst*) strain DC06T2-4 by co-incubation with endophytes R17 and R21 using an agar overlay assay. The overlay was tryptic soy agar (TSA) (1.50% agar) (50°C that was spiked with 0.1 mL of 2 x 10^7 CFU/mL *Pst*). This was poured over a 2-cm diameter circle of endophyte that had grown for 48 hours on a hard TSA agar base (1.50% agar), incubated for 48 hours, and assessed for the presence of a zone of inhibition.

<table>
<thead>
<tr>
<th>1.50% agar base with endophyte</th>
<th>1.50% agar overlay with <em>Pst</em></th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agar only</td>
<td>Agar only</td>
<td>0^a</td>
</tr>
<tr>
<td>Agar only</td>
<td><em>Pst</em></td>
<td>0</td>
</tr>
<tr>
<td>R17</td>
<td>Agar only</td>
<td>0</td>
</tr>
<tr>
<td>R17</td>
<td><em>Pst</em></td>
<td>8</td>
</tr>
<tr>
<td>R21</td>
<td>Agar only</td>
<td>0</td>
</tr>
<tr>
<td>R21</td>
<td><em>Pst</em></td>
<td>0</td>
</tr>
<tr>
<td><strong>sem</strong>^b</td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>

^a^ Means in the same column followed by the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD. Data from three independent trials with eight replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

^b^ sem = standard error of the mean for all ls means in the same column.
Table 4.8 Disease incidence of bacterial speck and growth parameters (relative chlorophyll, plant height, and root and foliage weight) of fertilized tomato cv. TSH4 following inoculation with buffer or the endophytes R17 and R21. Plants were fertilized 10, 15 and 21 days after seeding (DAS) with 20 (three experiments) or 80 mL (one experiment) of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Endophytes were inoculated using the seed soak + seed drench + seedling drench inoculation method at concentrations of 1 x 10^7 CFU/ml (R17) or 1 x 10^8 CFU/ml (R21). Plants receiving 10 mM MgCl₂ were used as a control. Plants were inoculated with *P. syringae* pv. *tomato* (*Pst*) 20 DAS. The incidence of bacterial speck lesions on all leaflets on the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with *Pst*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease incidence (lesions/cm²)</th>
<th>Relative chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>3.9 a</td>
<td>24.2 a</td>
<td>8.2 a</td>
<td>224.0 a</td>
</tr>
<tr>
<td>R17</td>
<td>2.6 b</td>
<td>25.0 a</td>
<td>7.7 b</td>
<td>248.1 a</td>
</tr>
<tr>
<td>R21</td>
<td>2.4 b</td>
<td>25.4 a</td>
<td>7.5 b</td>
<td>233.9 a</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Foliage</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>sem b</td>
<td>0.27</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>14.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.30</td>
</tr>
</tbody>
</table>

a Means in the same column followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials with four replications (two trials) or 10 replications (one trial) of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

b sem = standard error of the mean for all ls means in the same column.
Table 4.9 The effect of seed soak, seed drench and/or seedling drench application methods of R17 and R21 on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were inoculated with the seed soak, seed drench, seedling drench, seed soak + seed drench or seed soak + seed drench + seedling drench methods using $1 \times 10^7$ CFU/ml (R17) or $1 \times 10^8$ CFU/ml (R21) in 10 mM MgCl$_2$. Plants receiving 10 mM MgCl$_2$ were used as a control. Plants were fertilized 10, 15 and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Plants were inoculated with *P. syringae pv. tomato* (*Pst*) 20 DAS. The incidence of bacterial speck lesions on all leaflets of the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with *Pst*.

<table>
<thead>
<tr>
<th>Seed soak treatment (0 DAS)</th>
<th>Seed drench treatment (0 DAS)</th>
<th>Seedling drench treatment (10 DAS)</th>
<th>Disease incidence (lesions/cm$^2$)</th>
<th>Relative chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>2.9 a$^a$</td>
<td>25.4 a</td>
<td>9.5 a</td>
<td>312.8 a 152.0 a</td>
</tr>
<tr>
<td>R17</td>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>1.7 b</td>
<td>26.3 a</td>
<td>9.9 a</td>
<td>299.0 a 143.3 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>R17</td>
<td>MgCl$_2$</td>
<td>1.6 b</td>
<td>26.8 a</td>
<td>9.6 a</td>
<td>356.4 a 206.8 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>R17</td>
<td>2.3 ab</td>
<td>26.4 a</td>
<td>9.6 a</td>
<td>375.0 a 197.4 a</td>
</tr>
<tr>
<td>R17</td>
<td>R17</td>
<td>MgCl$_2$</td>
<td>2.3 ab</td>
<td>25.2 a</td>
<td>9.5 a</td>
<td>309.8 a 167.3 a</td>
</tr>
<tr>
<td>R17</td>
<td>R17</td>
<td>R17</td>
<td>2.2 ab</td>
<td>25.7 a</td>
<td>9.5 a</td>
<td>320.0 a 153.8 a</td>
</tr>
<tr>
<td>R21</td>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>2.1 ab</td>
<td>26.7 a</td>
<td>9.7 a</td>
<td>323.5 a 181.0 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>R21</td>
<td>MgCl$_2$</td>
<td>2.1 ab</td>
<td>25.0 a</td>
<td>9.6 a</td>
<td>332.5 a 165.8 a</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>MgCl$_2$</td>
<td>R21</td>
<td>2.1 ab</td>
<td>27.0 a</td>
<td>10.4 a</td>
<td>373.6 a 208.9 a</td>
</tr>
<tr>
<td>R21</td>
<td>R21</td>
<td>MgCl$_2$</td>
<td>2.2 ab</td>
<td>25.9 a</td>
<td>9.7 a</td>
<td>341.4 a 165.4 a</td>
</tr>
<tr>
<td>R21</td>
<td>R21</td>
<td>R21</td>
<td>1.9 ab</td>
<td>25.8 a</td>
<td>9.8 a</td>
<td>312.0 a 170.4 a</td>
</tr>
</tbody>
</table>

sem$^b$ 0.11 1.04 0.33 38.37 31.10

$^a$ Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Dry weight for foliage and root weight is from two independent trials with five replications of each treatment because samples from one trial were destroyed.

$^b$ sem = standard error of the mean for all ls means in the same column.
The effect of raw and pelleted seed with seed soak and/or seed drench application methods of R17 treatment on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were inoculated using the seed soak, seed drench, or seed soak + seed drench method using 1 x 10⁷ CFU/ml in 10 mM MgCl₂. Plants receiving 10 mM MgCl₂ were used as a control. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. Plants were inoculated with *P. syringae* pv. *tomato* (*Pst*) at 20 DAS. The incidence of bacterial speck lesions on all leaflets of the second and third youngest leaves, relative chlorophyll content, plant height, dry root weight and foliar dry weight were determined at five days post inoculation with *Pst*.

<table>
<thead>
<tr>
<th>Seed soak treatment (0 DAS)</th>
<th>Seed drench treatment (0 DAS)</th>
<th>Seed type</th>
<th>Disease incidence (lesions/cm²)</th>
<th>Relative chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MgCl₂</td>
<td>Raw</td>
<td>2.5 a</td>
<td>24.6 a</td>
<td>6.4 a</td>
<td>189.2 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>MgCl₂</td>
<td>Pellet</td>
<td>2.1 ab</td>
<td>25.0 a</td>
<td>6.9 a</td>
<td>209.6 a</td>
</tr>
<tr>
<td>R17</td>
<td>MgCl₂</td>
<td>Raw</td>
<td>1.7 bc</td>
<td>23.7 a</td>
<td>7.0 a</td>
<td>221.3 a</td>
</tr>
<tr>
<td>R17</td>
<td>MgCl₂</td>
<td>Pellet</td>
<td>1.6 bc</td>
<td>23.8 a</td>
<td>6.4 a</td>
<td>218.0 a</td>
</tr>
<tr>
<td>R17</td>
<td>R17</td>
<td>Raw</td>
<td>1.5 c</td>
<td>24.0 a</td>
<td>6.8 a</td>
<td>187.4 a</td>
</tr>
<tr>
<td>R17</td>
<td>R17</td>
<td>Pellet</td>
<td>1.5 c</td>
<td>23.6 a</td>
<td>6.6 a</td>
<td>185.2 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>R17</td>
<td>Raw</td>
<td>1.5 c</td>
<td>23.8 a</td>
<td>6.3 a</td>
<td>203.9 a</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>R17</td>
<td>Pellet</td>
<td>1.4 c</td>
<td>24.4 a</td>
<td>6.9 a</td>
<td>208.7 a</td>
</tr>
</tbody>
</table>

sem b 0.17 0.71 0.22 19.00 9.21

a Means in the same column followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from two independent trials with seven replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

b sem = standard error of the mean for all ls means in the same column.
Figure 4.1 Comparison of four commercial processing tomato cultivars for the effect of R17 seed drench treatment on the a) incidence of bacterial speck lesions, b) relative chlorophyll, c) plant height, d) dry root weight, and e) foliar dry weight. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench at 0 DAS at $1 \times 10^7$ CFU/mL in10 mM MgCl$_2$. Control plants received 10 mM MgCl$_2$. Plants were inoculated with P. syringae pv. tomato at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves. Data for each variable is shown for the nontreated control ( ).
and R17 (≡). Errors bars represent standard error of the mean. Bars with the same letter for the same
cultivar are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with
six replications of each treatment was pooled together because ANOVA showed no treatment x trial
interaction. Results for cv. H2401 (most variables) and cv. H9553 (relative chlorophyll) were inconsistent
and are presented in Table A.9.
Table 4.11 Effect of different doses of R17 as a seed drench treatment on the incidence of bacterial speck disease incidence and growth parameters (relative chlorophyll, plant height, foliar dry weight, and root dry weight) of fertilized tomato cv. TSH4. R17 was applied as a seed drench at zero days after seeding (DAS) at 0, 1x10^5, 1x10^6, 1x10^7, 1x10^8 or 1x10^9 cfu/mL in 10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. Plants were fertilized 10, 15 and 21 DAS with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. Plants were inoculated with \textit{P. syringae pv. tomato} at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves.

<table>
<thead>
<tr>
<th>Variable</th>
<th>R17 concentration (cfu/mL)</th>
<th>Regression equation</th>
<th>R^2</th>
<th>P</th>
<th>sem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease incidence (lesions/cm^2)</td>
<td></td>
<td>y = 4.85 – 0.22x + 0.01x^2</td>
<td>0.47</td>
<td>&lt;.0001</td>
<td>0.27</td>
</tr>
<tr>
<td>Relative chlorophyll</td>
<td></td>
<td>y = 23.1 + 0.1x</td>
<td>0.29</td>
<td>0.1813</td>
<td>0.65</td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td>y = 7.1 + 0.0x</td>
<td>0.32</td>
<td>0.1374</td>
<td>0.25</td>
</tr>
<tr>
<td>Foliar dry weight (mg/plant)</td>
<td></td>
<td>y = 78.1 + 0.8x</td>
<td>0.65</td>
<td>0.5944</td>
<td>15.00</td>
</tr>
<tr>
<td>Root dry weight (mg/plant)</td>
<td></td>
<td>y = 161.7 + 12x</td>
<td>0.54</td>
<td>0.3242</td>
<td>13.50</td>
</tr>
</tbody>
</table>

^a Regression analysis was completed in SAS proc reg and proc glm using data from three independent trial with six replications per trial. X = LOG10 (R17 concentration).
Figure 4.2 The duration of the plant response to R17 (---) or 10 mM MgCl₂ (→) seed drench treatment as measured by the a) incidence of bacterial speck lesions, b) relative chlorophyll, c) plant height, d) dry root weight, and e) foliar dry weight in fertilized tomato cv. TSH4. Plants were fertilized 10, 15, 21 and 26 DAS (plants inoculated with *P. syringae* pv. *tomato* at 25 days after seeding (DAS) only) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench 0 DAS at 1 x 10⁷ CFU/mL in 10 mM MgCl₂. Control plants received 10 mM MgCl₂. Plants were inoculated with *Pst* 15, 20
or 25 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves. Data points with the same letter at the same time point are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from three independent trials with six replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. Error bars represent standard error of the mean.
Table 4.12 The effect of R17 seed drench treatment on the incidence of bacterial speck lesions, *P. syringae pv. tomato* (*Pst*) population (LOG CFU) per cm\(^2\) and *Pst* population (LOG CFU) per lesion on terminal leaflets of the third youngest leaves of fertilized tomato cv. TSH4. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. R17 was applied as a seed drench 0 DAS at \(1 \times 10^7\) CFU/mL in 10 mM MgCl\(_2\). Plants were inoculated with *Pst* 20 DAS, and disease incidence assessed at five days post inoculation. Plants receiving 10 mM MgCl\(_2\) were used as a control.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease incidence (lesions/cm(^2))</th>
<th>Population/cm(^2) (LOG10 CFU)</th>
<th>Population/lesion (LOG10 CFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl(_2)</td>
<td>4.5 a (^a)</td>
<td>6.62 a</td>
<td>1.60 b</td>
</tr>
<tr>
<td>R17</td>
<td>2.6 b</td>
<td>6.51 a</td>
<td>2.81 a</td>
</tr>
<tr>
<td>sem (^b)</td>
<td>0.48</td>
<td>0.17</td>
<td>0.33</td>
</tr>
</tbody>
</table>

\(^a\) Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD. Data from three independent trials with three (two trials) or two (one trial) replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

\(^b\) sem = standard error of the mean for all ls means in the same column on LOG10 scale.
Figure 4.3 The effect of R17 seed drench treatment on a) the incidence of bacterial speck symptoms, b) mean lesion size, c) mean lesion circumference, and d) percent leaf area with lesions on the terminal leaflet of the third youngest leaf of fertilized tomato cv. TSH4. Disease incidence and mean lesion size of 10 to 15 lesions is shown for the nontreated control (■) and R17 (■). Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench 0 DAS at $1 \times 10^7$.
CFU/mL in 10 mM MgCl₂. Control plants received 10 mM MgCl₂. Plants were inoculated with *P. syringae* pv. *tomato* 20 DAS, and disease incidence assessed at five days post inoculation. Errors bars represent standard error of the mean. Bars with the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD. Data from two independent trials with seven (experiment 1) and six (experiment 2) replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.
Table 4.13 Comparison of R17 seed drench treatments with wt and rifampicin-resistant strains on disease incidence of bacterial speck and growth parameters of fertilized tomato cv. TSH4. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17 was applied as a seed drench at 0 DAS at 1 x 10^7 CFU/mL in 10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. Plants were inoculated with *P. syringae pv. tomato (Pst)* 20 DAS, and disease incidence assessed at five days post inoculation (DPI) on all leaflets of the second and third youngest leaves. The relative chlorophyll content, plant height, dry root weight and foliar dry weight were also determined at five DPI.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Disease incidence (lesions/cm²)</th>
<th>Relative chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
<th>Foliage</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>4.3 a</td>
<td>24.6 a</td>
<td>7.4 a</td>
<td>170 a</td>
<td>125 a</td>
<td></td>
</tr>
<tr>
<td>R17-wt</td>
<td>2.6 b</td>
<td>23.0 a</td>
<td>7.1 a</td>
<td>147 a</td>
<td>93 a</td>
<td></td>
</tr>
<tr>
<td>R17-RfpA</td>
<td>2.9 b</td>
<td>24.2 a</td>
<td>7.4 a</td>
<td>166 a</td>
<td>109 a</td>
<td></td>
</tr>
<tr>
<td>R17-RfpB</td>
<td>2.7 b</td>
<td>23.5 a</td>
<td>7.0 a</td>
<td>141 a</td>
<td>92 a</td>
<td></td>
</tr>
<tr>
<td>R17-RfpC</td>
<td>2.7 b</td>
<td>23.8 a</td>
<td>6.9 a</td>
<td>148 a</td>
<td>96 a</td>
<td></td>
</tr>
<tr>
<td>sem b</td>
<td>0.39</td>
<td>0.59</td>
<td>0.22</td>
<td>12.3</td>
<td>14.0</td>
<td></td>
</tr>
</tbody>
</table>

*a* Means in the same column followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials for with five or six replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction. For disease incidence data, data was log transformed to meet assumptions of ANOVA and the back transformed means are shown. One outlier was removed for the disease incidence data.

*b* sem = standard error of the mean for all ls means in the same column.
Table 4.14 Populations of rifampicin resistant R17 mutant R17-RfpC in leaves, roots and rhizosphere of tomato cv. TSH4. Plants were fertilized at 10, 15 and 21 days after seeding (DAS) with 80 mL of 1.26 g/L 20-20-20+micronutrients fertilizer solution. R17-RfpC was applied as a seed drench at 0 DAS at 1 x 107 CFU/mL in10 mM MgCl2. Plants receiving 10 mM MgCl2 were used as a control. R17-RfpC populations in rhizosphere growing media, roots and cotyledon leaves at 10 DAS or the rhizosphere growing media, roots and terminal leaflet on the second and third youngest leaves at 25 DAS. Samples were from 10 (10 DAS) or five (25 DAS) plants per replicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaves (CFU/g fresh tissue)</th>
<th>Roots (CFU/g fresh tissue)</th>
<th>Rhizosphere (CFU/g dry soil)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 DAS</td>
<td>25 DAS</td>
<td>10 DAS</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0 b  a</td>
<td>0 a</td>
<td>0 b</td>
</tr>
<tr>
<td>R17-RfpC</td>
<td>6.3 a</td>
<td>0 a</td>
<td>1.3 x 10²</td>
</tr>
<tr>
<td>sem b</td>
<td>0.11</td>
<td>.</td>
<td>0.12</td>
</tr>
</tbody>
</table>

a Means in the same column followed by the same letter are not significantly different at P ≤ 0.05, Tukey’s HSD. Data from three independent trials with four replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

b sem = standard error of the mean (LOG10) for all ls means in the same row.
Figure 4.4 Neighbour joining tree of partial 16S sequences (1160 bp) from representative members of the *E. cloacae* complex, other *Enterobacter* sp., closely related *Enterobacteriacea*, and R21.
Figure 4.5 Neighbour joining tree of partial \textit{rpoB} sequences (934 bp) from representative members of the \textit{E. cloacae} complex, other \textit{Enterobacter} sp., closely related \textit{Enterobacteraeae}, and R21.
Figure 4.6 Neighbour joining tree of partial 16S sequences (1234 bp) from representative members of the *B. cereus* group, other *Bacillus* sp., and R17.
Figure 4.7 Neighbour joining tree of partial gyrB sequences (696 bp) from representative members of the B. cereus group, other Bacillus sp., and R17.
4.4 Discussion

Screening bacterial endophytes from tomato resulted in the isolation of two strains with putative induce resistance against *Pst*. The most effective was *B. mycoides/weihenstephanensis* strain R17, which could be applied to the soil resulting in a reduction of the incidence of bacterial speck in tomatoes in growth room conditions by up to 51%. Many species of *Bacillus* have been shown to induce resistance in plants, including *B. mycoides* (Choudhary & Johri, 2009). In tomato, bacterial endophytes that have induced resistance against *Pst* are *B. pumilus* and *B. amyloliquefaciens* that reduced lesion incidence by 56% and 62%, respectively (Lanna-Filho et al., 2017), *B. pumilus* SE34 and *B. amyloliquifaciens* IN937a that reduced lesion incidence by 63% and 49%, respectively (Ji et al., 2006), and *B. megaterium* that reduced lesion incidence by 54% (Barretti et al., 2009). These levels of induced resistance are similar or slightly higher than that achieved in this study.

Many bacterial endophytes can affect plant growth in tomato (Pillay & Nowak, 1997, Amaresan et al., 2012, Barretti et al., 2009, Lanna-Filho et al., 2017), and application of strain R17 affected plant growth based on increases in foliar dry weight in two experiments, reductions in plant height in one experiment, changes in relative chlorophyll in two experiments and increases in root dry weight in one experiment. However, these effects were inconsistent among experiments, and thus no firm conclusions about growth promotion or inhibition can be made. Bacterial endophytes in tomato are not always associated with plant growth promotion, such as *B. pumilus* and *B. amyloliquefaciens*, which reduced plant height and total dry weight in greenhouse assays (Lanna-Filho et al., 2017).

As this study demonstrates, identification of an endophyte with putative induced resistance is only the starting point. The effectiveness of strain R17 was influenced by a number of factors including the inoculation timing, inoculation method, and bacterial concentration and host genotype. This shows that using an endophyte is dependent on a number of factors related to both the bacterial endophyte and its host.

4.4.1 Isolation of endophytes from domestic and wild tomato

Screening for phenotypically diverse endophytes from roots of the dwarf cv. Tiny Tim and the commercial processing cv. TSH4 yielded only two colony types, with some variation in colony size within each group. However, a higher diversity of colonies was expected as Xia et al. (2015) obtained 32 endophyte species from an unspecified tomato cultivar and Upreti and Thomas (2015) found 27 different endophyte species from cvs. Arka Abha and Arka Vikas. Root endophytes typically infect roots from the soil, and soil traits can greatly affect root endophyte populations (Long et al., 2010). The low endophyte diversity in this study may be due to the use of field soil from an agricultural research range that is
heavily managed resulting in different total soil carbon, soil nitrogen, and a C:N ratio than found in a natural system, which are key elements affecting microbial diversity (Trivedi et al., 2016). Another factor was that the soil was pasteurized to obtain an internal temperature of 60°C for 30 min, which was done as the majority of emerging cv. Tiny Tim seedlings died with symptoms of damping off in the growth room grown in unpasteurized soil. Pasteurization will reduce inoculum of soil borne plant pathogens and other microbes in the soil, selecting for heat tolerant bacteria, such as Bacillus spp., which are able to withstand temperatures of up to 100°C (Baker, 1962, Baker & Olsen, 1964), Dawson et al. (1965). However, soil pasteurization does not explain the low diversity of endophytes obtained from cv. TSH4 roots growing in the same soil directly in the field. Another possible explanation for low diversity is that some endophyte species did not survive tissue surface sterilization during isolation. Perhaps the method of Upreti and Thomas (2015) to use Na₂S₂O₃ to remove residues of the sterilizer, chloramine, from root tissue prior to maceration would have helped to remove residual bleach in the current study. In addition, serial dilutions and spread plating was used, which is commonly used for recovery of culturable endophytes from plants (Rasche et al., 2006, Goodwin & Gao, 2017, Mahaffee & Kloeper, 1997). However, Thomas et al. (2012) demonstrated that spread plating of endophytes can be variable resulting in reduced and inconsistent recovery compared to spotting dilutions and then tilting the plate to spread the solution without a spreader.

In order to increase the diversity of bacterial endophytes, isolations were also made from the wild tomato species S. arcanum, S. chmieleskii and S. cheesmaniae grown in a non-cultivated soil (i.e., a grass buffer strip). Non-agricultural soils with perennial plants may have more total soil carbon and nitrogen with a different C:N which can increase bacterial diversity and abundance (Trivedi et al., 2016). While the same colony types were obtained as with cvs. Tiny Tim and TSH4, there were also other colony phenotypes. While this study was not designed to evaluate the effect of host species or soil on bacterial endophyte diversity of tomato, this indicates that more diverse tomato genotypes and soil types provide a greater chance to obtain different bacterial endophytes. This is consistent with reports of organically cultivated soils having higher diversity of endophytes in corn, tomato, melon, and pepper compared to soils in conventional agricultural production (Xia et al., 2015), and reports of differences in plant physiology, such as ET production, affecting the bacterial endophyte community in tobacco roots (Long et al., 2008).

4.4.2 Screening for activity of endophytes for induced resistance and plant growth promotion

Bacterial endophytes isolated from domesticated and wild tomato were screened for induced resistance and plant growth promotion in the commercial processing tomato cv. TSH4. In addition, a collection of endophytes known to induce resistance against Colletotrichum orbiculare in N. benthamiana
(Goodwin & Gao, 2017) was included, as well as commercial formulations of \textit{B. subtilis} QST713 and \textit{B. subtilis} MBI600. \textit{B. subtilis} QST713 is marketed as Serenade Max and \textit{B. subtilis} MBI600 is marketed as Promix PGX for disease suppression and plant growth promotion (Boriss, 2016, Nava-Diaz, 2006). In initial screening tests, no response for disease or plant growth promotion variables was observed for any endophytes or the two commercial products. It is possible that the endophytes effective against a fungal pathogen of \textit{N. benthamiana} will not function against a bacterial pathogen of tomato. For Serenade Max, foliar applications of the bacterium for bacterial spot and speck control in tomato were inconsistent (Roberts et al., 2008, Trueman, 2015), and this organism may act more by directly attacking pathogens rather than as an elicitor of resistance. For Promix PGX, it may also act primarily by directly attacking pathogens as it is sold as part of a growing medium and is described as controlling root infections by \textit{Fusarium, Pythium} and \textit{Rhizoctonia}. Growth promotion by \textit{B. subtilis} MBI600 was observed in tomato in field experiments but not greenhouse experiments, and thus its response may also vary with environmental conditions (Nava-Diaz, 2006).

Although there were no significant differences, some of the endophytes in the initial screenings appeared promising despite a high level of variability in the incidence of bacterial speck symptoms and plant growth. One source of variability could have been the endophyte inoculation method which involved dipping extracted bare root seedlings in the bacterial suspension nine DAS. This may have triggered a mechanical stress response which could affect endophyte colonization or the host response. Mechanical stress in plants is associated with increases in ET biosynthesis in plants (Druege, 2006), and changes in ET status affect the plant defense response (Pieterse et al., 2009a) and endophyte colonization (Iniguez et al., 2005).

Five endophytes with the greatest numeric reductions in disease were selected for further evaluation by soaking the seed in the bacteria and then applying the bacterial solution as a soil drench after seeding and again 10 DAS (seed soak + seed drench + seedling drench) without removal of seedlings from the growing medium. Using the modified endophyte inoculation technique, endophytes R17 from \textit{S. arcanum} and R21 from \textit{S. cheesmaniae} reduced the incidence of bacterial speck symptoms, although it did not result in any any consistent alterations in plant growth. Inoculation of tomato seeds and seedlings with bacterial endophytes from oilseed rape and grape, watermelon and papaya, respectively, reduced the severity of wilt symptoms caused by \textit{F. oxysporum} f. sp. lycopersici (Nejad & Johnson, 2000) or \textit{R. solanacearum} (Thomas & Upreti, 2014).

While the reduction in disease incidence was greater with the root dip method compared to the seed soak + seed drench + seedling drench method (45% versus 27% for R17, and 42% versus 27% for R21), the variability was less with the latter method. The root dip method may have been more effective because of better penetration into the roots. Zakria et al. (2008) and Bressan and Borges (2004)
demonstrated that higher bacterial endophyte densities are achieved with bare root inoculation compared to rhizosphere inoculation in rice, and root pruning inoculation compared to seed, soil drench, foliar, and seed + soil drench inoculation in maize, respectively, possibly because of increased entry via wounding.

Another factor that may have made the results less variable with the seed soak + seed drench + seedling drench compared to the root dip method is that it allowed for 20 days instead of 10 days for endophyte colonization prior to challenge with Pst. In other studies of bacterial endophytes on tomato, Pst challenge occurred 10 days after a 24 h seed soak endophyte inoculation (Lanna-Filho et al., 2017), but 2 weeks after seedling drench endophyte inoculation (Fujita et al., 2017) and up to 7 weeks after seed soak + seedling drench endophyte inoculation (Ji et al., 2006).

4.4.3 Identification of strains R17 and R21

Sequencing the 16S rDNA region of endophyte R21 resulted in the closest matches being E. ludwigii, E. cloacae, and P. agglomerans. One concern with 16S sequence data is that there are very large numbers of such sequences submitted to GenBank resulting in many unvalidated 16S rDNA sequences with incorrect identifications (Woo et al., 2008). Also, 16S sequencing may not always provide enough information to identify many bacterial species (Woo et al., 2008). The rpoB gene encoding the RNA polymerase beta subunit was also sequenced for R21 as it is considered a more informative sequence for identification of species within the E. cloacae complex (Paauw et al., 2008, Yousaf et al., 2011). The rpoB sequence analysis showed that R21 is most closely related to all E. ludwigii and some E. cloacae isolates entered in GenBank, which are both members of the E. cloacae complex. Enterobacter species fall within class Gammaproteobacteria of the Proteobacteria, which is a common phylum containing bacterial endophytes (Bulgarelli et al., 2013, Rosenblueth & Martinez-Romero, 2006, Hardoim et al., 2008, Hallmann & Berg, 2006). E. ludwigii is a relatively new species, being first described by Hoffmann et al. (2005), and was formerly known as the genovar cluster V in the E. cloacae complex. It is associated with human clinical specimens as well as plants (Hoffmann et al., 2005). In plants, E. ludwigii was isolated from the rhizosphere of Lolium perenne and provided plant growth promotion after seed inoculation and also had in vitro antagonistic effects against F. solani (Shoebitz et al., 2009), and was isolated as an endophyte from roots of tomato cv. Arka Ananya and had antagonistic activity against R. solanacearum (Upreti & Thomas, 2015) E. ludwigii was also isolated as an endophyte in roots and shoots of Italian ryegrass and birdsfoot trefoil and was able to degrade hydrocarbons after colonization of the rhizosphere and plants tissues of Italian ryegrass, birdsfoot trefoil, and alfalfa growing in soil spiked with diesel fuel (Yousaf et al., 2011). E. cloacae is associated with bulb rot of onion (Schroeder et al., 2009) and mulberry (Zhu et al., 2010). However, E. cloacae can also be beneficial to plants, and it is possible that E. cloacae isolates described before Hoffmann et al. (2005) may now be classified as E. ludwigii. E.
*E. cloacae* was isolated from the rhizosphere of cotton, and root inoculation resulted in increased biomass of tomato seedlings (Mayak et al., 2001), and an endophytic *E. cloacae* from roots of tomato cv. Arka Abha exhibited antagonism against the causal agent of bacterial wilt, *R. solanacearum*, in vitro (Uperti & Thomas, 2015). Thus, it is not surprising that *E. ludwigii* could be isolated *S. arcanum* and that it could induce resistance against *Pst* when inoculated into domesticated tomato.

Sequencing the 16S rDNA region of endophyte R17 gave the closest sequence matches to *B. mycoides* and *B. weihenstephanensis*, which are both members of the *B. cereus* group. For the reasons described previously for R21, additional sequencing was done to identify R17 to species. In this case, the gene chosen was *gyrB* encoding the DNA gyrase B subunit, which is considered relatively effective at distinguishing between species of the *B. cereus* group (La Duc et al., 2004, Wang et al., 2007). The *gyrB* sequencing confirmed that R17 is very closely related to both *B. mycoides* and *B. weihenstephanensis* but did not provide any evidence that R17 is more closely related to one species than the other. The creation of *B. weihenstephanensis* as a separate species within the *B. cereus* group is relatively recent and is based on clustering of psychrotolerant strains of *Bacillus* sp., which can grow at 4 to 7°C and had 2 nt differences in the 16S rDNA region (Lechner et al., 1998). The sequence including the 2 nt of R17 matches that of the psychrotolerant *B. weihenstephanensis* type strain 10204, and R17 does grow at 4°C. More recently, several *B. mycoides* and *B. cereus* strains confirmed to grow at 7°C and containing specific signature sequences in the 16S rRNA, *cspA, glpF, gmK, purH* and *tpi* characteristic of *B. weihenstephanensis*, were proposed for reclassification to *B. weihenstephanensis* (Soufiane & Côté, 2013). However, based on whole-genome sequence-based Genome BLAST Distance Phylogeny, Liu et al. (2015) proposed that *B. mycoides* and *B. weihenstephanensis* are not genetically distinct and should be considered the same species. Another reason for questioning the creation of *B. weihenstephanensis* was that a group of bacterial strains isolated from the tomato rhizosphere were classified as *B. weihenstephanensis* based on the 16S sequence and multi-locus sequence typing of seven housekeeping genes but showed mesophilic growth (Hollensteiner et al., 2016). Thus, whether *B. mycoides* and *B. weihenstephanensis* should be considered separate species remains unclear, and so R17 was classified as *B. mycoides/weihenstephanensis*.

The colony morphology of *B. mycoides* is described as chains of cells forming curving radial filaments on agar, also known as rhizoid or mycoidal growth (Di Franco et al., 2002). Lechner et al. (1998) stated that *B. mycoides* had rhizoidal growth and *B. weihenstephanensis* had non-rhizoidal growth. However, isolates of *B. mycoides* from a peat bog in Germany had motile cells and non-rhizoid colony morphology (von Wintzingerode et al., 1997), and isolates of *B. weihenstephanensis* from the tomato rhizosphere had two colony morphologies with one type being rhizoidal and similar to *B. mycoides* and the other type being non-rhizoidal having circular to weakly irregular colonies with entire or undulate edges.
edges similar to *B. thuriengiensis* (Hollensteiner et al., 2016). Psychrotolerant *B. weihenstephanensis* isolates from soil in Denmark had a colony morphology like that of *B. weihenstephanensis* instead of *B. mycoides* (Thorsen et al., 2006). Logan and De Vos (2009) stated that the ability to form rhizoid colonies by *B. mycoides* may be lost, indicating that it is an unreliable taxonomic characteristic, and Soufiane and Côté (2013) speculated that genes responsible for colony morphology in *B. mycoides* may be transferred through horizontal gene transfer. Strain R17 showed circular to weakly irregular colonies with entire or undulate edges and was motile in TSB. *B. mycoides* is widespread in the environment, including the rhizosphere (Buyer, 1995) and phyllosphere (Bargabus et al., 2002), whereas *B. weihenstephanensis* is described from dairy products and refrigerated food (Soufiane & Côté, 2013, Meer et al., 1991, Larsen & Jørgensen, 1997) but more recently from soil (Hollensteiner et al., 2016). This is the first report of *B. mycoides* or *B. weihenstephanensis* as an endophyte, and it may be that motility and colony morphology is affected by source material. Future research needs to determine if *B. mycoides* and *B. weihenstephanensis* isolates colonizing plant tissues (i.e., dead sphagnum moss in a bog or live plant roots) differ from those coming from other environments.

*Bacillus* sp. fall within class Bacilli within phylum Firmicutes, which is a common phylum containing bacterial endophytes that can induce disease resistance (Bulgarelli et al., 2013, Rosenblueth & Martinez-Romero, 2006, Hardoim et al., 2008, Hallmann & Berg, 2006). *B. mycoides* isolate J, which was isolated from the phyllosphere of sugar beet, reduced symptoms of Cercospora leaf spot (*C. beticola*) in sugar beet by approximately 70% when applied to leaves, but effects on growth promotion were not reported (Bargabus et al., 2004). In tomato, a commercial product containing *B. mycoides* isolate J, marketed as LifeGard, was recently registered in Canada for foliar application to suppress *Xe, A. solani,* and *P. infestans,* and partially suppress *Pst* (Bargabus et al., 2003, Bargabus et al., 2002, FRAC, 2017, Certis, 2017b). Gene expression analysis was not completed but a form of induced resistance is implicated due to increased activity of oxidative burst, chitinase and B-glucanase was reported for the isolate on sugar beets (Bargabus et al., 2003). For *B. weihenstephanensis,* three strains from ginseng rhizosphere reduced the severity of *P. cactorum* when applied to soil, but effects on growth promotion were not evaluated (Lee et al., 2015). Similarly, some isolates of *B. weihenstephanensis* from the tomato rhizosphere were antagonistic against *V. dahliae* and *Verticillium longisporum in vitro,* but effects on induced resistance and plant growth were not evaluated (Hollensteiner et al., 2016). Reports of *Bacillus* sp. implicated in induced resistance in tomato include *B. pumulis* against *Pst* and *F. oxysporum* f. sp. *radices-lycopersici* (Lanna-Filho et al., 2017, Ji et al., 2006, Benhamou et al., 1998), *B. amyloliquefaciens* against *Pst* (Lanna-Filho et al., 2017, Ji et al., 2006) and *B. megaterium* against *A. solani* and *Pst* (Barretti et al., 2009). None of these studies report positive effects of these endophytes on plant growth, although these measurements of plant growth were only reported by Lanna-Filho et al. (2017) and Barretti et al.
Thus, R17 belongs to a genus of bacteria commonly associated with induced resistance but not plant growth promotion in tomato.

4.4.4 **Optimization of bacterial endophyte treatment**

To increase the effectiveness of seed soak + seed drench + seedling drench application of strains R17 and R21, three applications of fertilizer instead of no fertilizer was used with the tomato seedlings because low nitrogen in tissues was reported to reduce constitutive levels of defense related enzymes and the induced level of peroxidase and chitinase in *A. thaliana* treated with the SAR-inducer ASM (Dietrich et al., 2004) and also reduced the effectiveness of mycorrhiza-induced resistance by the arbuscular mycorrhizal fungus, *Rhizophagus irregularis*, against *B. cinerea* in tomato (Sanchez-Bel et al., 2016). With fertilized tomato seedlings, the disease reduction with R17 and R21 was 33% and 38%, respectively, which was higher than the 27% reduction observed in non-fertilized plants, indicating that increased control was possible. However, comparison of the level of induced resistance in non-fertilized versus fertilized tomatoes cannot be made because disease assessments were measured at five DPI in fertilized plants instead of seven DPI in non-fertilized plants. This was done to reduce the variability because it was more accurate to count lesions at five DPI because fewer lesions had coalesced, thus providing a more accurate number. However, applications of fertilizer did not affect plant growth promotion by either endophyte.

Plant height was reduced with R17 and R21 inoculation compared to the MgCl₂ control, although this was not associated with a reduction in foliar or root biomass. Host fitness costs are most often associated with SAR because plant resources are allocated immediately to a defense response (Walters & Heil, 2007), as opposed to ISR, which is associated with priming of host defenses for pathogen attack (van Hulten et al., 2006, Conrath et al., 2006). However, Lanna-Filho et al. (2017) observed reductions in plant height, total dry weight, and growth rate in five week old tomato grown from seed inoculated with endophytes *B. pumilus* and *B. amyloliquefaciens*, but these effects were not as strong as similar effects caused by the application of ASM. The current study was only 3.5 weeks in duration, which is short for measuring growth rate, and thus the longer term effects of R17 and R21 were not evaluated.

The first aspect examined to increase the effectiveness of the endophytes was to further modify the endophyte inoculation technique. For strain R17, the most effective reduction in disease incidence was as a seed soak or seed drench treatment compared to inoculation as a seedling drench, seed soak + seed drench or seed soak + seed drench + seedling drench. Thus, it appears that multiple applications are not beneficial for inducing resistance as well as latter applications once the seedling has developed. R17 may be better adapted to colonize tomato plants at the time of seed germination relative to other developmental stages. During seed germination, the spermosphere, which is the environment surrounding the seed under
the influence of seed carbon deposition, releases root exudates that attract some microorganisms, including *Bacillus* sp., within a few hours of sowing (Nelson, 2004). Also, endophyte community diversity in other plant species changes with host developmental stage (Andreote et al., 2010, Monteiro et al., 2011, Kuklinsky-Sobral et al., 2004), which may be due to changes in host defense mechanisms that affect colonization (Andreote et al., 2010) or other factors, such as the level of soluble carbohydrates, calcium and phenolic compounds (Hunter et al., 2010) or starch content (Inceoglu et al., 2010). It may be that R17 is better at responding to root exudates during seed germination (Baudoin et al., 2002) or less able to colonize tomato tissues during later stages due to selection by the host for bacteria that are required for growth during later developmental stages (Qiao et al., 2017). Seed soaking or seed drench applications in tomato using bacterial endophytes also reduced the severity of *Pst* in other studies (Lanna-Filho et al., 2017, Ji et al., 2006). However, a soil drench inoculation of one-week tomato seedlings with an endophytic *Azospirillum* sp. resulted in induced resistance to *Pst* in tomato (Fujita et al., 2017), and multiple applications of *B. pumilus* SE34 by seed treatment and soil drenches to young tomato seedlings also resulted in induced resistance against *Pst* (Ji et al., 2006). There are no reports of induced resistance by inoculation with bacterial endophytes past the seedling stage (i.e. > 6 weeks). This indicates that endophytes may differ as to which inoculation method is best, and each researcher must determine this empirically. Although the inoculation method affected the level of disease incidence, there was no effect on plant growth using any inoculation method.

In contrast, strain R21 showed no significant control of *Pst* incidence regardless of the endophyte inoculation technique. The lack of efficacy of all R21 treatments indicate that the efficacy of R21 was inconsistent even using the seed soak + seed drench + seedling drench technique where it was effective in only one set of experiments. Herman et al. (2008) also reported inconsistent effects of the mixture of the PGPR *B. subtilis* GB03 and endophyte *B. amyloliquefaciens* IN937a for *Pst* control in tomato, which was associated with inconsistent induction of *PR1a* and *PR1b* after pathogen inoculation and an increase in *Pin2* expression beginning 12h prior to pathogen inoculation. The inconsistency justified discontinuing studies on strain R21.

As application to seeds was most effective, but commercial tomato seeds are typically coated with proprietary materials, such as combinations of clay and sand and other inter materials to improve handling and act as carriers for pesticide seed treatments (Sundstrom, 2002), the seed soak and seed drench inoculation methods for R17 were evaluated again comparing raw and pelleted seed. No differences were observed. This was expected because the seed coatings generally contain inert materials such as sand and clay, which would not be expected to have antimicrobial properties, and no known pesticides that could diffuse with the root exudates during the seed soaking or when the seed is germinating in the soil and negatively affect bacterial populations, including the R17 cells applied to the
seed or soil. Other reports of microorganisms in tomato using inoculation at the time of seedling do not specify the use of raw or pelleted seed (Lanna-Filho et al., 2017, Ji et al., 2006, Herman et al., 2008), and so this appears to be the first study to show that commercial seed coating does not affect the ability of endophytes to reduce disease, at least in tomato. There was also no effect of seed coating on plant growth, which was also similar to previous experiments.

Comparing different doses of R17 using the seed drench method showed that the relationship between inoculum dose and the level of disease reduction was quadratic rather than linear, and there was no significant relationship for inoculation concentration and any of the plant growth variables measured. In contrast, there was a significant linear relationship between the initial population density of the rhizobacteria and endophyte *B. pumilus* SE34 and rhizobacteria *P. fluorescens* 89B61 inoculums (ranging from $10^3$ to $10^9$ CFU/g soil mix) and tomato seedling growth and induced resistance against late blight disease (Yan et al., 2000).

The optimum concentration of approximately $1 \times 10^7$ CFU/mL in this study was generally lower than that used in several other studies of endopytes reducing disease in tomato. By comparison, $3 \times 10^5$ to $7 \times 10^8$ CFU/mL was optimum for plant growth promotion in tomato using the endophyte *Pseudomonas* sp. PsJN (i.e., *B. phytofirmans* PsJN) applied as seedling root soak in a gnotobiotic system (Pillay & Nowak, 1997). For induced resistance in tomato against *Pst*, *Azospirillium* sp. 510 was applied as a seedling soil drench at $1 \times 10^9$ CFU/ml (Fujita et al., 2017), *B. pumulis* and *B. amyloquefaciens* were applied as a seed soak at $1 \times 10^8$ CFU/ml (Lanna-Filho et al., 2017), and *B. pumilus* SE34 and *B. amyloliquifaciens* IN937a were applied as a seed treatment at $1 \times 10^9$ CFU/ml (Ji et al., 2006). However, (Ji et al., 2006) also used *B. pumilus* SE34 and *B. amyloliquifaciens* IN937a as seedling soil drench of tomato at $1 \times 10^7$ CFU/ml to induce resistance against *Pst*, and Li et al. (2016) found that the relatively low concentration of $1.21 \times 10^6$ CFU/ml was most effective for growth promotion by an endophytic *Bacillus* sp. pp02 in hybrid pennisetum when applied as a soil drench. Thus, the concentration used in this study is at least within the range used by others.

Most studies do not describe how the endophyte inoculum concentrations were chosen. However, Pillay and Nowak (1997) compared several inoculum concentrations of the well studied endophyte strain, *Pseudomonas* sp. PsJN (i.e., *B. phytofirmans* PsJN) in tomato and showed that root surface populations increased linearly with increasing inoculum densities between $10^7$ to $10^8$ CFU/ml, while root endophyte populations did not increase linearly with inoculum concentration but were highest at approximately $4 \times 10^8$ CFU/ml. Shoot endophyte population were not affected by the inoculum concentration. Inoculum concentration may thus play less of a role in latter stages as the endophyte invades the plant, and other factors become more important, which may explain why there was a limited range of populations ($10^5$-$10^6$ CFU/g tissue) inside roots and shoots. The population size within tissues was proposed to result from
limitations of nutrients and microniche s for the bacteria inside the plant. The inoculum concentrations that best resulted in colonization of inner roots gave best plant growth promotion, and Pillay and Nowak (1997) proposed a stimulation threshold of the endophyte population was needed for the plant response. Thus, the optimum concentration for inoculation with bacterial endophytes appears to be variable and may depend on the host, bacterial endophyte, inoculation method and growing environment.

A screening of five commercial processing tomato cultivars revealed that R17 had some effects on particular parameters of plant growth for certain cultivars unlike previous experiments. Further work is needed to discover why certain plant growth parameters were sometimes affected by R17. Kloeppe et al. (1989) stated that inconsistencies in promoting plant yield with PGPR results from the effects of many aspects of the environment on the interaction between the introduced bacteria and the plant and soil microflora. Thus, similar inconsistencies with host-endophyte interactions for plant growth promotion may be at play in this work.

Among the tomato cultivars, R17 caused a reduction in bacterial speck symptoms in cv. TSH4, and not in cvs. H5108, H9553, H2401 and TSH33. The differential response of the tomato cultivars to inoculation with R17 and challenge with Pst is not surprising. The responses of A. thaliana and wheat to induced resistance by rhizobacteria are genotype dependent (Ton et al., 2001, Ton et al., 2002, Maketon et al., 2012). In tomato, growth promotion and induced resistance to B. cinerea following inoculation with the rhizosphere fungi T. harzianum T22 and T. atroviride P1 differed between two inbred processing tomato lines, the landrace Corbarino, the advanced breeding line SM26 and the wild tomato S. habrochaites (Tucci et al., 2011). In the above cases, the differences in response were related to different expression of host defense genes. Thus, if induced resistance is the mode of action of R17, cv. TSH4 may have a stronger induction of defense genes in response to R17 than the other cultivars tested. Another possibility is that cv. TSH4 is better adapted for colonization by R17. Endophyte colonization can vary depending on tomato genotype. For example, a more diverse population of endophytes colonize the bacterial wilt resistant cv. Arka Abha compared to the susceptible cv. Arka Vikas (Upreti & Thomas, 2015). Similarly, colonization of the spermosphere by 24 seed inoculated B. cereus strains differed among inbred tomato lines (Simon et al., 2001). There is limited information available on the pedigrees of the cultivars included in this study. For the Heinz cultivars, there is a possibility that they share some common ancestry; however, full information on their backgrounds is not publically available (G. Collier, personal communication). The responsive cv. TSH4 and non-responsive cv. TSH33 are from Tomato Solutions (www.tomatosolutions.ca) and are from different parents but no additional information on their backgrounds is publically available (J. Dick, personal communication). Since cv. TSH4 was the only responsive cultivar identified in this study, it would be useful to know if it has any unique ancestors in its background that allow for better colonization of R17, a unique host defense response or both.
The duration of the induced resistance response in cv. TSH4 by R17 showed that it declined from 15 to 20 DPT until there was no disease reduction by 25 DPT. For induced resistance against *Pst* in tomato, this is longer than the 10 DPT produced by the endophytes, *B. pumulis* and *B. amyloquefaciens* (Lanna-Filho et al., 2017) but shorter than the 7 weeks post treatment due to the endophyte, *B. subtilis* (Herman et al., 2008). A factor affecting the results from 25 DPT in this work was that the host susceptibility to *Pst* progressively declined from 15 to 25 DPT, perhaps due to age-related resistance. Age-related resistance has been described in many plant-pathogen interactions (Develey-Rivière & Galiana, 2007). For *Pst*, it has been noted in *A. thaliana* and is independent of ISR or SAR, but was dependent upon SA (Kus et al., 2002). In tomato, this is the first report for *Pst*, but it has been reported for *C. michiganensis* subsp. *michiganensis* (Sharabani et al., 2013), *P. infestans* (Shah et al., 2015) and *C. fulvum* (Panter et al., 2002).

The decline in the duration of the disease response by R17 could be due to the population of R17 diminishing over time, which was demonstrated in the study of R17 colonization after inoculation. Pillay and Nowak (1997) proposed that endophyte populations need to reach a stimulation threshold to achieve plant response for improved growth, and root colonization at $10^4$ CFU/g fresh weight of cucumber by *P. fluorescens* G8-4 (89B61) was associated with induced resistance to *C. orbiculare* on leaves at 14 days after emergence (Kloepper et al., 1992). However, the extent of plant colonization required by endophytes to reduce disease has not been well established (Hardoim et al., 2015). For example, while seed inoculation of cucumbers with the PGPRs, *S. marcescens* 90-166 and *P. putida* 89B-27, resulted in a greater reduction in disease symptoms by *C. orbiculare* at the fifth leaf stage compared to the first leaf stage, the population of the bacteria colonizing plant roots dropped from approximately $10^8$ and $10^{10}$ CFU/g fresh weight at seven days after planting for 89B-27 and 90-166, respectively, to approximately $10^3$ CFU/g fresh weight at 28 days after planting for both bacteria (Liu et al., 1995a). As rhizosphere bacteria can induce resistance without colonization of internal tissues (Kloepper & Ryu, 2006, Bent, 2006), it is also possible that internal colonization is not even required by endophytes to induce resistance. In the case of R17, populations declined over time both inside tomato tissues and the rhizosphere.

Another reason for the decline in R17 effectiveness over time could be that the effects of R17 on the host decline over time. For example, induced resistance by endophytes and PGPRs is most often associated with ISR involving priming of defense gene expression (i.e., stronger and faster induction following infection) mediated by ET and JA-dependent signaling pathways, which is usually considered to be longer lasting than that mediated by SA-dependent signaling pathways (Pieterse et al., 2009, Ton et al., 2006). For example, protection lasted up to five weeks against *C. orbiculare* in cucumber after seed inoculation with the PGPRs, *S. marcescens* 90-166 and *P. putida* 89B-27 (Liu et al., 1995a). However, the number of abiotic and biotic stress genes showing up- and down-regulation of expression using
microarrays of *Theobroma cacao* at three, seven and 14 days after inoculation with the endophytic fungus *Colletotrichum tropicale* demonstrated that the resistance response can change over time (Mejía et al., 2014). Mauch-Mani et al. (2017) hypothesize that the duration of defense priming may be dependent on the frequency and intensity of the stress pressure, which indicates that signaling needs periodic stimulation to be maintained at similar levels. While plants can induce JA signaling by stresses, they are also capable of suppressing it by switching off JA signaling for a subset of genes through the conversion of JA to 12-hydroxyjasmonic acid (Miersch et al., 2008). In addition, SA appears to be important for induced resistance caused by some PGPRs (Maurhofer et al., 1998). Lanna-Filho et al. (2017) also associated induced resistance against *Pst* in tomato due to the endophytes, *B. pumilus* and *B. amyloliquefaciens*, with SA-dependent signaling pathways based on increases in defense enzymes including PAL up to four days prior to pathogen challenge. Induction of gene expression via SA is well known to decline over time. For example, foliar applications of the SAR activator ASM to tomato resulted in increased expression of the defense genes *PR1a* and *PR1b*, but this declined to baseline levels within one week (Herman et al., 2007). Thus, it is possible that the decline in the duration of the response to R17 was related to changes in the defense signaling response to the endophyte, which could also be combined with the effect of the declining population of R17 possibly reducing the frequency and intensity of the activation of the resistance.

### 4.4.5 Direct antimicrobial effects of promising endophytes

It is possible that the ability of R17 to reduce disease incidence is due to a direct antimicrobial effects against *Pst*, since R17 showed some in vitro activity against *Pst* using two in vitro assays. However, due to the limited ability of R17 to colonize leaf tissue as an endophyte, the likelihood is low that the antimicrobial compounds were the primary explanation for it reducing the incidence of *Pst*. This is the first report of direct antimicrobial effects of a strain of *B. mycoides/weihenstephanensis* against *Pst*. The production of antibiotics by *Bacillus* sp. group is reported for many species against 80 bacteria or fungal organisms, including many bacteria and plant pathogens (Shafi et al., 2017). Lipopeptides belonging to the surfactin, iturin and fengycin (or plipastatin) families are the most common antibiotics produced by *Bacillus* sp., and have shown activity against many plant pathogenic bacteria, fungi and oomycetes (Ongena & Jacques, 2008). Lipopeptides have been described from *B. mycoides* including cerexin A1 (Cochrane et al., 2015) as well as bacteriocin-like substances (Abriouel et al., 2011). Genome analysis of 13 strains of *B. weihenstephanensis* revealed gene clusters for bacteriocin and microcin production, and some strains had gene clusters for other peptides including lanthipeptides and lassopeptides (Hollensteiner et al., 2016). The antifungal activity of some *B. weihenstephanensis* isolates
from the tomato rhizosphere was associated with the siderophore bacillibactin and mycolytic chitinases (Hollensteiner et al., 2016). Given the widespread ability of Bacillus sp. to suppress the growth of other microorganisms, it is most likely that the antimicrobial effects of *B. mycoides/weihenstephanensis* strain R17 are a result of several antibacterial compounds.

The direct antimicrobial effects of the other disease resistance activating strain in this study, R21, against *Pst* were less clear as it only showed activity using one of the two in vitro assays. However, that was sufficient to conclude that this is the first report of direct antimicrobial effects of *E. cloacae/ludwigii* against *Pst*. The antimicrobial effects of *Enterobacter* sp. are also widely reported. For example, the endophyte *E. cloacae subsp. cloacae* from pepper has antagonistic activity against *Collectotricum capsici*, *S. sclerotiorum*, *Alternaria* sp., *D. bryoniae*, *F. oxysporum*, and *R. solanacearum* (Liu et al., 2013), *E. cloacae* and *E. ludwigii* from tomato are antagonistic against *R. solanacearum* (Upreti & Thomas, 2015), and *E. ludwigii* from *L. perenne* decreased spore germination and produced a zone of inhibition against *F. solani in vitro* (Shoebitz et al., 2009). The antimicrobial activity of *E. cloacae* subsp. *cloacae* was associated with the bacteriocin, colicin V, as well as siderophores, and chitinases (Liu et al., 2013), and the antimicrobial activity of *E. cloacae* was linked to the presence of hydrogen cyanide (Upreti & Thomas, 2015). Species of Enterobacter from soil also produce antimicrobial lipopeptides (Mandal et al., 2013). Thus, there are a variety of potential antimicrobial factors being produced by *E. ludwigii* strain R21.

### Colonization of R17 inside tomato tissues

To confirm that R17 colonizes tomato tissue as an endophyte, rifampicin resistant R17 mutants were created, which appeared to be identical to the original strains relative to reducing bacterial speck incidence and not affecting plant growth. In roots, the population of R17-RfpC did not exceed $1.3 \times 10^2$ CFU/g fresh tissue, which is relatively low compared to other studies which found root bacterial endophyte populations ranging from $10^2$ to $10^{10}$ CFU/g fresh weight (Hallmann & Berg, 2006). However, that population dropped by two orders of magnitude between 10 and 25 DPT. In the rhizosphere, the R17-RfpC population was approximately $10^6$ CFU/g dry soil, which is lower than that of the total population of rhizobacteria of $10^7$ to $10^{10}$ CFU/g dry soil in barley, wheat, and canola (Lupwayi et al., 2004), and $10^8$ CFU/g soil or higher in watermelon (An et al., 2011). However, the percentage of culturable *Bacillus* sp., ranged from 17% in 70-day old plants to 36% in 28-day old plants in the cucumber rhizosphere (Mahaffee & Kloepper, 1997), and approximately 10% of the potato rhizosphere were Firmicutes (Berg et al., 2005). Thus, the R17-RfpC population in the rhizosphere as a component of the total population of rhizobacteria is reasonable. Between 10 and 25 DPT, the R17-RfpC population dropped by 50% in the rhizosphere indicating that it was out-competed by the endemic rhizobacteria.
These declines would not however preclude R17 from inducing resistance. The endophytes *B. pumilus* SE34 and rhizobacteria *P. fluorescens* 89B61, which induced resistance against late blight of tomato (Yan et al., 2000), were incorporated into the growing medium at the time of planting, but their populations dropped in the tomato rhizosphere by approximately one half to one order of magnitude, similar to R17, by 28 days after planting (Yan et al., 2003). As R17 both colonize plant tissues and the rhizosphere, one possibility is that the induced resistance by R17 may have occurred due to its rhizosphere colonization thus acting more like a PGPR, instead of its internal root colonization, although both may have been involved.

In leaves, R17-RfpC was only detected in cotyledon leaves at 10 DPT but not true leaves at 25 DPT. Thus the bacterium has a limited ability to spread and persist in above ground tissues. The absence of R17 in leaf tissue at 25 DPT is consistent with Romero et al. (2014) who found few endemic Firmicutes present in tomato leaf tissues at 30 DAS. Since R17-RfpC was found in cotyledon leaves at low numbers at 10 DPT, it is possible that R17-RfpC colonized the cotyledons directly when the germinating seed was treated, rather than infecting the roots and then spreading internally through the plant like that of a competent endophyte (Hardoim et al., 2008). On the other hand, vertical spread of *B. polymyxa* and *B. subtilis* in plant tissues was reported in spruce (Shishido et al., 1999) and cacao (Shishido et al., 1999), indicating that they were competent endophytes. Thus, although colonization through the xylem vascular is thought to take several weeks (Hardoim et al., 2015), it is also a possibility for colonization of the cotyledons by R17.

Another possible reason for the decline in R17 populations inside plant tissues following inoculation is changes in the physiology of the host. Endophyte colonization is dependent on a series of interactions with the host plant including the host recognition of MAMPs and the elicitation of MTI, ETS and ETI (Zamioudis and Pieterse 2012). Thus, the host can have a significant impact, and several studies have shown that endophyte colonization is influenced by plant developmental stage. For example, in soybean root endophyte populations were higher at the leaf senescence stage compared to the flowering stage, which was higher than the vegetative stage (Kuklinsky-Sobral et al., 2004). In potato, the population of *Pseudomonas* spp. and *Actinobacteria* was lower at plant senescence than flowering or juvenile stages (van Overbeek and van Elsas 2008), and 73% of the variation observed in endophyte genetic fingerprints was explained by plant growth stage. Total endophyte population in cucumbers roots increased during the first three weeks post-emergence, but then stabilized at $10^5$ CFU/g fresh weight (Mahaffee & Kloepper, 1997), and in wheat, populations of *B. subtilis* peaked before heading and then steadily declined, unlike populations of other endophytes that remained relatively constant throughout the wheat life cycle (Comby et al., 2016). Thus, R17 populations may have been declined due to changes in the interaction of endophytes with tomato as the plants grew.
4.4.7 Effect of R17 on bacterial speck lesion incidence versus \textit{Pst} population

Typically, the \textit{Pst} population in leaf tissues is related to the percentage of leaves with symptoms, which has been demonstrated in \textit{A. thaliana} (Vallad et al., 2003), or the \textit{Pst} population is a severity disease index based on the number of lesions per leaflet, which occurs in tomato (Baysal et al., 2007, Scarponi et al., 2001). However, in this study the \textit{Pst} population per cm\(^2\) in R17 inoculated plants was equivalent to the control, even though the number of lesions per leaflet was reduced. This was related to a higher \textit{Pst} population per lesion in R17 treated plants than the control. This is in contrast the endophytes \textit{B. pumilis} and \textit{B. amyliloquefaciens}, which reduced \textit{Pst} population on tomato leaf surfaces by three to four orders of magnitude and reduced the number of lesions per leaflet, but \textit{Pst} populations inside the leaves were not measured (Lanna-Filho et al., 2017), and the endophyte \textit{Azospirillum} sp. B510 which reduced both \textit{Pst} population growth \textit{in planta} and disease severity in tomato (Fujita et al., 2017).

However, sometimes reductions in plant disease symptoms are not associated with reductions in pathogen population (Hammerschmidt, 2009, Summermatter et al., 1995). Inoculation of \textit{P. syringae pv. syringae} induced resistance in \textit{A. thaliana} through an incompatible interaction on lower leaves, which resulted in reduced necrosis in upper leaves after a later challenge, but the populations of \textit{P. syringae pv. syringae} in the challenged leaves remained unchanged (Summermatter et al., 1995). In addition, induced resistance resulting in lower levels of necrosis by \textit{Xcv} or \textit{Pst} on challenged leaves activated by \textit{Xcv}, avirulent \textit{Xcv}, or \textit{Pst} on different leaves on the same plant was not related to reduction in populations of \textit{Xcv} and \textit{Pst} on the challenged leaves (Block et al., 2005). Block et al. (2005) described a reduction in symptoms without an associated reduction in pathogen population as systemic acquired tolerance. It appears that R17 in the current study causes a reaction similar to systemic acquired tolerance, except the number of lesions and not total necrosis, is reduced.

The lack of correlation between disease incidence and \textit{Pst} population in this study appeared to be due to the lesions on R17 inoculated plants being larger than lesions on control plants. This is similar to what was found for PABA in Chapter 2, and R17 may suppress initial infections so that fewer lesions develop, but then lesion development occurs more rapidly post-infection. Although lesion size for \textit{Pst} infected plants is rarely reported, this was surprising since induced resistance by the SAR inducer ASM reduced bacterial speck lesion diameter in tomato (Scarponi et al., 2001).

The mechanism of disease reduction by R17 is unknown; however, induced resistance associated with endophytes is mostly related to ISR, which involves control of necrotrophs through JA and ET signalling pathways, unlike SAR, which generally controls biotrophs and is associated with SA signalling pathways (Pieterse et al., 2009, Ton et al., 2006). In the current study, one explanation for the fewer lesions in R17 treated plants could be because R17 primes or triggers a defense response prior to \textit{Pst} entry.
into the plant. *Pst* must penetrate the leaf tissue through wounds or natural openings, such as stomata. Part of the defense response against foliar infection by bacteria includes stomatal closure, which is triggered by bacterial PAMPs, positively regulated by SA versus negatively regulated by JA and can be suppressed by the pathogen using mechanisms, such as the T3SS effector AvrRpt2 and the phytotoxin coronatine (Melotto et al., 2008, Melotto et al., 2017). Therefore, it is possible that R17 prevents *Pst* from entering the apoplast by increasing stomatal closure if the induced resistance mechanism involved SA, which would reduce the number of infection sites and result in fewer lesions. This idea is supported by the finding that the rhizobacterium *B. subtilis* FB17 accelerated stomatal closure in *A. thaliana* against *Pst* for 48 h post-inoculation in an ABA and SA-dependent manner (Kumar et al., 2012).

Following penetration, the biotrophic phase of *Pst* occurs as the pathogen grows in the apoplastic fluid and releases T3SS effectors to suppress PTI (Preston, 2000, Cunnac et al., 2009, Munkvold et al., 2009). Induced resistance due to R17 could have triggered defenses related to limiting biotrophic growth of *Pst*, and thus prevented infection that would subsequently develop into lesions. Induction of SA signalling can suppress JA/ET defense signaling (Gimenez-Ibanez & Solano, 2013). For example, infection by Pst induced SA-mediated defenses in *A. thaliana* resulting in the suppression of expression of the JA/ET dependent genes and increasing susceptibility to the necrotroph, *A. brassiccola* (Spoel et al., 2007). Therefore, if increased resistance due to R17 is mediated through SA targeting *Pst* biotrophic growth, then this could suppress JA-related defenses against necrotrophic growth result in larger lesions, which is due to *Pst* necrotrophic growth. Like stomatal resistance, this explanation implies that R17 induces SA-dependent defence signalling. Although less frequent than associations with ISR, there is evidence for *Bacillus* sp. with SA-dependent defense signaling. *B. pumulis* and *B. amyloquefaciens* induced higher PAL activity after *Pst* inoculation (Lanna-Filho et al., 2017), which is associated with SAR, Ahn et al. (2002) found that *B. amyloquefaciens* induces both SA and JA-pathways by measure of *PR1a*, PDF1.2 and PAL gene expression, and Niu et al. (2011) found that *B. cereus* AR156 primes enhanced expression of SA, JA and ET-dependent genes in *A. thaliana* challenged with *Pst*. To assess the role of SA with R17, the ability of R17 to induce resistance in SA-deficient plants, like nahG tomatoes (Brading et al., 2000), should be determined to see if the level of induced resistance is affected.

### 4.4.8 Conclusions

In this study, the ability of two bacterial endophytes, *B. mycoides/weihenstephanensis* strain R17 and *E. ludwigii/cloacae* strain R21, to reduce symptoms of bacterial speck in tomato was demonstrated. The endophytes, which were isolated from two wild species of tomato, are members of genera often associated with endophytes (Bulgarelli et al., 2013, Rosenblueth & Martinez-Romero, 2006, Hardoim et al., 2008, Hallmann & Berg, 2006) and in the case of *B. mycoides/weihenstephanensis*, induced resistance...
(Bulgarelli et al., 2013, Rosenblueth & Martinez-Romero, 2006, Hardoim et al., 2008, Hallmann & Berg, 2006). R17 and R21 also demonstrated antimicrobial effects against \textit{Pst} in vitro, which were first reports for these organisms against this plant pathogen, although both species have shown antibacterial activity against a range of other microorganisms. However, the physical separation of R17 and R21, which were applied to seed and soil, from the leaves that were assessed for bacterial speck incidence suggested that induced resistance was the mechanism of disease suppression. This should be confirmed with additional research on the molecular mechanisms of this response. Neither endophyte showed consistent effects on plant growth promotion showing that endophytes beneficial for disease are not always linked to plant growth promotion (Lanna-Filho et al., 2017).

Many factors affected the ability of R17 to suppress bacterial speck. Among the factors tested, endophyte inoculation method, host genotype, endophyte concentration and duration of resistance, the most critical ones were host genotype and inoculation method. No disease reduction occurred unless a particular genotype was used. This is potentially a very limiting factor in the application of R17 as many tomato genotypes have been developed for specific commercial purposes, such as high soluble solids content, maturation time, pest resistance and fruit colour (Berry & Uddin, 1991, Bai & Lindhout, 2007). Duration of the effect was also a factor that was critical to achieve any level of suppression and appears to be approx. 3 weeks. The implication from this is that repeated applications of R17 may be needed to maintain the effect, which would increase costs. Despite examining a number of factors, the maximum level of \textit{Pst} symptom reduction ever achieved was 51%. This would not provide a commercially acceptable level of control in commercial production as bacterial speck is a polycyclic disease with multiple infection cycles during the season. In polycyclic disease cycles, disease accumulates in an exponential manner and so it is unlikely that reducing infections by only half would have a significant impact on total disease over a growing season (Arneson, 2001).

To help explain the disease suppression, R17 colonization of the tomatoes following treatment and \textit{Pst} populations in treated and non-treated plants were examined. R17 colonization declined in the tomato rhizosphere, root and foliar tissue over time. While it is unclear how endophyte colonization is related to the effect observed here (Hardoim et al., 2015, Liu et al., 1995b, Kloepper & Ryu, 2006, Bent, 2006), rapidly declining populations may mean that R17 would need to be reapplied in the field. The practical use of R17 as a disease management tool may also be limited because although it reduced the number of bacterial speck lesions, these lesions were larger than those in control plots, and as a result, did not reduce the populations of \textit{Pst} or the total leaf surface area with lesions. To control polycyclic diseases like bacterial speck, a control agent must be able to limit the rate of reproduction or the duration of the epidemic, as discussed above. R17 treatments would likely not limit the inoculum for subsequent infections or delay the epidemic by delaying initial infections and thus ultimately not control field
epidemics of the disease. Overall, while R17 can suppress \textit{Pst}, it has limited potential for field use unless issues, such as the level of disease control, duration of disease control and inability to reduce \textit{Pst} populations, can be addressed.
Chapter 5: General Discussion

Bacterial speck and bacterial spot remain serious diseases of field tomatoes, especially in production years with frequent or intense rain events. Copper is the traditional tool for management of these diseases. However, in a review of 45 studies evaluating copper efficacy for bacterial spot or speck control, 56% of the studies reported less than 50% disease reduction, while only 38% of studies reported more than 60% disease reduction (Griffin et al., 2017). The activity of copper in field tomatoes may be limited by poor coverage, which can result when there is a heavy canopy, and/or the presence of copper tolerant bacteria. Widespread tolerance to copper by Pst, Xg, and Xp is reported in major tomato growing areas, including Ontario (Abbasi et al., 2015, Cuppels & Elmhirst, 1999, Griffin et al., 2017).

This has motivated the search for more effective disease management tools. Active ingredients currently registered and available for use against bacterial speck or bacterial spot on field tomatoes in Canada include kasugamycin (Kasumin 2L), B. subtilis QST 713 (Serenade Opti, Serenade Max, Cease), copper hydroxide (Coppercide WP, Kocide 2000, Parasol WG), copper octanoate (Cueva), extract of giant knotweed (Regalia MAXX) and B. mycoides isolate J (LifeGard) (OMAFRA, 2014, Certis, 2017b, Bayer, 2017, Bayer, 2014a, Bioworks, 2016). The registration of these products, including the induced resistance activators B. subtilis QST 713, B. mycoides isolate J, and extracts from R. sachalinensis (Regalia MAXX) suggests that many companies consider them to be promising tools for bacterial speck and spot management in tomato (PMRA, 2011, PMRA, 2016d, Certis, 2017b, Bayer, 2017, Bayer, 2014a). However, the efficacy of B. subtilis QST 713, extracts from R. sachalinensis and ASM has proven to be quite variable under field conditions (Obradovic et al., 2004, Roberts et al., 2008, Wilson et al., 2002, Trueman, 2015, Louws et al., 2001), while there is no publically available data on the efficacy of B. mycoides isolate J. Furthermore, ASM application sometimes results in host fitness costs (Walters & Heil, 2007, Lanna-Filho et al., 2017, Louws et al., 2001, Damicone & Trent, 2003, Lange & Smart, 2005, Pontes et al., 2016, Kunwar et al., 2017), and the registrant for the commercial formulation of ASM withdrew the product from the Canadian market because of declining sales in 2016 (Filotas, 2015).

The need for more effective control products are clear from a survey that showed that copper use has declined (J. LeBoeuf, pers. communication) and defense activators have not been widely adopted by Ontario processing tomato growers for bacterial spot and speck management because of a lack of perceived benefits due to poor or inconsistent field performance (J. LeBoeuf, pers. communication). Pesticide application, including copper and defense activators, are not expected to have significant use in managing tomato bacterial disease in Ontario because of copper tolerance and poor performance in field trials (Trueman & LeBoeuf, 2015). Thus, while induced resistance holds promise as an alternate disease management tool, more information is needed to make defense activators effective in the field.
In this thesis, an attempt was made to improve the efficacy of ASM by first treating tomatoes with the PGR UNI, which is also associated with increasing stress tolerance in plants (Al-Rumaih & Al-Rumaih, 2007, Duan et al., 2008, Senaratna et al., 1988, Zhang et al., 2007). The hypothesis was that ASM is sometimes ineffective because of excess stress created by the compound, and that stress could be reduced by UNI to allow for induced resistance. However, there was little evidence of fitness costs by ASM, and when evidence for fitness costs was observed, UNI did not prevent yield loss.

There may be other ways to make ASM more consistently effective against speck and spot on field tomatoes in Ontario. One alternative would be to combine ASM with biological ISR activators, like B. subtilis GB03 and B. amyloliquefaciens IN937a for bacterial speck in tomato, where control was better than ASM alone in one of three greenhouse experiments (Herman et al., 2008). In this case, ASM could be combined with B. mycoides/weihenstephanensis strain R17, which was putatively found to be effective in inducing resistance in this thesis. Another possibility is to combine ASM with copper based nanomaterials, such as core-shell copper, multi-valent copper and fixed quarternary ammonium copper, since the nano sized copper materials are toxic to copper tolerant strains of Xp (Strayer-Scherer et al., 2017). Another way to possibly improve the effectiveness of ASM is to use the product only on tomato genotypes that can respond with increased resistance considering that only one out of eight tomato breeding lines showed increased resistance to Pst following seed applied ASM (Goodwin et al., 2017b). Future work could examine additional tomato genotypes, both with ASM alone and ASM combined with UNI, B. mycoides/weihenstephanensis strain R17 and copper based nanomaterials. Yet another way to make ASM more effective would be to increase the time between applications more than the Canadian Actigard label recommendation of seven days (PMRA, 2011). The interval period may be related to the duration of induced defense gene expression as Herman et al. (2007) showed that defense gene expression induced by ASM returns to baseline levels after seven days in three fresh market cultivars. However, Pontes et al. (2016) showed that a slightly longer interval of eight to 10 days provided the maximum bacterial spot reduction using response-surface curve analysis for processing tomatoes. These differences may be because Pontes et al. (2016) used the processing tomato cv. H9992 compared to the fresh market cultivars Supersonic, Rutgers, and Rio Grande used by Herman et al. (2007). A longer interval for ASM may have been better using cvs. TSH4 and H9909 in this thesis. Defense gene expression induced by ASM among different tomato genotypes can vary in intensity and duration (Goodwin et al., 2017b, Herman et al., 2007), and thus future work needs to examine defense gene expression in cvs. TSH4 and H9909 following ASM application to help determine whether a seven day interval is appropriate. While combining ASM with other compounds and defense activators, limiting the range of cultivars on which it should be applied and adjusting application intervals for each cultivar may limit the use of ASM, at least it could give greater confidence of success in those cultivars where it is effective.
Abiotic factors can also influence gene expression and host fitness effects of ASM (Dietrich et al., 2004, Dietrich et al., 2005) and there is a limited ability to control most abiotic stresses in the field, thus, any disease management system using ASM would have to be sufficiently robust to be effective in a relatively wide range of environments. Future studies need to examine all the parameters included in this thesis under a range of environmental conditions that might reasonably be expected by field tomatoes in Ontartio. ASM will likely only achieve acceptance by tomato growers if the risks associated with its use is greatly reduced.

Many other chemical compounds, such as tiadinil, thiamine and PABA, are also reported to induce resistance against plant pathogens but have not been developed as commercial products (Schreiber & Desveaux, 2008, Song et al., 2013). In this thesis, PABA was selected for evaluation against Pst in tomato because it reduced the severity of Xe/Xp in peppers and P. carotovorum subsp. carotovotrum in tobacco (Song et al., 2013, Yang et al., 2011b). Although PABA applications reduced the incidence of bacterial speck lesions in growth chamber experiments, the maximum level of control was a 43% reduction in lesion incidence, and there was no effect on the total leaf surface area with bacterial speck or Pst population or disease severity or tomato yield in one field experiment. This occurred despite examining application method, timing of Pst inoculation after application, number of applications, concentration and host genotype to increase PABA performance. A limitation of the growth chamber experiments in this thesis is that only young plants were examined, and so additional research should be undertaken to better understand the effectiveness of PABA in older tomato plants. In addition, only six tomato genotypes were tested for their response to PABA, and future work could examine many more genotypes as well as breeding for a stronger response to PABA applications (Michelmore et al., 2017). Field performance of PABA in the current study may have been reduced because a non-formulated version of PABA was used. Pesticides are exposed to variable environmental conditions in the field, including heavy dews, rain, heat, and UV light, that can reduce their effectiveness, which would not be the case in the protected environment of a growth chamber (Oliver & Hewitt, 2014). Typically, formulations of pesticides sprayed onto plants contain UV stabilizers to prevent photodegradation, adhesives to lengthen the duration of effectiveness and surfactants to improve coverage (Oliver & Hewitt, 2014). Future experiments should examine different formulations of PABA under field conditions.

In addition to chemical activators, there are many reports of using living organisms as biological activators to induce plant disease resistance (Bent, 2006, Hardoim et al., 2015). Bacterial endophytes were studied in this thesis as potential biological activators for management of bacterial speck. While PGPRs are available as commercial biological activators of resistance, such as B. subtilis QST 713 (Serenade Max, Serenade Opti, Cease) and B. subtilis GB03 and B. amyloliquefaciens (BioYield) (Fousia et al., 2016, Herman et al., 2008, Bayer, 2017, Bayer, 2014a, Bioworks, 2016), bacterial endophytes are
appealing because they can internally colonize the plants where they are protected from the environment and potentially a single infection can lead to endophyte establishment for the life of the plant. Two bacterial endophytes, *E. cloacae/ludwigi* R21 and *B. mycoides/weihenstephanensis* R17, from wild tomato species were identified as causing putative induced resistance because application resulted in a reduction in bacterial speck lesion incidence. R17 was the most consistent in growth room experiments. However, the maximum level of control was a 51% reduction in lesion incidence despite examining many factors, including inoculation timing, inoculation method, bacterial concentration and host genotype, to increase the level of control. Future work that could possibly increase that level of control would include examining more tomato cultivars to find ones that show a greater level of response since R17 induced a response against *Pst* in only one of five commercial cultivars tested.

Unlike chemicals, biological activators need to be able to grow and persist with the crop. R17 colonization in growth chamber experiments declined within a few weeks of inoculation, but it was not clear if colonization is required for the response to occur, or if the response in older seedlings declined for other reasons such as developmental resistance. If colonization of internal tissues by R17 is required to elicit an effect, then the ability of R17 to reduce bacterial speck symptoms may be limited over longer periods. In addition, studies are needed on the ability of R17 to induce a response in tomato at different growth stages as only young plants were tested in the growth room, and confirm induced resistance as the mechanism of the response. This should include field trials.

Considering that the level of disease control was similar between PABA and *B. mycoides/weihenstephanensis* R17 in the growth room but PABA was ineffective in the field, *B. mycoides/weihenstephanensis* R17 may not be effective in the field. If it were to be effective, then for a bacterial activator to be commercialized, it must also be evaluated for their environmental and toxicological impact, ability to be produced on a commercial scale and suitability for long term storage (Montesinos, 2003). There are reports of *B. mycoides* being pathogenic to farmed channel catfish (Goodwin et al., 1994), but regulatory agencies in the USA and Canada did not identify any other concerns regarding environmental or human health impacts in a review of *B. mycoides* isolate J (PMRA, 2016b). However, work is still needed to confirm the safety of *B. mycoides/weihenstephanensis* R17. *B. mycoides* isolate J has been commercialized as LifeGard and this could serve as a model for future work on large scale production (Certis, 2017b). For storage, *Bacillus* spp., such as R17, are spore forming and thus can survive for long periods as well as enabling the bacteria to be resistant to environmental stress making it easier to formulate in a commercial product (Rahman, 2016). Future work could examine endospore production by R17. Finally, it was unexpected that the only bacterial endophytes that affected disease incidence in this study originated from wild tomato species. This suggests that more work should be done examining wild tomato species as sources of bacterial endophytes that may provide benefits to
domesticated tomato.

In this study, disease incidence was measured in growth chamber trials by calculating the number of lesions per unit area of tomato leaf tissue. It was initially assumed that this measure would correlate with a reduction in the pathogen population in leaf tissues; however, for both PABA and R17, there was no reduction in *Pst* population per unit area of leaf. This effect was associated with fewer but larger lesions resulting in a higher *Pst* population per lesion than control plants. Such results may occur because PABA and R17 may limit initial infections of *Pst* (i.e. reduce the number of lesions) but then have little effect on the subsequent increase in pathogen population and lesion size. Since *Pst* enters plants via natural openings or wounds, additional research on stomatal defense in the *Pst*-tomato pathosystem would help elucidate the mechanisms of this response. Recent research shows that stomatal defense is dependent on MAMPs, PAMPs and hormonal signalling, but it can be counterbalanced by pathogen effectors and phytotoxins, as well as environmental factors (Melotto et al., 2017). There is a dearth of information on the role of plant defense activators in stomatal defense, and thus further research on PABA and R17 on stomatal closure in tomato would further our understanding of this response as it relates to induced resistance.

One possible reason that *Pst* populations and lesion size increased faster in plants treated with PABA or R17 than in control plants could be related to cross-talk between the SA and JA/ET signaling pathways resulting in a decline in JA/ET-related defenses if SA-related defenses are activated and vice versa (Gimenez-Ibanez & Solano, 2013, Song et al., 2013, Goodwin et al., 2017a, Spoel et al., 2007, Walters et al., 2011a). The effect of PABA is clearly related to SA in plants (Song et al., 2013, Tazhoor, 2014, Goodwin et al., 2017a, Yang et al., 2011b), and so future work needs to determine if that may be suppressing JA/ET-related defenses. One approach is to examine the effects of PABA and R17 on lesion number, lesion size and *Pst* population in tomato mutant lines deficient in SA, JA or ET signaling, such as ETR mutants deficient in ET perception (Lashbrook et al., 1998), NahG transformants in which SA is degraded (Brading et al., 2000, Di et al., 2017), and def1 mutants in which JA signalling is compromised (Di et al., 2017). This would help uncover if the lesion number response is dependent on a particular pathway. Since *Pst* DC3000 infects *A. thaliana*, which has mutants and combinations of mutants for large numbers of genes, then the nature of PABA or R17 activated resistance could be very extensively examined if PABA and R17 have similar effects on *Pst* in that pathosystem.

The lack of relationship observed between lesion density and *Pst* population for R17 and PABA-activated resistance in this study indicates a need for this to be considered in future research on *Pst* and possibly other diseases where resistance is evaluated based on lesion number. Growth chamber and greenhouse studies on plant defense activators and *Pst* normally evaluate disease severity using a variety of methods including visually with photos (Fujita et al., 2017, Block et al., 2005), rating scales based on
severity or the number of lesions per leaflet (Herman et al., 2008) or the absolute number of lesions per leaflet (Lanna-Filho et al., 2017). However, those do not account for variation in leaflet size and the difference in lesion size observed in this work was so small that it required very careful image analysis. Few studies determine if defense activators actually reduce pathogen population or growth in plant tissues as opposed to just symptom development (Lanna-Filho et al., 2017, Herman et al., 2008). In future research, it may be informative to test several methods of assessment for disease incidence or severity.

Because R17 and PABA-activated resistance do not reduce *Pst* populations, then they may not even have an impact on bacterial speck epidemiology in the field unless they induced very high levels of resistance. This is because the polycyclic nature of bacterial speck would still allow the pathogen population to build over time in the field even if symptom reductions were observed. This could possibly even be observed in growth room experiments if continued for a prolonged time with overhead irrigation or misting to simulate rainfall. Thus, a possible future experiment would be to grow tomatoes inoculated with *Pst* until maturity in growth rooms with overhead irrigation and then use methods of disease assessment like in this thesis to test if R17 and PABA reduce disease progress over time.

Although effects regarding the activity and optimization of the activators ASM, PABA, R17, and R21 were found in this study, practical management of bacterial speck and spot remains a challenge. In recent years, only products labelled for suppression, which is defined as having commercial benefit (i.e. 60 to 80% reduction), or partial suppression, which is defined as less than 60% reduction have been registered for bacterial speck or spot control in tomato (PMRA, 2011, Bayer, 2017, Bayer, 2014a, Bioworks, 2016, Certis, 2017b, PMRA, 2016d, Certis, 2017a, PMRA, 2016f). Based on the results of field efficacy tests of defense activators in Canada and the USA, it is unlikely that such products alone will provide economic control of bacterial speck and spot (Trueman, 2015, Roberts et al., 2008). However, plant defense activators could be part of a true systems approach that involves all tenants of integrated pest management (IPM). Furthermore, the increasing demand by consumers for organic or sustainably produced food (Lamichhane et al., 2015, Dewen et al., 2017), the withdrawal or proposed withdrawal of long approved pesticides because of health and safety or environmental concerns in Canada and Europe (Lamichhane, 2017, Hillocks, 2012, PMRA, 2016e, PMRA, 2016c) and shifts in corporate and government policy toward mandatory IPM (Lamichhane et al., 2015, Bonduelle, 2015) support this approach.

Research is still needed to improve bacterial disease management in field tomato. Traditional breeding approaches have yielded few cultivars with commercially acceptable levels of host resistance against multiple races of *Pst* or races and species of BSX. However, new opportunities in plant breeding include breeding for non-blighting traits, identification of resistance quantitative trait loci, and the introduction of resistance genes using CRISPR technology (USDA, 2015). Breeding for specific effector
targets that are conserved across *Pst* races or BSX races and species, may also lead to the development of tomato cultivars with more durable resistance (Timilsina et al., 2016). Sanitation is another option. Research on the role of contaminated seedling transplant trailers and transplanting equipment in bacterial spot epidemics in Ontario includes the evaluation of cleaning and sanitation tools to prevent spread of BSX (OPVG, 2017a). It may also be possible to control bacterial speck and spot by modifying the plant environment by introducing mulch cover crops as this increased the yield of commercial grade squash by reducing the population of pathogenic *P. syringae* in plots (Toussaint et al., 2012), or with the strategic use of windbreaks, as suggested to limit spread of citrus canker (*Xanthomonas campestris* pv. *citri*) in Florida (Tamang et al., 2010). Despite these efforts, more work is needed on agents that can control *Pst* and *Xg* infections in the field. The use of antibiotics in commercial plant agriculture is effective, but unlikely to be approved in Canada and has resulted in relatively rapid evolution of resistance in bacterial pathogens in other jurisdictions (Ritchie & Dittapongpitch, 1991, Araujo et al., 2012). Therefore, defense activators will continue to be investigated as one option, although this thesis has shown some of the challenges in developing and using these tools.


Chen L, Qian J, Qu S, et al., 2008. Identification of specific fragments of HpaG(Xooc), a harpin from Xanthomonas oryzae pv. oryzicola, that induce disease resistance and enhance growth in plants. Phytopathology 98, 781-91.


Fu ZQ, Yan S, Saleh A, et al., 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486, 228-32.


Nava-Diaz C, 2006. Role of plant growth-promoting rhizobacteria in integrated disease management and productivity of tomato. Columbus, Ohio, USA: Ohio State University, Ph.D. Thesis.


219


Potlakayala SD, Reed DW, Covello PS, Fobert PR, 2007. Systemic acquired resistance in canola is linked with pathogenesis-related gene expression and requires salicylic acid. Phytopathology 97, 794-802.


Appendix

**Table A.1** Effect of different para-aminobenzoic acid (PABA) concentrations and application volumes on the relative chlorophyll content in tomato cv. H9909 inoculated with *P. syringae pv. tomato* (*Pst*). PABA was applied to the root zone with a pipette 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* 20 DAS. Relative chlorophyll was measured at five days post inoculation.

<table>
<thead>
<tr>
<th>PABA (mM)</th>
<th>Volume applied (mL)</th>
<th>PABA applied (mg)</th>
<th>Relative chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0</td>
<td>0.0</td>
<td>21.5 ab a</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>1.4</td>
<td>24.1 a</td>
</tr>
<tr>
<td>1.0</td>
<td>20</td>
<td>2.7</td>
<td>21.5 ab</td>
</tr>
<tr>
<td>1.0</td>
<td>40</td>
<td>5.5</td>
<td>19.7 b</td>
</tr>
<tr>
<td>4.5</td>
<td>10</td>
<td>6.2</td>
<td>21.6 ab</td>
</tr>
<tr>
<td>4.5</td>
<td>20</td>
<td>12.3</td>
<td>22.8 ab</td>
</tr>
<tr>
<td>4.5</td>
<td>40</td>
<td>24.7</td>
<td>21.0 ab</td>
</tr>
<tr>
<td>9.0</td>
<td>10</td>
<td>12.3</td>
<td>20.4 ab</td>
</tr>
<tr>
<td>9.0</td>
<td>20</td>
<td>24.7</td>
<td>21.1 ab</td>
</tr>
<tr>
<td>9.0</td>
<td>40</td>
<td>49.4</td>
<td>22.7 ab</td>
</tr>
</tbody>
</table>

$^a$ Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

$^b$ sem = standard error of the mean for all ls means in the same column.
Table A.2 Effect of different PABA concentrations on the relative chlorophyll content in tomato cv. H9909 inoculated with *P. syringae pv. tomato* (*Pst*). Plants were coated with 0, 0.01, 0.1, 0.5, 1, 4, 9, or 18 mM para-aminobenzoic acid (PABA) 10 and 15 days after seeding (DAS). Plants were coated with a fine mist of $2 \times 10^7$ CFU/ml of *Pst* 20 DAS. Relative chlorophyll was measured at five days post inoculation.

<table>
<thead>
<tr>
<th>PABA (mM)</th>
<th>Relative chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>24.8 a</td>
</tr>
<tr>
<td>0.01</td>
<td>24.8 a</td>
</tr>
<tr>
<td>0.05</td>
<td>25.8 a</td>
</tr>
<tr>
<td>1.00</td>
<td>24.5 a</td>
</tr>
<tr>
<td>4.00</td>
<td>24.6 a</td>
</tr>
<tr>
<td>9.00</td>
<td>25.3 a</td>
</tr>
<tr>
<td>18.00</td>
<td>25.9 a</td>
</tr>
</tbody>
</table>

| sem b     | 0.94                 |

a Means in the same column followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from two independent trials with five replications of each treatment was pooled together because ANOVA showed no treatment x trial interaction.

b sem = standard error of the mean for all ls means in the same column.
Table A.3 The incidence of bacterial speck and bacterial spot on green tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 green fruit harvested in a 2m section of each plot was evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial speck on fruit (%)</th>
<th>Bacterial spot on fruit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.0 a</td>
<td>8.8 a</td>
</tr>
<tr>
<td>UNI</td>
<td>5.3 a</td>
<td>9.5 a</td>
</tr>
<tr>
<td>ASM</td>
<td>2.7 a</td>
<td>10.5 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>5.3 a</td>
<td>11.0 a</td>
</tr>
<tr>
<td>sem b</td>
<td>1.15</td>
<td>2.99</td>
</tr>
</tbody>
</table>

*a* Means in the same column followed by the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD.

*b* sem = standard error of the mean for all ls means in the same column.
**Table A.4** Incidence and severity of anthracnose symptoms on red tomato fruit, cv. TSH4, harvested from plots treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit was collected from all red fruit harvested in a 2m section of each plot, stored for three days at room temperature, and then assessed for anthracnose symptoms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence (%)</th>
<th>DSI a</th>
<th>2013</th>
<th>2012</th>
<th>2011</th>
<th>2013</th>
<th>2012</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.7 a b</td>
<td>16.5 a</td>
<td>2.5 a</td>
<td>4.7 a</td>
<td>7.1 a</td>
<td>1.0 a</td>
<td>0.7 a</td>
<td>1.3 a</td>
</tr>
<tr>
<td>UNI</td>
<td>16.0 a</td>
<td>21.0 a</td>
<td>7.5 a</td>
<td>7.5 a</td>
<td>10.0 a</td>
<td>4.1 a</td>
<td>1.6 a</td>
<td>2.5 a</td>
</tr>
<tr>
<td>ASM</td>
<td>16.0 a</td>
<td>17.5 a</td>
<td>2.0 a</td>
<td>8.8 a</td>
<td>7.3 a</td>
<td>0.9 a</td>
<td>1.5 a</td>
<td>1.9 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>10.7 a</td>
<td>23.0 a</td>
<td>4.5 a</td>
<td>6.5 a</td>
<td>9.0 a</td>
<td>2.9 a</td>
<td>1.5 a</td>
<td>2.4 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sem c</td>
<td>5.28</td>
<td>9.53</td>
<td>2.24</td>
<td>3.66</td>
<td>4.96</td>
<td>1.62</td>
<td></td>
</tr>
</tbody>
</table>

*a DSI = disease severity index and was calculated using the equation the following equation: DSI = \( \sum \frac{\text{(class no.) (no. of fruit in each class)}}{\text{(total no. fruit per sample) (no. classes -1)}} \times 100. \) Fruit were sorted into the following classes: 0 = no lesions, 1 = one lesion, 2 = two to three lesions, 3 = four or more lesions.

*b Means in the same column followed by the same letter are not significantly different at \( P \leq 0.05 \), Tukey’s HSD.

*c sem = standard error of the mean for all ls means in the same column.
Table A.5 Number of days after transplanting to begin inflorescence, fruit set, and ripening for tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. Five plants per plot were monitored at seven to 12 day intervals after transplanting.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First inflorescence (^a)</th>
<th>First fruit set (^a)</th>
<th>First fruit ripening (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UNI</td>
<td>35 a</td>
<td>33 a</td>
<td>33 a</td>
</tr>
<tr>
<td>ASM</td>
<td>35 a</td>
<td>35 a</td>
<td>33 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>35 a</td>
<td>33 a</td>
<td>30 a</td>
</tr>
</tbody>
</table>

\(^a\) First inflorescence, fruit set, and fruit ripening were defined as plants with open flowers, visible fruit approximately 1 cm in diameter, and any pink or red colouration on fruit, not including fruit with blossom end rot.

\(^b\) Means in the same column followed by the same letter are not significantly different at \(P \leq 0.05\), Tukey’s HSD.

\(^c\) sem = standard error of the mean for all ls means in the same column.
Table A.6 The incidence of bacterial speck and bacterial spot on green tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 green fruit harvested in a 2m section of each plot was evaluated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bacterial speck on fruit (%)</th>
<th>Bacterial spot on fruit (%)</th>
<th>Bacterial speck on fruit (%)</th>
<th>Bacterial spot on fruit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.0 a</td>
<td>9.9 b</td>
<td>8.2 a</td>
<td>24.0 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>5.5 a</td>
<td>2.7 c</td>
<td>8.0 a</td>
<td>28.0 a</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>3.0 a</td>
<td>8.0 bc</td>
<td>10.5 a</td>
<td>28.5 a</td>
</tr>
<tr>
<td>ASM</td>
<td>3.5 a</td>
<td>17.5 a</td>
<td>10.5 a</td>
<td>23.5 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>4.0 a</td>
<td>11.0 b</td>
<td>4.3 a</td>
<td>35.5 a</td>
</tr>
<tr>
<td>UNI</td>
<td>5.5 a</td>
<td>NT</td>
<td>32.0 a</td>
<td>NT</td>
</tr>
<tr>
<td>sem b</td>
<td>1.65</td>
<td>1.52</td>
<td>2.63</td>
<td>4.17</td>
</tr>
</tbody>
</table>

*a* Means in the same column followed by the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD. *NT* = not tested.

*b* sem = standard error of the mean for all ls means in the same column.
Table A.7 Incidence and severity of anthracnose symptoms on red tomato fruit, cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. A subsample of 50 red fruit was collected from all fruit harvested in a 2m section of each plot, stored for three days at room temperature, and then assessed for anthracnose symptoms.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TSH4 Incidence (%)</th>
<th>H9909 Incidence (%)</th>
<th>DSI a</th>
<th>TSH4 Incidence (%)</th>
<th>H9909 Incidence (%)</th>
<th>DSI a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.5 a c</td>
<td>20.5 a</td>
<td>12.0 a</td>
<td>7.3 a</td>
<td>4.7 a</td>
<td>4.6 a</td>
</tr>
<tr>
<td>CuOH</td>
<td>8.0 a</td>
<td>12.5 a</td>
<td>12.5 a</td>
<td>3.8 a</td>
<td>5.3 a</td>
<td>4.6 a</td>
</tr>
<tr>
<td>CuOH + UNI</td>
<td>9.0 a</td>
<td>15.0 a</td>
<td>15.0 a</td>
<td>3.9 a</td>
<td>5.5 a</td>
<td>8.0 a</td>
</tr>
<tr>
<td>ASM</td>
<td>5.0 a</td>
<td>4.0 a</td>
<td>9.5 a</td>
<td>1.8 a</td>
<td>1.1 a</td>
<td>4.1 a</td>
</tr>
<tr>
<td>ASM + UNI</td>
<td>7.0 a</td>
<td>4.0 a</td>
<td>13.0 a</td>
<td>2.6 a</td>
<td>1.0 a</td>
<td>5.9 a</td>
</tr>
<tr>
<td>UNI</td>
<td>8.5 a</td>
<td>NT</td>
<td>NT</td>
<td>3.0 a</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>sem d</td>
<td>2.54</td>
<td>4.05</td>
<td>4.59</td>
<td>2.57</td>
<td>3.67</td>
<td>1.42</td>
</tr>
</tbody>
</table>

a DSI = disease severity index and was calculated using the equation following equation: DSI = \( \frac{\sum \text{[(class no.) (no. of fruit in each class)]}}{[(\text{total no. fruit per sample}) (\text{no. classes -1})]} \times 100. \) Fruit were sorted into the following classes: 0 = no lesions, 1 = one lesion, 2 = two to three lesions, 3 = four or more lesions.

b Data in this column were square root transformed to meet assumptions of ANOVA. The back-transformed means are presented here.

c Means in the same column followed by the same letter are not significantly different at \( P \leq 0.05, \) Tukey’s HSD.

d sem = standard error of the mean for all ls means in the same column, except for the DSI for treatment Control, TSH4, 2012 the sem is 1.68.
Table A.8 Number of days after transplanting to begin inflorescence, fruit set, and ripening for tomato cv. TSH4 and H9909, harvested from plots treated with CuOH, uniconazole (UNI) and acibenzolar-S-methyl (ASM) in the greenhouse and inoculated with *P. syringae* pv. *tomato* and *X. gardneri*, Ridgetown, ON, 2011-2013. Five plants per plot were monitored at seven to 12 day intervals after transplanting.

<table>
<thead>
<tr>
<th>Growth stage</th>
<th>Cultivar</th>
<th>Year</th>
<th>Control</th>
<th>CuOH + UNI</th>
<th>ASM + UNI</th>
<th>UNI</th>
<th>sem b</th>
</tr>
</thead>
<tbody>
<tr>
<td>First inflorescence</td>
<td>TSH4</td>
<td>2011</td>
<td>32 a</td>
<td>30 a</td>
<td>32 a</td>
<td>32 a</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>36 a</td>
<td>36 a</td>
<td>32 a</td>
<td>33 a</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>29 a</td>
<td>27 a</td>
<td>27 a</td>
<td>27 a</td>
<td>28 a</td>
</tr>
<tr>
<td></td>
<td>H9909</td>
<td>2011</td>
<td>32 a</td>
<td>31 a</td>
<td>28 a</td>
<td>33 a</td>
<td>30 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>35</td>
<td>33</td>
<td>35</td>
<td>35</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>27 a</td>
<td>27 a</td>
<td>27 a</td>
<td>28 a</td>
<td>26 a</td>
</tr>
<tr>
<td>First fruit set</td>
<td>TSH4</td>
<td>2011</td>
<td>41 a</td>
<td>39 a</td>
<td>38 a</td>
<td>41 a</td>
<td>39 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>37 a</td>
<td>36 a</td>
<td>35 a</td>
<td>38 a</td>
<td>37 a</td>
</tr>
<tr>
<td></td>
<td>H9909</td>
<td>2011</td>
<td>41 a</td>
<td>43 a</td>
<td>39 a</td>
<td>43 a</td>
<td>38 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>35 ab</td>
<td>35 ab</td>
<td>34 ab</td>
<td>36 a</td>
<td>35 ab</td>
</tr>
<tr>
<td>First fruit ripening</td>
<td>TSH4</td>
<td>2011</td>
<td>70 a</td>
<td>69 a</td>
<td>69 a</td>
<td>69 a</td>
<td>69 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>78 a</td>
<td>78 a</td>
<td>77 a</td>
<td>77 a</td>
<td>77 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>H9909</td>
<td>2011</td>
<td>71 a</td>
<td>69 a</td>
<td>69 a</td>
<td>71 a</td>
<td>69 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2012</td>
<td>77 a</td>
<td>78 a</td>
<td>78 a</td>
<td>78 a</td>
<td>78 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2013</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
<td>70</td>
</tr>
</tbody>
</table>

a First inflorescence, fruit set, and fruit ripening were defined as plants with open flowers, visible fruit approximately 1 cm in diameter, and any pink or red colouration on fruit, not including fruit with blossom end rot.

b sem = standard error of the mean for all ls means in the same row. NA = not analysed because all observations were the same.

c Means in the same row followed by the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD.
Figure A.1. Lesion size was determined by a) taking a photo (12.2 MB, 4288 x 2824 pixels) of each leaflet was taken using a Nikon D300s camera with a ruler for reference, b) uploading, enlarging and printing each photo in colour, c) tracing lesion circumference using a fine point marker on acetate, and d) scanning lesion circumference images and analyzing using Image J.
Figure A.2 Leaf counts and early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 treated with uniconazole (UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with \textit{P. syringae} pv. \textit{tomato} and \textit{X. gardneri}, Ridgetown, ON, in 2013. The a) total number of symptomless and symptomatic leaves, and b) \% of leaves with symptoms in the nontreated control (\arrowright), UNI (\rightarrow), ASM (\rightarrow), and ASM + UNI (\rightarrow) is shown for a 1.24 m² area. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. NS = no significant difference.
Figure A.3 Leaf counts and early season progress of bacterial spot and speck symptoms in tomato cv. TSH4 and H9909 treated with uniconazole.
(UNI) in the greenhouse and acibenzolar-S-methyl (ASM) in the field and inoculated with *P. syringae* *pv. tomato* and *X. gardneri*, Ridgetown, ON, in 2013. Figures represent a) the total number of symptomless and symptomatic leaves in cv. TSH4, b) the % of leaves with symptoms in cv. TSH4, c) the total number of symptomless and symptomatic leaves in cv. H9909, and d) the % of leaves with symptoms in cv. H9909, in the nontreated control (→), CuOH (←), CuOH + UNI (→), ASM (←), ASM + UNI (→), and UNI (→) treatments is shown for a 1.24 m² area. Error bars represent standard error of the mean. Data points on the same date with the same letter are not significantly different at *P* ≤ 0.05, Tukey’s HSD. NS = no significant difference.
Figure A.4 Standard dilution curves of a) R17 and b) R21 endophytes of A600 versus CFU/ml. A standard growth curve was developed for R17 and R21 by plating dilutions of the bacteria grown overnight in tryptic soy broth and then diluted in sterile distilled water to 0.2, 0.4, 0.6, 0.8, and 1.0 absorbance (OD = 600). Serial dilutions at each absorbance level were plated onto tryptic soy agar and counted at 48 hours after incubation at approximately 22°C. From this, a formula was developed for R17: population (CFU/mL) = [(9x10^7) (absorbance)] - 1x10^7 (R^2 = 0.93) and for R21: population (CFU/mL) = [(2x10^9) (absorbance)] - 2x10^8 (R^2 = 0.89). Values at 1.0 absorbance for R21 were omitted because using the values from 0.200 to 0.800 provided a curve with a higher R^2 value. Second values for R21 at 0.4 absorbance are missing due to missing plots because of plate contamination.
Table A.9 Response of commercial processing tomato cvs. H2401 (all variables) and H9553 (relative chlorophyll) for the effect of endophyte R17 treatment on the incidence of bacterial speck lesions, relative chlorophyll, plant height, dry root weight, and foliar dry weight. Plants were fertilized 10, 15, and 21 days after seeding (DAS) with 80 mL of 20-20-20+micronutrients fertilizer solution mixed at a concentration of 1.26 g/L. R17 was applied as a seed drench 0 DAS at $1 \times 10^7$ CFU/mL in 10 mM MgCl$_2$. Plants receiving 10 mM MgCl$_2$ were used as a control. Plants were inoculated with *P. syringae pv. tomato* at 20 DAS, and disease incidence assessed at five days post inoculation on all leaflets of the second and third youngest leaves.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Exp.</th>
<th>Disease incidence (lesions/cm$^2$)</th>
<th>Relative Chlorophyll</th>
<th>Height (cm)</th>
<th>Dry weight (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MgCl$_2$</td>
<td>R17- treated</td>
<td>sem</td>
<td>MgCl$_2$</td>
</tr>
<tr>
<td>H2401</td>
<td>1</td>
<td>4.7 a</td>
<td>2.7 b</td>
<td>0.41</td>
<td>27.4 a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.7 a</td>
<td>3.5 a</td>
<td>0.81</td>
<td>28.2 a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.8 a</td>
<td>3.4 a</td>
<td>0.29</td>
<td>28.0 a</td>
</tr>
<tr>
<td>H9553</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>26.7 a</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.3 b</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28.4 a</td>
</tr>
</tbody>
</table>

$^a$ sem = standard error of the mean for all ls means for the same group in the same row.

$^b$ Means in the same row and group followed by the same letter are not significantly different at $P \leq 0.05$, Tukey’s HSD. Data from different trials was not pooled together because ANOVA showed significant treatment x trial interactions, except for cv. H2401 where there was no interaction for relative chlorophyll or height. Missing variables for cv. H9553 is presented in Figure 4.1 because there were no significant treatment * trial interactions.