Copper tolerance in *Pseudomonas syringae* pv. *tomato* isolates from tomato crops in Eastern Australia.

by

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Thesis

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Supervisory panel: Philip Brown (Central Queensland University), Cherie Gambley (Queensland Department of Agriculture and Fisheries) and Yujuan Li (Central Queensland University)

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Abstract

Pseudomonas syringae pv. *tomato* causing bacterial speck disease in tomatoes is a significant threat to commercial field tomato production in most growing regions of Australia and globally. Infection of crops with this pathogen can cause significant reductions in fruit quality and yields. There are limited pesticide control options available for bacterial diseases in tomato, with copper-based bactericides currently one of the few registered products globally. The state of Queensland (QLD) in Australia produces approximately 69% of Australia's fresh market outdoor tomatoes estimated at a value of AUD$122 billion. *P. syringae* pv. *tomato* consistently threatens tomato production in QLD and other Eastern Australian states and many producers report copper products fail to adequately control disease progression. To date no studies have tested for copper tolerance in *P. syringae* pv. *tomato* in QLD Australia, despite reports of tolerance in many other countries. This study found that 100% of the *P. syringae* pv. *tomato* isolates collected were tolerant to copper and this tolerance was linked to the presence of *cop* genes in their genetic profiles. This is the first systematic study of copper tolerance prevalence in Eastern Australia, particularly QLD, and the first study analysing the genetic basis of copper tolerance in Australian *P. syringae* pv. *tomato*.

Published copper tolerance and copper efficacy studies on bacterial disease control report a wide range of response data, generated under varying field and laboratory conditions, making it difficult to draw strong conclusions from individual studies. Therefore, a systematic literature review was completed, investigating the prevalence of copper tolerance, the relative efficacy of copper for the control of disease and the identification of key emerging alternative products to copper for disease control. Results highlighted that copper tolerance is a global issue, which is affecting the usefulness of copper-based products for the control of bacterial diseases. A large range of alternative products for disease control were identified and the efficacy of eight key products were evaluated. However, there was a limited volume of published efficacy data available of for these alternative products, particularly for the control of disease caused by *P. syringae* pv. *tomato*.

The systematic literature review also identified inconsistencies with *in vitro* copper tolerance screening methodology for *P. syringae* pv. *tomato* in current literature, particularly in relation to the appropriate media to use, copper tolerance thresholds and inadequate reporting of media pH and/or pH adjustment steps. The effect of media and pH on copper tolerance results was therefore investigated, including the use of a pH buffering agent. Copper tolerance thresholds with different media were found to vary significantly and outside of a specific pH range, copper tolerance data was unreliable. A recommended methodology for copper tolerance screening was developed and published. This refined methodology was used to screen *P. syringae* pv. *tomato* isolates from a number of geographically distinct regions of QLD, New South Wales (NSW) and Victoria (VIC).

To date, no published studies are available on copper tolerance stability in *P. syringae* pv. *tomato.* Understanding the stability or biological fitness of copper tolerance in *P. syringae* pv. *tomato* can provide valuable insights into how copper-based disease control programs could be modified to mediate or even reduce the prevalence copper tolerance. Therefore, the stability of copper tolerance in study isolates was investigated through *in vivo* experiments. Findings suggested that copper tolerance may not be stable in all isolates when copper selection pressure is removed *in vivo.*

Despite a general consensus that plasmid *cop* genes are essential for copper tolerance in *P. syringae* pv. *tomato*, the gene and protein characterisation work undertaken to form these conclusions is solely based on isolates collected in America. Additionally, Australian *P. syringae* pv. *tomato* are yet to be genetically characterised. Polymerase Chain Reaction (PCR) assays and genomic analysis were used to explore the genetic basis of copper tolerance in Australian isolates, with a particular focus on the *cop* genes. Genetic analysis identified putative Cop-protein coding regions on a Cop operon and a CopA/B complex in Australian isolates. The analysis also suggested that the Cop operons may be located on either plasmid or chromosomal DNA, depending on the isolate studied. This study is the first detailed investigation of the genetic basis of tolerance in this species outside of America.

This study presents a range of novel findings which are of significance to both the scientific community and the agricultural industry. The presence of widespread copper tolerance has serious implications for commercial tomato producers. Bacterial disease management programs need to be revised to mediate resistance development and provide a more environmentally sustainable approach to crop production.

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DECLARATION OF AUTHORSHIP AND ORIGINALITY

I, the undersigned author, declare that all of the research and discussion presented in this thesis is original work performed by the author. No content of this thesis has been submitted or considered either in whole or in part, at any tertiary institute or university for a degree or any other category of award. I also declare that any material presented in this thesis performed by another person or institute has been referenced and listed in the reference section.

Karina Griffin

Date: 16 July 2018

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Karina Griffin

Date: 16 July 2018

Table of Contents

Tables and Figures

Publications and Presentations

Publications Relevant to the Thesis

All publications included in this thesis were primarily written by myself, I collected, analysed and collated all information within each. Each of the my supervisors, Philip Brown, Cherie Gambley and Yujuan Li, were involved in proof reading and refinement of sentence structure, however, their contributions varying between publications. Signed declarations outlining the contribution of supervisors and other authors is included within each Chapter where a publication is present.

Griffin, K., Gambley, C., Brown, P., & Li, Y. (2017). Copper tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomona*s spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review. *Crop Protection,* 96, 144-150. doi:10.1016/j.cropro.2017.02.008

Griffin, K, Brown, P & Gambley, C. (2018). Media pH and media type can significantly affect the reliability of *in vitro* copper tolerance assessments of *Pseudomonas syringae* pv. *tomato. Journal of Applied Microbiology*, 125, 216-226. doi: 10.1111/jam.13753

Griffin, K, Campbell, P. & Gambley, C. (2019). Genetic basis of copper tolerance in Australian *Pseudomonas syringae* pv. *tomato*. *Australasian Plant Pathology*. Online 12 Jun 2019.

Additional Publications Relevant to the Thesis but not Forming Part of it

Nature of Candidate Contribution: As the primary author I collected and analysed all data and wrote all the content in the publication listed below.

Nature of Co-authors Contributions: Each of the other authors listed were involved in proof reading and some refinement of sentence structure.

Griffin, K., Gambley, C., Brown, P., & Li, Y. 2016. *Are in vitro methods for determining copper tolerance in Pseudomonas syringae* pv. *tomato giving us the best result?* Poster presented at the V International Symposium on Tomato Diseases: Perspectives and Future Directions in Tomato Protection, Malaga Spain. International Society of Horticultural Science (ISHS).

Griffin, K., Brown, P & Gambley, C. 2017. *Copper tolerance in Pseudomonas syringae* pv. *tomato from tomatoes in Queensland, Australia.* Oral presentation at the Science Protecting Plant Health 2017 Conference, Brisbane, Australia. Australasian Plant Pathology Society (APPS) and CRC Plant Biosecurity.

Griffin, K & Gambley, C 2016, *Review Of Alternative Treatments For Bacterial Spot And Speck Control*. Short document and presentation at Department of Agriculture and Fisheries Tomato Grower Information Session Stanthorpe QLD. Document also distributed to tomato producers and industry representatives not present at the session.

Griffin, K 2018, *Research Summary: Copper tolerance in Pseudomonas syringae* pv. *tomato causing bacterial speck disease in tomatoes*. Distributed to tomato producers and industry representatives.

The following publication was produced as a grower information factsheet by Cherie Gambley and Clinton McGrath for the Queensland Department of Agriculture and Fisheries. I contributed to this factsheet by adding some additional information and proofreading.

Gambley, C., McGrath, C. & Griffin, K. 2016 *Is copper copper? Limitations in the control of foliar bacterial diseases in capsicum, chilli and tomato crops*. Factsheet Queensland Department of Agriculture and Fisheries.

Abbreviations

aa	Amino acid
ANOVA	Analysis of variance
ASCT	Agricultural Scientific Collections Trust
BA	Publication on a biological or alternative product to copper
BBCH	Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie
BOM	Australian Bureau of Meteorology
bp	Base-pair
CE	Copper efficacy publication
cfl	*C*orono*f*acate *l*igase
CFU	Colony Forming Units
cop	*Cop*per resistance/tolerance genes
CuOH	Copper hydroxide
CuOx	Copper oxide
CuOxy	Copper oxychloride
CR	Copper resistance or tolerance publication
CuSO	Copper sulfate
CV	Coefficient of Variation
cv.	Cultivar
CYE	Casitone Yeast Extract Glucose Agar
CYEG	Casitone Yeast Extract Glycerol Agar
DAI	Days After Inoculation
DAT	Days After Transplanting
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphates

EBDC	Ethylene bis-dithiocarbamates
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium Bromide
g ai/kg	Grams active ingredient per kilogram of product
g ai/L	Grams active ingredient per litre of product
gapA	*G*lycer*a*ldehyde-3-*p*hosphate dehydrogenase
gltA (cts)	Citrate synthase
GNA	Glucose Nutrient Agar
gyrB	DNA *gyr*ase *B*
HKY	Hasegawa-Kishino-Yano substitution model
$hrpZ_{Pst}$	*H*ypersensitivity *r*esponse and *p*athogenicity-associated *Z* gene
HSD	Tukey's Honestly Significance Difference
JC69	Jukes-Cantor, 69 substitution model
K80	Kimura 2-parameter substitution model
KB	King's B medium
KOH	Potassium hydroxide
MaxLH	Maximum Likelihood
MBMM	2-(N-morpholino)ethanesulfonic acid buffered minimal medium
MES	2-(N-morpholino)ethanesulfonic acid
MGY	Mannitol Glutamate Yeast Extract
MIC	Minimum Inhibitory Concentration
MLST	Multilocus Sequence Typing
MMCC	Copper-Complexing Minimal Medium, also known as CYEG
MOPS	3-N-morpholinopropanesulfonic acid
MSError	Mean Square Error

NA	Nutrient Agar
N/A	Not applicable or not available
NCBI	National Center for Biotechnology Information
NG	No Growth
NGS	Next Generation Sequencing
NS	No Sequence
NSW	New South Wales
NT	Not Tested
nt	Nucleotide
NYGA	Nutrient Yeast Extract Glycerol Agar
OD	Optical Density
PAMDB	Plant Associated and Environmental Microbes Database
PCR	Polymerase Chain Reaction
Pcop	plasmid-borne cop promoter
PcopH	chromosomal *cop* homolog promoter
PDA	Potato Dextrose Agar
PG	Phylogroup
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	Pathovar
QLD	Queensland
QLD DAF	Queensland Department of Agriculture and Fisheries
rpoD	Sigma factor 70 proteins
RTP	Research Training Program
SPA	Sucrose Peptone Agar
spp.	Species plurales (relating to many species)

Spr	Spring
Sum	Summer
T_A	Annealing temperature
TBE	Tris/Borate/ Ethylenediaminetetraacetic acid buffer
USA	United States of America
UTC	Untreated Control
VIC	Victoria
YDC	Yeast Dextrose Carbonate Agar

Chapter 1 - Introduction

1.1 Research Overview

Tomatoes (*Solanum lycopersicum* L.) are an important vegetable crop and are grown commercially in most countries of the world. Protecting the crop from pest and disease damage is critical for successful commercial production. Members of the *Pseudomonas syringae* species complex are generally pathogenic and cause disease in most crop types from grains and vegetables to tree crops (Lamichhane *et al.* 2015). Worryingly, reports of disease outbreaks caused by *P. syringae* are increasing in frequency (Lamichhane *et al.* 2015). Climate change is also contributing to a significant shift in the prevalence of plant diseases, with some areas likely to get higher disease pressure as this shift occurs (Juroszek & Von Tiedemann 2011).

Tomatoes are susceptible to several different bacterial diseases. Of these *P. syringae* pv. *tomato* (Young *et al.* 1978), causing bacterial speck, and *Xanthomonas* spp., causing bacterial spot, are of importance in outdoor tomato production. Prior to 2004, the *Xanthomonas* species causing bacterial spot were generally identified as *X. campestris* pv. *vesicatoria* (Jones *et al.* 2004; Potnis *et al.* 2015). It is now known there are five distinct *Xanthomonas* species causing disease in tomato and pepper, *X. euvesicatoria*, *X. perforans*; *X. gardneri*, *X. vesicatoria* and *X. arboricola* (Jones *et al.* 2004; Mbega *et al.* 2015; Vauterin *et al.* 1995). In this study, for simplicity, they are collectively referred to as *Xanthomonas* spp. Symptoms of bacterial speck and spot disease are very difficult to separate visually, with both being characterised by the formation of dark lesions or spotting on the plant foliage (Miller & Jones 2014) (Figure 1-1). Lesions can also form on fruit, reducing quality and providing infection points for secondary pathogens such as rots. Plant stems and flowers are also susceptible to infection and lesion development.

Figure 1-1: Symptoms of bacterial speck disease on tomato foliage and fruit caused by infection with *Pseudomonas syringae* pv. *tomato*.

In moderate to severe infections bacterial speck and spot can cause defoliation, reducing photosynthetic area and consequently the crop production potential (Miller & Jones 2014). The earlier that infection takes places the more likely that the disease will cause significant fruit quality and yield reductions. *P. syringae* pv. *tomato* infection of tomato seedlings one month after transplanting had significantly greater fruit yield and quality reductions compared to infection two and three months after transplanting (Yunis *et al.* 1980). Similarly, *X. vesicatoria* infection of mature tomato plants one month prior to harvest (approximately two months after transplanting) resulted in 75% of plant foliage being affected and significant yield reduction, whereas infection two weeks prior to harvest resulted in only 35% of foliage affected with no significant yield reductions (Dougherty 1978). Time of infection also affects lesion development on fruit, with only green fruit being susceptible, this is attributed to the drop in pH on the fruit surface as it ripens which supresses infection (Yunis *et al.* 1980).

Given the similarities in infection symptoms of these diseases, primary producers will often refer to them collectively as bacterial leaf spot, and the causative species is often not diagnosed. *Xanthomonas* spp. and *P. syringae* pv. *tomato* can occur together, however, *P. syringae* pv. *tomato* was shown to proliferate more rapidly than *Xanthomonas* in mixed inoculations *in vitro* and *in vivo* (Shenge, Mabagala*, et al.* 2008). No survey data is reported on the relative prevalence of *Xanthomonas* spp. and *P. syringae* pv. *tomato* in commercial tomato cropping regions of Australia. However, anecdotal evidence indicates that *Xanthomonas* spp. is predominantly in the northern dry tropic growing regions of Bowen, whilst both *Xanthomonas* spp. and *P. syringae* pv. *tomato* have been identified in the subtropical and temperate climate regions of QLD such as Bundaberg, Gatton and the Granite Belt regions (pers. comm. Cherie Gambley, Chris Monsour). *P. syringae* pv. *tomato* is reported to be more prevalent in cooler regions, particularly in southern Australian production areas around Echuca in Victoria (VIC), where tomatoes are grown under field conditions (pers. comm. Ann Morrison). The climatic conditions and geographical location of these regions and the physiological state of the plants are likely to contribute to the prevalence of *Xanthomonas* spp. or *P. syringae* pv. *tomato* infection (Yunis *et al.* 1980).

The optimum conditions for *P. syringae* pv. *tomato* are temperatures ranging from 18 to 24 °C, whereas *Xanthomonas* spp. thrive in warmer temperatures from 24 to 30 °C (Miller & Jones 2014). Both *Xanthomonas* spp. and *P. syringae* pv. *tomato* spread with water in the form of fog, dew, hail, rain and irrigation (Pietrarelli *et al.* 2006), as well as high relative humidity (Yunis *et al.* 1980). Infected seed and crop debris are a major source of inoculum for infecting crops, volunteer plants and contaminated equipment can also carry the bacteria (Lamichhane *et al.* 2015; Miller & Jones 2014). The tomato growing season in the dry tropics (e.g. Bowen district) is over autumn and winter with hot temperatures more conducive to *Xanthomonas* spp. at the beginning of the season, whilst in

temperate areas (e.g. Granite belt district) the growing season is over spring and summer with cooler temperatures more conducive to *P. syringae* pv. *tomato* at the beginning of the season (Figure 1-2).

Bacterial diseases consistently threaten tomato cropping systems in QLD, with climatic conditions conducive to disease spread, low rotation farming systems and year round production in some areas. There are limited pesticide control options available for bacterial diseases in tomato, with copper based bactericides currently one of the few registered products globally. There are also currently no commercially available cultivars that carry resistance to *P. syringae* pv. *tomato* suitable for QLD conditions. Recently in Australia, one alternative product to copper, based on a strain of *Bacillus subtilis,* was registered for suppression of bacterial diseases in tomato. This product it is not yet widely used but is expected to be incorporated into disease management programs. In many bacterial disease control programs for tomatoes, copper bactericides are used regularly with the first application generally going on within 7 days of transplanting and subsequent applications every 7 days, on average, for almost the entire life cycle of the crop which is 12-14 weeks for field grown tomatoes. The application interval can vary from 5 to 10 days depending on localised disease pressure and farm management plans. Copper bactericides are also used extensively in agricultural production to control a range of bacterial and fungal diseases in many crop types. These intensive usage patterns of copper products are highly conducive to the development of copper pesticide resistance or tolerance in bacterial species. Hence, it is not surprising that copper-tolerant strains of *Xanthomonas* spp. and *P. syringae* pv. *tomato* from tomato crops have been identified globally (e.g. Pernezny *et al.* 1995; Shenge, Wydra*, et al.* 2008). Copper-tolerant strains of *Xanthomonas* spp. affecting pepper cropping systems have also been reported (e.g. Mirik *et al.* 2007).

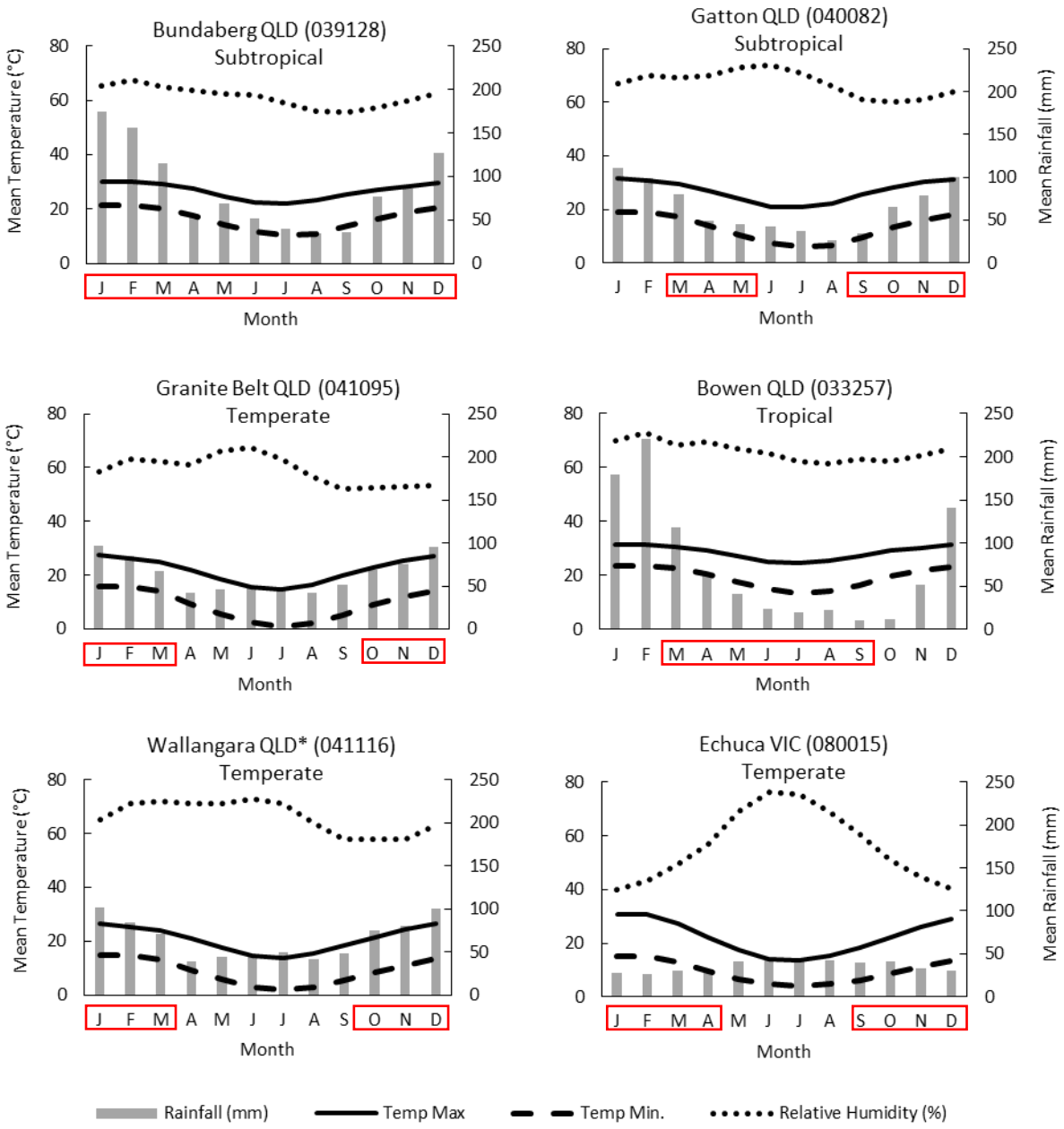

Figure 1-2: Average climatic conditions of field tomato growing regions in the Eastern States of Australia. Boxed regions around the months indicate the normal tomato growing season for that region. Information obtained from the Australian Bureau of Meteorology (BOM), climate data statistics online (BOM n.d, continuous), mean values recorded from at least the last 30 years. Relative humidity values align to the left hand y-axis. The BOM weather station site identifier is included in brackets after each region name, the climate zone of each region is also included. *Note: Wallangarra was the closest BOM data recording station to the New England NSW sampling site.

Despite evidence for copper tolerance in *Xanthomonas* spp. and *P. syringae* pv. *tomato* of tomatoes, only three studies have reported on copper tolerance in these species in Australia over the last 20 years (Hall *et al.* 2011; Martin *et al.* 2004; Tesoriero *et al.* 1997). None of these studies report on tolerance in *P. syringae* pv. *tomato* isolates from QLD. Agricultural commodities estimates by the Australian Bureau of Statistics (ABS 2017) state that QLD produced approximately 69% of Australia's fresh market outdoor tomatoes with close to 64,000 tonnes from 1,634 ha, worth a gross value of $122 billion (2015-16 financial year). Hence, copper tolerance in *P. syringae* pv. *tomato* affecting QLD tomato production is of significant importance to the industry.

Given the potential yield losses caused by *P. syringae* pv. *tomato,* understanding the prevalence and mechanisms of copper tolerance is vital, especially as the disease control options are limited. A greater understanding of copper tolerance in Australia has the potential to inform more effective and robust bacterial disease management strategies for primary producers. Factors explored in this research, including *in vitro* copper tolerance screening methodology and the genetic basis of copper tolerance, also have wide reaching applications to the global scientific community.

1.2 Research Objectives

This thesis investigated copper tolerance in *P. syringae* pv. *tomato* from outdoor or field produced fresh market tomatoes. The key research questions of this study were:

- Is there evidence in published literature that demonstrates the prevalence of copper tolerance in *P. syringae* pv. *tomato* and *Xanthomonas* spp. globally?
- How does the presence of copper tolerance affect the efficacy of copper-based products for bacterial disease control?
- What are the key emerging alternative products to copper for the control of bacterial diseases and how effective are they?
- Are current *in vitro* screening methods reliable for identifying copper tolerance in *P. syringae* pv. *tomato*?
- Is there copper tolerance in *P. syringae* pv. *tomato* from Eastern Australian tomato production regions and how widespread is this tolerance?
- Is copper tolerance in *P. syringae* pv. *tomato* stable or unstable when selection pressure from the use of copper bactericides is removed?
- Is the genetic basis of copper tolerance analogous to systems previously characterised in American isolates?

1.3 Thesis Structure

This is a thesis investigates copper tolerance in *P. syringae* pv. *tomato* collected from commercial tomato properties in Eastern QLD between 2015 and 2017.

Chapter 2 is a systematic literature review, providing a background to the global prevalence of copper tolerance and how this affects the efficacy of copper based products for the control of bacterial spot and speck disease of tomato, caused by *Xanthomonas* spp. and *P. syringae* pv. *tomato*, respectively. *Xanthomonas* spp. of both tomatoes and peppers were included in the analysis, given their similarity in proliferation and control to *P. syringae* pv. *tomato*. The inclusion of these provided a larger volume of studies to compare, as fewer publications are available on *P. syringae* pv. *tomato* relative to those examining *Xanthomonas* spp. Copper tolerance screening methodology between these species are also similar. Alternatives products for bacterial spot and speck disease control were also identified in this review. A review of literature relevant to content in Chapters 4-6 is also included in the introduction and discussion of these chapters.

Chapter 3 describes the general materials and methods used in the study. This chapter provides supplementary detail of the standard methodology used in the thesis, which is not included in other chapters of the thesis. It also provides details on preliminary method development required to optimise some of the experimental processes.

Through the analysis of papers for the systematic literature review (Chapter 2), *in vitro* methodology for the determination of copper tolerance was compared to gather the information necessary to develop a method to be used in this study. This comparison highlighted some aspects of the methodology that were inconsistent across the literature and factors that were not recorded even though they could significantly affect the results obtained. The major contributors to the accuracy of published copper tolerance results were the media type used and the pH of that media. Chapter 4 is a published article investigating these factors on *in vitro* copper tolerance assessments of *P. syringae* pv. *tomato*. This part of the thesis study also involved *in vivo* testing to confirm the copper tolerance identified *in vitro*.

Once the methodology for the *in vitro* copper tolerance testing for *P. syringae* pv. *tomato* was finalised (Chapter 4), all study isolates were screened for copper tolerance *in vitro* (Chapter 5). The stability of this tolerance was then explored through *in vivo* experiments to determine if copper tolerance levels changed if selection pressure was removed (Chapter 5). Study isolates were collected from a range of commercial outdoor tomato crops between 2015 and 2017. *P. syringae* isolates provided by the Plant Pathology Herbarium of Biosecurity Queensland Department of Agriculture and Fisheries (QLD DAF), collected from 1970-80, were also screened to determine whether copper tolerance was present in

the past. The Bundaberg and the Granite Belt regions of QLD were the main areas sampled, with a small number of isolates from the Gatton region of QLD, New England region of New South Wales (NSW) and Echuca region of VIC.

Literature suggests that copper tolerance in *P. syringae* pv. *tomato* is mediated by the copper detoxifying activity of a group of proteins encoded by the copper metabolising (*cop*) genes, which are present on plasmid deoxyribonucleic acid (DNA; Bender & Cooksey 1987,1987). Given the high incidence of copper tolerance identified in study isolates (Chapter 5), it was hypothesised that isolates were also carrying Cop proteins. Therefore, the genetic bases of copper tolerance in Australian *P. syringae* pv. *tomato* was investigated through Polymerase Chain Reaction (PCR) assays and next generation sequencing (NGS) analysis (Chapter 6).

The final chapter of this thesis, Chapter 7, is a discussion integrating the most significant findings of each thesis chapter of this study, presenting prospective future research and the overall conclusions.

Chapter 2 - Systematic Literature Review

2.1 Overview

This chapter is a published systematic literature review, and the article is included in its entirety. The only change that has been made is the numbering of tables so that they conform with the rest of the thesis.

This article provides background information on the global prevalence of copper tolerance and how this affects the efficacy of copper based products for the control of bacterial spot and speck disease of tomato, caused by *Xanthomonas* spp. and *P. syringae* pv. *tomato*, respectively. *Xanthomonas* spp. of both tomatoes and peppers were included in the analysis, given their similarity in proliferation and control to *P. syringae* pv. *tomato*. The inclusion of these provided a larger volume of studies to compare, as fewer publications are available on *P. syringae* pv. *tomato* relative to those examining *Xanthomonas* spp. Copper tolerance screening methodology between these species were also very similar. Alternatives products for bacterial spot and speck disease control were also identified and discussed in this review.

Bibliographic Information

Griffin, K., Gambley, C., Brown, P., & Li, Y. (2017). Copper tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomona*s spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review. *Crop Protection, 96*, 144-150. doi:10.1016/j.cropro.2017.02.008

This article is no longer within its embargo period.

Declaration of Authorship

Title of Paper: Copper tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomonas* spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review

Status: Published 10 February 2017

Nature of Candidate Contribution: As the primary author I collected and analysed all data and wrote all the content.

Nature of Co-authors Contributions: Each of the other authors listed were involved in proof reading and refinement of sentence structure.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Karina Griffin

Date: 16 July 2018

Copper tolerance in *Pseudomonas syringae* pv. *tomato* and *Xanthomonas* spp. and the control of diseases associated with these pathogens in tomato and pepper. A systematic literature review

2.2 Abstract

A large body of literature exists on the use, efficacy and pathogen resistance associated with the application of copper-based products to control bacterial diseases of Solanaceous crops. In particular, the bacterial pathogens *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al*. which cause bacterial speck in tomato (*Solanum lycopersicum*) and multiple *Xanthomonas* spp. which cause bacterial spot in tomato and pepper (*Capsicum annum*). There is also an increasing number of studies reporting on alternative products to copper, either biological or chemical, for control of the diseases caused by these pathogens. This systematic literature review provides a synthesis of the published data reporting on the prevalence of copper tolerance, how this affects the efficacy of copper-based bactericides and key emerging alternative products to copper for disease control. A total of 133 publications were analysed within these three areas. Of all the *P. syringae* pv. *tomato* and *Xanthomonas* spp. isolates screened, 78.5% and 54.0% respectively, were identified as copper-tolerant. However, the methods used for in vitro screening of the isolates varied between studies, with evidence that differences in methodology may affect test results. In studies on efficacy of copper products when applied alone, rather than in a mixture with other compounds, only 38% reported highly effective control of bacterial speck and spot. The remaining studies reported variable, weak or no control. Even though the bacteria present weren't specifically screened for copper tolerance in the majority of these studies, the efficacy data suggest tolerance was present. Around 100 different alternative products to copper for control of *P. syringae* pv. *tomato* and *Xanthomo*nas spp. were identified in the literature. Of these, acibenzolar-s-methyl, *Pseudomonas fluorescens*, *Bacillus subtilis*, bacteriophages, *Pseudomonas syringae* Cit7 and the antibiotics streptomycin, oxytetracycline and kasugamycin were the most studied, efficacy data for these products from the analysed publications are reported.

2.3 Introduction

The plant diseases bacterial speck (caused by *Pseudomonas syringae* pv. *tomato* (Okabe) Young *et al*. and bacterial spot (caused by *Xanthomonas* spp.) can cause significant reductions in field crop yields of tomato (*Solanum lycopersicum*) and pepper (*Capsicum annum)*. *P. syringae* pv. *tomato* and *Xanthomonas spp.* can cause dark lesions on the foliage and fruit of plants (Miller and Jones, 2014). In moderate to severe infections, lesions coalesce and cause leaf death, reducing photosynthetic capacity, and consequently crop yield. Control of *P. syringae* pv. *tomato* and *Xanthomonas* spp. can be challenging with a limited range of chemical control options or resistant cultivars available.

Currently, chemical control predominantly relies on copper-based bactericides, which are used in both non-organic and organic agriculture production systems. Copper-tolerant populations of *P. syringae* pv. *tomato* and *Xanthomonas spp.* are identified worldwide (e.g. Marco and Stall, 1983; Martin *et al.*, 2004; Pernezny *et al.*, 1995; Shenge, Wydra *et al.*, 2008), leading to interest in alternative control strategies. There is a growing body of data on biological and other alternative products for bacterial speck and spot control, with some now produced and registered commercially (Byrne *et al.*, 2005). However, continued use of copper-based products is likely, due to its low cost and widespread availability.

Copper tolerance in *P. syringae* pv. *tomato* is mediated by the pPT23D plasmid which carries the six genes, *cop*A*,-B,-C,-D,-R* and *-S* (Mellano and Cooksey, 1988a; Mellano and Cooksey, 1988b; Mills *et al.*, 1993). The *cop* genes encode proteins that are proposed to sense, bind and transport copper ions, resulting in their detoxification (Bondarczuk and Piotrowska-Seget, 2013; Puig and Thiele, 2002). Genes involved in copper tolerance in *Xanthomonas* spp. are not as well defined as those of *P. syringae* pv. *tomato* (Basim *et al.*, 2005). Plasmids carrying copper tolerance in *Xanthomonas* spp. were identified (Bender *et al.,* 1990; Cooksey *et al.*, 1990; Stall *et al*., 1986), but their size and restriction enzyme profiles vary (Voloudakis *et al.*, 1993). Variation in plasmid identity and presence could be attributed to the different *Xanthomonas* species that cause bacterial spot symptoms in tomato and pepper. Prior to 2004, the pathogens causing these diseases were generally identified as *X. campestris* pv. *vesicatoria* (Jones *et al.*, 2004; Potnis *et al.*, 2015; Vauterin *et al.,* 1995). It is now known there are five distinct *Xanthomonas* species causing disease in tomato and pepper. These are *X. euvesicatoria* Jones *et al*., *X. perforans* Jones *et al*.; *X. gardneri* Jones *et al*., *X. vesicatoria* Vauterin *et al*. and *X. arboricola* Vauterin *et al*. 1995 (Jones *et al.*, 2004; Mbega *et al.,* 2015; Vauterin *et al.,* 1995). The specific plasmid profiles of these five species, and individual races or populations within each species, are largely unknown.

Over the last 25 years an increasing number of publications have reported copper tolerance in *P. syringae* pv. *tomato and Xanthomonas* spp. and reduced efficacy of copper-based products for controlling these pathogens. Published copper tolerance studies and product efficacy studies on bacterial disease control report a wide range of response data, generated under varying field and laboratory conditions, making it difficult to draw strong conclusions from individual studies. This systematic literature review aims to provide a synthesis of published data on the prevalence of copper tolerance and how this relates to the efficacy of copper-based bactericides. Managing copper tolerance in *P. syringae* pv. *tomato and Xanthomonas* spp. will increasingly rely on emerging alternative products, either biological or chemical. Therefore, this review also aims to identify some

key emerging products and discuss how their efficacy compares to that of copper-based bactericides.

2.4 Methods

The review strategy adopted in this study was based on steps outlined by Pickering and Byrne (2014). Briefly, selected databases were searched using specific key words to generate a publication list which was then screened for relevance using the eligibility criteria detailed below. Relevant data were then extracted and analysed.

2.4.1 Eligibility criteria

Literature was confined to studies examining either: 1) copper tolerance and/or resistance of *P. syringae* pv. *tomato* or *Xanthomonas* spp. from tomato or pepper (denoted CR); 2) efficacy of copper-based compounds for the control of disease caused by *P. syringae* pv. *tomato* or *Xanthomonas* spp. in tomato and/or pepper (denoted CE); or 3) efficacy of alternative compounds for the control of disease caused by *P. syringae* pv. *tomato* or *Xanthomonas* spp. in tomato and/or pepper (denoted BA).

Selection was mostly limited to studies published in the English language. Only publications reporting quantitative data on copper tolerance and product efficacy were selected. Literature reviews and book chapters/sections were excluded. Any publications presenting results previously published were also excluded. There was no limit on the age of publications. Literature searching was completed by March 2016. If publications analysed multiple bacterial species, subspecies or pathovars from a number of plant families, only information relevant to *P. syringae* pv. *tomato* or *Xanthomonas* spp. of tomato and pepper was recorded.

2.4.2 Literature search strategy

The American Phytopathological Society (APS) Journals database, Science Direct and Scopus were the primary databases used to identify publications. CAB Abstracts and Google Scholar were used for citation searches. Database searches were done in two parts: Part One to find copper tolerance/resistance studies[1] and Part Two to find studies on the efficacy of copper and/or other alternatives for disease control. In Part Two, three different search terms were used to target copper[2], alternative products[3] and biologicals[4].

[1] copper toleran* OR copper resist* AND “Pseudomonas syringae pv. tomato” OR Xanthomonas AND plant* AND tomato OR pepper
[2] control AND copper AND “Pseudomonas syringae pv. tomato” OR Xanthomonas AND plant* AND tomato OR pepper
[3] control AND “Pseudomonas syringae pv. tomato” OR Xanthomonas AND plant* AND tomato OR pepper
[4] biological control AND “Pseudomonas syringae pv. tomato” OR Xanthomonas AND plant* AND tomato OR pepper

Publications from the searches were screened to remove duplicates. Upon review of abstracts any publications that did not fulfil the eligibility criteria were excluded from further analysis. Full text documents were searched for key words including; copper, resistance, tolerance, control, biological, tomato, pepper, *Pseudomonas syringae* and *Xanthomonas*. The full text was then read in detail to ensure all eligibility criteria were met and to categorise them into the three research areas (CR, CE and/or BA). The reference lists of 40 publications were reviewed and citation searches were completed to identify additional relevant publications.

2.4.3 Data extraction and analysis

After applying the eligibility criteria to search results, a total of 133 publications were identified (Appendix A). Relevant information was extracted and collated into three key areas 1) general information, 2) copper tolerance screening method and key findings and 3) efficacy of copper and/or alternatives for disease control (Table 2-1).

Table 2-1: Information extracted and collated from publications analysed in the present study.

General Information	**Copper tolerance screening method**	**Efficacy of copper and/or alternatives for disease control**
Author/s	Number of isolates screened	Testing situation i.e. *in vitro* (generally agar well diffusion assay) or *in vivo* (plant growth chamber, greenhouse or field)
Title	Experiment type (*in vitro* and/or *in vivo*)	
Year	Media type for *in vitro* testing	
Continent and country of study	Media pH	Treatments tested
Target crop	Type of copper used	Treatment performance
Bacterial species	Copper concentrations* used and how these were translated into definitions of copper tolerance	If inoculated, the copper tolerance status of bacterial strains
Research category (CR, CE and/or BA)	Positive controls	
	Percentage of tolerant isolates identified	

*Concentration units varied between studies, therefore all were converted to millimolar (mmol/L).

2.5 Results and Discussion

2.5.1 Prevalence of copper tolerance in *P. syringae* pv. *tomato* and *Xanthomonas* spp.

Forty-two publications investigated copper tolerance in *P. syringae* pv. *tomato* and *Xanthomonas* spp., with three publications presenting data on both. Only 10 studies focused on *P. syringae* pv. *tomato* with a total of 358 isolates screened for copper tolerance, the remaining studies investigated *Xanthomonas* spp., with 7938 isolates screened. The majority of isolates screened were from the American continent (Table 2-2). A large proportion of these were from the Caribbean, due to a large surveys completed by Ward and O'Garro (1992) and O'Garro (1998).

Table 2-2: Total number of isolates screened for copper tolerance in *Xanthomonas* spp. and *Pseudomonas syringae* pv. tomato within each continent, and the countries in which isolates were collected.

Continent/Country	*Xanthomonas* spp.	*P. syringae* pv. *tomato*
America		
Brazil	509	119
Canada	98	13
Caribbean	5660	0
Costa Rica, Guatemala & Nicaragua	124	0
Mexico	104	0
United States of America	938	24
Total[A]	*7433*	*156*
Europe		
Czech & Slovak Republic	11	80
Italy	38	0
Macedonia	90	0
Serbia	130	0
Total	*269*	*80*
Africa		
Ethiopia	64	0
Tanzania	30	56
Total	*94*	*56*
Australia		
Total	*75*	*64*
Asia		
Japan	0	2
Turkey	67	0
Total	*67*	*2*
Grand Total	*7938*	*358*

[A] = total refers to the total number of isolates screened on each continent.

Of all the *P. syringae* pv. *tomato* isolates screened 78.5% were identified as being copper-tolerant, and the incidence of copper tolerance within each study was greater than 50% of isolates. Of all the *Xanthomonas* spp. isolates screened, 54.0% were identified as being copper-tolerant and the

incidence of copper tolerance varied from 1-100%. Nine *Xanthomonas* spp. studies reported a tolerance incidence of 1-50% and five studies found only copper-sensitive isolates. A number of factors could contribute to the higher proportion and level of copper tolerance in *P. syringae* pv. *tomato,* such as crop management and rotation practices, and differences in the relative ability of *P. syringae* pv. *tomato* and *Xanthomonas* spp. to develop copper tolerance. Further investigation into these factors is warranted and the number of *P. syringae* pv. *tomato* screened for tolerance should be increased. When analysed at a regional level, no significant trends in the prevalence of copper tolerance were identified between continents or countries.

Whilst the trends highlighted above provide an interesting insight into the global prevalence of copper tolerance, Hasman *et al.* (2009) demonstrated that for *in vitro* screening, unless both the type of media and its pH is the same between studies there is limited scope for the direct comparison of copper tolerance levels. Of the studies reviewed, 93% use an *in vitro* technique with copper-amended agar media to screen bacterial isolates for copper tolerance. However, no one medium was used in these studies (Table 2-3). Additionally, 74% of studies do not report the pH of the media. Different ingredients and the pH of agar medium influence its capacity to complex copper, hence changing the availability of copper ions to react with the bacteria (Hasman *et al.*, 2009; Zevenhuizen *et al.*, 1979). The variability in copper tolerance results using different medium was demonstrated in *P. syringae* pv. *tomato* by Silva and Lopes (1995) and in *Xanthomonas* spp. of tomato and pepper by Mixon (2012) and Pernezny *et al.* (2008). Mixon (2012) also showed that *in vitro* copper tolerance of *Xanthomonas* spp. varied significantly within the narrow pH range of 6.2-7.0.

Table 2-3: Types of media used for in vitro screening of copper tolerance in P. syringae pv. tomato and Xanthomonas spp. of tomato and pepper in published studies that use copper-amended agar media plates. The number of studies using each media type is listed separately for the two pathogens.

Media type	***P. syringae* pv. *tomato* studies**	***Xanthomonas* spp. studies**
Mannitol glutamate yeast extract (MGY)	4	4
Casitone yeast extract glycerol agar (CYEG)	2	1
Potato dextrose agar (PDA)	1	0
Nutrient agar (NA)	0	11
Glucose nutrient agar (GNA)	1	2
King's B medium (KB)	1	0
Nutrient yeast extract glycerol agar (NYGA)	0	1
Sucrose peptone agar (SPA)	0	11
*KB, NA and CYEG[1]	1	0
*Casitone yeast extract glucose agar (CYE) and GNA[2]	0	1

*More than one media type used within a single study: [1] Silva and Lopes, 1995 and [2] = Pernezny *et al*., 2008

Given the variability in media type used and inconsistent reporting of media pH in studies documenting copper tolerance screening *in vitro*, the use of positive controls is desirable. Ideally, these would be reference isolates known to be copper-tolerant or sensitive, both *in vitro* and *in vivo*. However, of all 38 studies reporting on agar plate based copper tolerance screening, 23 studies reporting only *in vitro* results did not use positive controls. Whilst most of these studies used methods based on those developed with a positive control, there were differences in methodology that likely affected the copper tolerance results. For example, the study by Shenge, Wydra *et al.* (2008) cited methodology by Gore and O'Garro (1999), but used King' B medium (KB) in place of the nutrient agar (NA) used by Gore and O'Garro (1999). Silva and Lopes (1995) reported that more isolates grew on KB compared to NA at the same concentration of copper sulphate, suggesting that the copper tolerance results by Shenge, Wydra *et al.* (2008) cannot validly be assumed to reflect tolerance equivalent to Gore and O'Garro (1999). Additionally, only 4 of these 23 *in vitro* studies confirmed copper tolerance *in vivo*. To enable the direct comparison of copper tolerance levels worldwide and even within countries we recommend that a standardised screening method be adopted, particularly in regards to the type of media and its pH.

There was a notable increase in the number of studies investigating copper tolerance in the 1990s, with 6232 of the 7938 *Xanthomonas* spp. isolates and 277 of the 358 *P. syringae* pv. *tomato* isolates being screened in publications from this decade. This is likely due to a number of key copper tolerance publications in the late 1980s and early 1990s that raised awareness of the issue. Although there was a reduction in the number of isolates being screened for copper tolerance after the 1990s there was an increase in the number of papers reporting on copper efficacy, with 83% of studies in this area published between 2000 and 2015. As would be expected, this time period is also when a majority of studies on biological or alternatives products were published (95%).

2.5.2 Efficacy of copper products for the control of bacterial speck and spot

The efficacy of crop protectant products to control bacterial speck and spot disease was investigated in 97 publications, using *in vitro* and/or *in vivo* experimental designs. Of these, 63 studies included an investigation of copper efficacy and 84 included biological or alternative products. The most common copper-based bactericide formulations tested were copper hydroxide ($Cu(OH)_2$) with 26 studies, and copper oxychloride ($Cu_2(OH)_3Cl$) with 14 studies. Tribasic copper sulphate ($Cu_3H_2O_{10}S_2$) and copper oxide (Cu_2O) were less commonly tested with 9 and 4 studies, respectively. Some studies also tested copper salts of fatty and rosin acids, copper ammonium carbonate, and copper ammonium sulphate. Copper was generally applied alone or in tank mixtures with manganese/zinc ethylene bis-dithiocarbamates (EBDC; commonly called mancozeb).

There were 45 studies measuring the efficacy of copper applied alone, 38% of these reported good disease control with copper (e.g. Fousia *et al.*, 2015; Wilson *et al.*, 2002). However, 41% of studies reporting good control were published prior to 1988 (e.g. Jones and Jones, 1985; Yunis *et al.*, 1980), suggesting a lower prevalence of copper tolerance compared to studies published after 1990. Good control was defined as a 60-100% reduction in disease relative to an inoculated or naturally infected untreated control. No significant disease control was reported in 18% of studies (e.g. Ji *et al.*, 2006; Jones *et al.*, 1993). The remaining 56% of studies reported significantly lower disease levels when tomato or pepper were treated with copper products alone compared to inoculated untreated controls. However, overall disease reduction was fair (approximately 50% reduction relative to a control) to weak (less than 50% reduction) and it is unlikely that the level of control provided would be commercially acceptable. For example, Worthington *et al*. (2012) reported a final mean disease severity rating of 5.32 in the inoculated untreated control and 4.19 with copper hydroxide applications. While statistically different this only represents a 10-15% reduction in the percentage of diseased foliage. A similar trend was observed by Byrne *et al.* (2005) with copper hydroxide having 22.9% disease severity compared to 34.7% in the untreated control. Additionally, bacterial speck or spot control with copper applied alone was sometimes variable between different experimental sites or seasons, with control reported in some instances and no control in others (do Carmo *et al.,* 2001; Buonaurio *et al.*, 2002; Trueman, 2013).

The vast majority (97%) of copper efficacy experiments were inoculated with *Xanthomonas* spp. or *P. syringae* pv. *tomato*. However, the copper tolerance status of these isolates was not mentioned in 62.5% of studies published after copper tolerance was identified in the late 1980s. Jones *et al.* (1991) compared the efficacy of copper alone on both sensitive and tolerant *Xanthomonas campestris* pv. *vesicatoria* isolates. Although disease severity was significantly lower than the untreated control with the tolerant isolate (57% reduction), there was a greater reduction (80%) when a sensitive isolate was used. This suggests that in the 56% of studies reporting fair to weak disease control with copper products, copper tolerance in *Xanthomonas* spp. and/or *P. syringae* pv. *tomato* existed in those isolates.

Copper used with an EBDC can provide control of bacterial speck and spot even when copper-tolerant populations are present (Huang *et al.*, 2012). A total of 36 studies used copper with EBDC, 31 of these reported significant control of bacterial speck or spot. Inadequate control of bacterial speck and spot was reported in 2 studies (Miller *et al.*, 2007; Obradovic *et al.*, 2004) and three showed that control was inconsistent (Balogh *et al.*, 2003; Huang *et al.*, 2012; Louws *et al.*, 2001). The most common copper formulation used in this mixture was copper hydroxide, with 30 studies in total. EBDC in

combination with copper oxychloride, tribasic copper sulphate and copper oxide was only tested in a few studies.

Even though copper/EBDC mixtures provide a useful alternative to copper applied alone for mediation of copper tolerance in *Xanthomonas* spp. or *P. syringae* pv. *tomato*, the application window is limited in countries such as Australia due to the 7-14 day harvest withholding period of EBDC (DuPont, 2012; Farmoz, 2012). Therefore, it cannot be applied over the harvesting period that can last for up to eight weeks in tomato and pepper crops. Additionally, there are environmental and health concerns about the accumulation of both copper and EBDC in the soil (Chibuike and Obiora, 2014; Gullino *et al.*, 2010; Wightwick *et al.*, 2010; Van-Zwieten *et al.,* 2004). These factors along with the presence of copper tolerance in *Xanthomonas* spp. or *P. syringae* pv. *tomato* has prompted a significant volume of research into the development of biological and other alternative products for disease control.

2.5.3 Alternative products for the control of bacterial speck and spot diseases

There were close to 100 different biological or alternative products tested within the studies reviewed. Products included strains of non-pathogenic bacteria, plant or compost extracts, essential oils, antibiotics, synthetic chemical compounds, bacteriophages and nanomaterials. Of these products, eight were most prevalent in publications. These were acibenzolar-s-methyl, *P.fluorescens*, *Bacillus subtilis*, bacteriophages, *P.syringae* Cit7 and the antibiotics streptomycin, oxytetracycline and kasugamycin.

Acibenzolar-s-methyl was the most prevalent of the alternative products tested in the studies identified for this review. Overall, 21 of 25 studies reported effective control of *P. syringae* pv. *tomato* and *Xanthomonas* spp. with acibenzolar-s-methyl applied alone, and overall performance was either better or equivalent to a copper/EBDC program (Buonaurio *et al.*, 2002; Graves and Alexander, 2002) . Mixtures with other products, such as copper hydroxide or biological products, were also highly effective (Buonaurio *et al.,* 2002; Wilson *et al.,* 2002). Acibenzolar-s-methyl was sometimes reported to have negative crop growth and yield effects, however, some trial sites within the studies were not affected (Pontes *et al.*, 2016; Louws *et al.,* 2001; Romero *et al.*, 2001). Phytotoxic effects including stunting and chlorosis were reported in greenhouse tomato and pepper but not in open field crops (Abbasi *et al.*, 2002; Abbasi *et al.*, 2003). However, although phytotoxic affects weren't specifically noted on the field grown peppers, there were some negative effects on yield (Abbasi *et al.*, 2002). Negative crop effects are reported to be mitigated by incorporating acibenzolar-s-methyl applications into a fortnightly program alternating with a copper-based bactericide or other products (Roberts *et al.*, 2008). As well as using a maximum of 7-8 applications of acibenzolar-s-methyl, followed by copper-based products later in the season (Pontes *et al.,* 2016). Since the early 2000s, acibenzolar-s-methyl

also seems to be the preferred control treatment in place of the more commonly used copper products, especially for studies on the efficacy of biological products (Abbasi *et al.*, 2002; Abo-Elyousr and El-Hendawy, 2008; Al-Dahmani *et al.*, 2003).

Two non-pathogenic *Pseudomonas* spp., *P. fluorescens* and *P. syringae* Cit7, are reported to be effective in bacterial speck and spot control in 71-75% of studies. Tank mixtures of these Pseudomonads with products such as acibenzolar-s-methyl, copper hydroxide, bacteriophage and *B. subtilis* were also shown to be highly effective against *Xanthomonas* spp. of tomato and pepper (Briceno-Montero and Miller, 2004; Ji *et al.*, 2006). *B. subtilis* strain QST713, is another non-pathogenic species, and is the active component of at least one commercial product. The overall efficacy of the *B. subtilis* strain QST713 for disease control was variable when applied alone, with three of six studies reporting a positive effect. Tank mixtures with copper hydroxide performed better, with five of six studies reporting control. However, interpretation of these results is difficult without details on the copper tolerance status of the *P. syringae* pv. *tomato* and *Xanthomonas* spp. isolates used. Three other *B. subtilis* based products tested in other studies did not provide effective disease control.

Bacteriophage mixtures specific to *Xanthomonas* spp. of tomato were shown to be highly effective in all five studies reporting significant suppression of bacterial spot disease. No bacteriophage studies relating to *P. syringae* pv. *tomato* were identified. Streptomycin, oxytetracycline and kasugamycin were the most common antibiotics tested. Streptomycin and oxytetracycline alone and in mixtures provided significant control in all of the studies but kasugamycin alone was only effective 50% of the time. Kasugamycin in mixtures tended to be effective for bacterial speck and spot control. However, use of antibiotics in horticulture is restricted in countries such as Australia, and resistance to streptomycin is also prevalent (Araújo *et al.*, 2012; Bouzar *et al.,* 1999), limiting the potential of these products.

Only 20 of the 91 studies on alternative products were tested on *P. syringae* pv. *tomato*. The remainder focused only on *Xanthomonas* spp. Similar trends were observed in copper tolerance and efficacy studies, with 10 of 41 and 19 of 63 studies, respectively. This bias in focus may indicate that *Xanthomonas* spp. are more prevalent on tomato and pepper worldwide compared to *P. syringae* pv. *tomato*, or that *Xanthomonas* spp. is considered to have a greater commercial impact on crops. Nonetheless, *P. syringae* pv. *tomato* can cause significant crop damage. There is a need for a larger volume of studies that test for copper tolerance in *P. syringae* pv. *tomato* and the efficacy of emerging alternative products for its control.

2.6 Conclusion

The synthesis of information in this review highlights that copper tolerance in *P. syringae* pv. *tomato* and *Xanthomonas* spp. is a global issue, which is affecting the usefulness of copper-based products for the control of bacterial speck and spot in tomato and pepper. However, the analysis also revealed a lack of consistency in methodology used to assess copper tolerance of bacteria *in vitro* is a hindrance to the completion of a more accurate assessment of the global prevalence of tolerance levels. It would be useful if a standardised screening method was adopted by researchers, enhancing comparisons between different regions and countries. A large range of alternative products for bacterial disease control were identified, with some of these now in commercial use. The effectiveness of these products is variable but there is potential for them to be integrated into disease management programs to mediate resistance development and provide a more environmentally sustainable approach to crop production.

2.7 Additional Information

This section was not included in the original publication.

This systematic literature review found that there was a much lower proportion of studies on *P. syringae* pv. *tomato* compared to *Xanthomonas* spp. Of all studies identified in the systematic review more than 75% were tomato focused rather than pepper, hence the lower proportion of studies on *P. syringae* pv. *tomato* was not attributed to more studies on pepper which is not susceptible to this pathogen. Few studies are available that investigate the relative prevalence of *P. syringae* pv. *tomato* versus *Xanthomonas* spp. in tomatoes. In a survey of tomato crops in the Czech and Slovak republic, Pernezny *et al.* (1995) report 86% of isolates were *P. syringae* pv. *tomato.* Also, *P. syringae* pv. *tomato* is reported to proliferate more rapidly than *Xanthomonas* in mixed inoculations, suggesting that *P. syringae* pv. *tomato* is more likely to be present in crops (Shenge, Mabagala*, et al.* 2008). However, of 166 bacterial isolates collected from tomato crops in Tennessee, United States of America (USA), none were *P. syringae* pv. *tomato,* with 162 identified as *Xanthomonas* spp. and 4 as *Clavibacter michiganensis* subsp. m*ichiganensis* (Mixon 2012). From these few studies it is evident the relative prevalence of *P. syringae* pv. *tomato* and *Xanthomonas* spp. differs between crops, the most likely reason for this is region specific climatic and environmental conditions. Such as the differences in state wide distribution of *P. syringae* pv. *tomato* relative to *Xanthomonas* spp. in Australia discussed in Chapter 1 (section 1.1). With a large amount of publications identified in the review being studies conducted in the USA, it is possible that *Xanthomonas* spp. is more prevalent in USA tomato growing regions, and subsequently there is more extensive reporting of copper tolerance and the efficacy of copper and copper alternatives for bacterial spot control than for bacterial speck.

Through the collection of isolates for this study (Chapters 4 and 5) it was observed that there was a lower prevalence of *P. syringae* pv. *tomato* in the Bundaberg region in the 2016-17 spring/summer growing period compared to the same period in 2015-16. In comparison, isolates collected from the Granite Belt region were exclusively *P. syringae* pv. *tomato*. These differences, along with the paucity of published studies, suggests there is significant scope for more detailed surveys of the relative prevalence of *P. syringae* pv. *tomato* and *Xanthomonas* spp. in tomato crops across difference regions.

Chapter 3 – General Materials and Methods

3.1 Chapter Overview

This chapter provides supplement detail of the standard methodology used in the thesis which is not included in the subsequent chapters. It also links to appendix sections containing details on preliminary method development and associated results of this development required to optimise experimental processes.

Plant material suspected to be infected with bacterial speck disease caused by *P. syringae* pv. *tomato,* or bacterial spot disease caused by *Xanthomonas* spp., was collected from commercial tomato properties. Standard bacterial isolation and purification methods (Section 3.2) were used to isolate bacterial colonies suspected to be *Pseudomonas* and *Xanthomonas* from the plant material. Biochemical testing was carried out on purified isolates to characterise the properties of the bacterial isolates, as well as providing a preliminary species identification (Section 3.3). Biochemical test results were able to identify isolates that were likely to be *P. syringae*. To confirm the species and pathovar identity genetic identification was required using PCR techniques (Section 3.5).

Colony forming units (CFU) are a unit of measurement used in bacterial methodology to estimate the number of viable cells present in a sample. When using bacterial suspensions in experiments it is useful to have consistent concentrations, as an estimation of CFU/mL, to improve the replicability of experiments. This estimation also provides perspective for interpretation of experimental results and subsequent publication. The optical density (OD) of a solution containing bacterial cells is proportional to cell density or the CFU/mL (Widdell 2010). Hence, the OD of a solution can be used to give an estimate of the number of CFU in a sample (Section 3.4).

Isolates confirmed to be *P. syringae* pv. *tomato* were used throughout the project to fulfil the research objectives (Section 1.2). Chapter 4 is a methodology paper which contains specific details on the investigation and development of *in vitro* copper tolerance screening methods, these methods are not repeated in this Chapter.

3.2 Bacterial isolation and purification

The isolation, purification and identification of *P. syringae* pv. *tomato* was required throughout the duration of this thesis study to provide isolates for copper tolerance testing and genetic analysis (Chapters 4, 5 and 6). Tomato leaves and/or fruit with disease symptoms suspected to be caused *P. syringae* pv. *tomato* were collected from commercial tomato properties from a number of different locations throughout Australia (Table 3-1). Sampling commenced in September 2015 and continued through to February 2017. Samples were collected year round depending on the location and climatic conditions conducive to disease development.

Table 3-1: Sample details of *Pseudomonas syringae* pv. *tomato* isolates collected for this study including the region, property, crop stage, season and year collected, and the tomato type and cultivar. This table is also included as part of Chapter 5 (Table 5-1).

Isolate ID	Region[a]	Property Number	Crop Stage (BBCH)	Collection time	Tomato type	Cultivar
BRIP66795	A	1	61	Spr 2015	Gourmet	unknown
BRIP66796	A	4	89	Spr 2015	Gourmet	Leon
BRIP66797	A	4	89	Spr 2015	Roma	unknown
BRIP66798	A	2	82	Spr 2015	Gourmet	Ninja
BRIP66799	A	2	82	Spr 2015	Gourmet	Stewart
BRIP66800*	A	6	69	Spr 2015	Gourmet	Stewart
BRIP66801	A	6	65	Spr 2015	Gourmet	Stewart
BRIP66802	A	6	64	Spr 2015	Gourmet	Stewart
BRIP66803	B	17	69	Sum 2015-16	Bush	Coltrane
BRIP66804	B	17	71	Sum 2015-16	Cherry	Cherry Bite
BRIP66805	B	17	81	Sum 2015-16	Gourmet	Montenegro
BRIP66806	B	17	83	Sum 2015-16	Ox Heart	Rugantino
BRIP66807	B	10	71	Sum 2015-16	Roma	Romeo
BRIP66808	C	15	82	Sum 2015-16	Bush	Entice
BRIP66809	C	15	82	Sum 2015-16	Gourmet	Ninja
BRIP66810	C	18	85	Sum 2015-16	Roma	Romeo
BRIP66811	C	15	73	Spr 2015	Bush	Entice
BRIP66812	A	2	72	Spr 2016	Gourmet	Ninja
BRIP66813	E	13	61	Sum 2016-17	Bush	H3402 mix
BRIP66814	D	12	81	Sum 2016-17	Gourmet	Rifle
BRIP66815	D	12	81	Sum 2016-17	Gourmet	Silviana
BRIP66816	D	12	81	Sum 2016-17	Roma	Romeo
BRIP66817	D	12	81	Sum 2016-17	Gourmet	Leon
BRIP66818	C	19	89	Sum 2016-17	Gourmet	unknown
BRIP66819	C	14	71	Sum 2016-17	Gourmet	unknown
BRIP66820	C	20	88	Sum 2016-17	Roma	Romeo
BRIP66821	C	23	81	Sum 2016-17	Roma	Romeo

[a] Regions: A= Bundaberg QLD, B= Gatton QLD, C= Granite Belt QLD, D= New England NSW, E= Echuca VIC. Abbreviations: BBCH = Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie, this is a standard rating scale based on plant stages of phenological development. Spr = Spring. Sum = Summer

*Experimental findings suggest this isolate may represent a unique variant of *P. syringae* and may not be pathovar tomato, discussed further in section 5.9 and Appendix E.

Leaves and fruit were sprayed with 70% ethanol prior to lesion isolation to sterilise the surface and remove any dust, then left to dry in sterile conditions. After drying, lesions were cut from plant material, 3-6 lesions were taken for each sample and small subsections of each lesion placed on a microscope slide with water to test for bacterial ooze or streaming. If the lesion section produced bacterial ooze the rest of the lesion was placed in microcentrifuge tubes with 0.5-1.0 mL of sterile deionised water and left to stand at room temperature for 5-30 min. A loop-full of solution was then plated on GNA (containing Difco™ Nutrient Agar with 0.5% w/v glucose pH 7.0), and incubated at 24 °C for 48-72 h.

Bacterial colonies that were circular, white/cream in colour and convex were suspected to be *Pseudomonas.* Individual colonies with these properties were selected from the agar plates and purified three times. Colonies that were circular, yellow, convex and mucoid were suspected to be *Xanthomonas* spp. and were also purified.

For short term storage bacteria were mixed in sterile deionised water and stored at approximately 4 °C. Stock solutions containing bacteria in 30% v/v sterile glycerol, were made up for long term storage of bacteria at -20 °C and -80 °C. Isolates were deposited in the Plant Pathology Herbarium of Biosecurity QLD DAF.

3.3 Biochemical and pathogenicity tests

Suspected *Pseudomonas* isolates were tested for gram negativity, catalase activity and fluorescence on King's B medium (King *et al.* 1954). They were also put through the LOPAT tests, which are a standard set of tests used for the preliminary profiling of *Pseudomonas* (Elkhalfi *et al.* 2013; Lelliott *et al.* 1966). These tests refer to the production of levan (L); oxidase (O); pectinolytic enzymes (P); arginine dihydrolase (A); and induction of a hypersensitive response in tobacco (T). Isolates suspected to be *P. syringae* (Table 3-2) were also tested for pathogenicity on tomato seedlings (T.P). Samples identified to be *P. syringae* were then tested using a *P. syringae* pv. *tomato* diagnostic PCR assay to confirm their identity (section 3.5).

Table 3-2: Reported outcomes of tests on suspected *Pseudomonas* spp. isolates, where '+' is a positive result and '-' is negative. N indicates that the bacteria is gram negative as determined by the KOH gram test. Adapted from Lelliott *et al.* (1966).

***Pseudomonas* Species**	**Gram Test**	**Cat**	**KB**	**L**	**O**	**P**	**A**	**T**	**T.P**
P. syringae	N	+	+	+	-	-	-	+	+
P. viridiflava	N	+	+	-	-	+	-	+	+
P. fluorescens	N	+	+	-	+	-	+	-	-
P. marginalis	N	+	+	+	+	+	+	-	-
P. tolaasii	N	+	+	-	+	-	+	-	-
P. cichorii	N	+	+	-	+	-	-	+	+
P. savastanoi	N	+	+	+ or -	+ or -	-	-	+ or -	+

Key to tests and abbreviations: Gram test (KOH test; see 3.3.1) where N= gram negative; Cat, catalase activity (3.3.2); KB, fluorescence on King's B medium (3.3.3); L, Leven production (3.3.4); O, oxidase production (3.3.5); P, pectinolytic activity (3.3.6); A, arginine dihydrolase production (3.3.7); T, tobacco hypersensitivity (3.3.8); and T.P, tomato pathogenicity (3.3.9).

Reference cultures of *P. syringae* pv. *tomato* (DAR 75965)*, P. syringae* pv. *syringae* (DAR 77319) *and P. marginalis* (DAR 65987) were provided by the Agricultural Scientific Collections Trust, New South Wales Australia (ASCT). These were used as controls for the biochemical tests. The Plant Pathology Herbarium of Biosecurity QLD DAF also provided reference bacterial cultures (Table 3-3). These were put through all the biochemical and most genetic tests, then used in copper tolerance testing to determine historical levels of copper tolerance (Chapter 5). A copper-tolerant isolate A1513R (Andersen *et al.* 1991) *P. syringae* pv. unknown and copper sensitive *P. syringae* pv. *tomato* isolate DC3000 (Buell *et al.* 2003), provided by the University of Florida, were used as controls for copper tolerance testing.

Table 3-3: Reference bacterial cultures provided by the Plant Pathology Herbarium of Biosecurity QLD DAF, including the accession number, *Pseudomonas* species (spp.), the host plant, the closest town to the collection location and the collection year.

Accession	*Pseudomonas* spp.	Host plant	Closest town to Collection Location (QLD)	Collection Year
BRIP34832	*P. syringae* pv. *striafaciens*	Oats	Wyaga	1981
BRIP34945	*P. viridiflava*	Tomato	Bundaberg	1985
BRIP38735	*P. syringae* pv. *tomato**	Tomato	Brisbane	1973
BRIP38746	*P. syringae* pv. *tomato*	Tomato	Caboolture	1973
BRIP38748	*P. syringae* pv. *syringae*	Bean	Brisbane	1974
BRIP38751	*P. cichorii*	Cabbage	Brisbane	1974
BRIP38826	*P. syringae* pv. *tomato**	Tomato	Nambour	1970
BRIP38831	*Pseudomonas sp.*	Sunflower	Nobley	1978
BRIP34814	*P. syringae* pv. *tabaci*	Tobacco	Bundaberg	1981
BRIP38744	*P. syringae* pv. *tomato*	Tomato	Brisbane	1973

*These specimens are listed in the herbarium as *P. syringae* pv. *tomato*, however, results obtained in this study suggest they were not pv. tomato. This is discussed further in section 5.9 and Appendix E.

Suspected *Xanthomonas* spp. were tested for gram reaction, catalase activity, oxidase production, growth on yeast dextrose carbonate agar (YDC; Wick & Hansen n.d) and tobacco hypersensitivity. Two reference cultures, *X. vesicatoria and X. euvesicatoria*, were provided by Rebecca Roach from the Ecosciences Precinct, Brisbane Australia (Roach *et al.* 2017). These references and isolates collected in this study were stored as frozen glycerol stocks for potential future analysis, for example comparing the copper tolerance mechanisms of *Xanthomonas* spp. to *P. syringae* pv. *tomato*. Given results obtained for *P. syringae* pv. *tomato* and time restrictions, no further testing was completed on these isolates.

3.3.1 Gram test

Drops of a 3% w/v solution of potassium hydroxide (KOH) were placed onto a plastic petri dish. Bacteria from an agar plate was picked up with a sterile toothpick and gently mixed into the KOH drops. The toothpick was then lifted slowly out of the mixture, if a fine string of mucous between the toothpick and mixture was present it was a positive result (Figure 3-1) and the bacteria were considered to be gram negative.

3.3.2 Catalase activity

Drops of 30% v/v hydrogen peroxide were placed onto a plastic petri dish. Bacteria from an agar plate was picked up with a sterile toothpick and placed in the drops. A positive result was indicated by the formation of bubbles in the hydrogen peroxide (Figure 3-1), no bubbles indicated negative catalase activity.

3.3.3 Fluorescence on King's B medium

A loop-full of bacterial solution was plated on King's B medium (King *et al.* 1954) containing 1.5 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 20 g/L Bacto™ Proteose Peptone #3, 1.5 g/L dipotassium hydrogen phosphate (K_2HPO_4), 10 mL/L glycerol and 15 g/L agar. Plates were incubated at 24 °C for 48-72 h, then observed under ultraviolet (UV) light, if the bacterial growth was fluorescent (Figure 3-1) it was considered a positive result. No fluorescence was a negative result.

3.3.4 Levan production

A loop-full of bacterial solution was plated onto a Levan media (Difco™ Nutrient Agar with 5% w/v sucrose) and incubated at 24 °C for 48-72 h. Bacteria capable of levan production grew white, mucoid and highly domed (Figure 3-1). If the bacterial growth was flat then it was considered a negative result.

3.3.5 Oxidase production

Bacteria from an agar plate was picked up with a sterile toothpick and smeared onto an oxidase test strip (product ID 40560, Sigma Aldrich). These test strips have one end that is saturated with N,N-dimethyl-1,4-phenylene diamine and alpha-naphtol solution. The activity of cytochrome oxidase in the bacteria causes a reaction with these chemicals, resulting in the production of indophenol blue, which is observed as a colour change (Figure 3-1). If there was no colour change it was considered a negative result.

3.3.6 Pectinolytic activity

A 10 µL aliquot of bacterial solution was spotted onto a petri dish containing Crystal Violet Pectate Medium (Product ID M1392, HiMedia) and incubated at 24 °C for 48-72 h. Bacteria capable of pectinolytic activity had a white transparent mucoid growth (Figure 3-1). If the bacteria did not grow it was considered a negative result.

3.3.7 Arginine dihydrolase production

Approximately 4 mL of molten sterile arginine dihydrolase medium, was dispensed into sterile 15 mL plastic tubes with lids. The medium contained 1.0 g/L Bacto™ Peptone, 5.0 g/L sodium chloride, 0.01 g/L Phenol red, 3.0 g/L Bacto™ Agar, 0.30 g/L dipotassium phosphate (K_2HPO_4) and 10.0 g/L L-Arginine HCl, Once set, the media was stab inoculated with either bacteria growth from plates or bacteria suspended in sterile water. Two tubes were inoculated for each isolate, one tube was then sealed with water agar and the other was left unsealed. Un-inoculated tubes served as a negative control. Tubes were then incubated at 27 °C for 3-7 days.

Arginine dihydrolase releases ammonium from arginine, which results in an increase in medium pH. The phenol red changes to a bright pink with this pH increase, whereas it is yellow at lower pH. Positive arginine dihydrolase activity is therefore indicated by a colour change to dark pink in the media of the

sealed tube (Figure 3-1). Sealed tubes that remained orange-pink in colour are considered a negative result. The unsealed tubes turned pink, which was used as a positive control for inoculation success.

3.3.8 Tobacco hypersensitivity

Methods described by Wick (2010) were used to test if bacterial isolates were pathogenic. A turbid solution of bacteria in sterile water was infiltrated into the underside of tobacco (*Nicotiana tabacum* cv. White Burley) leaves using a 1 mL syringe. Sterile deionised water and DAR 65987 (*P. marginalis*) were used as a negative control. Leaves were checked after 24-48 h for a hypersensitive result, which caused tissue necrosis in the infiltrated area (Figure 3-1). No necrosis of the leaf tissue indicated a negative result.

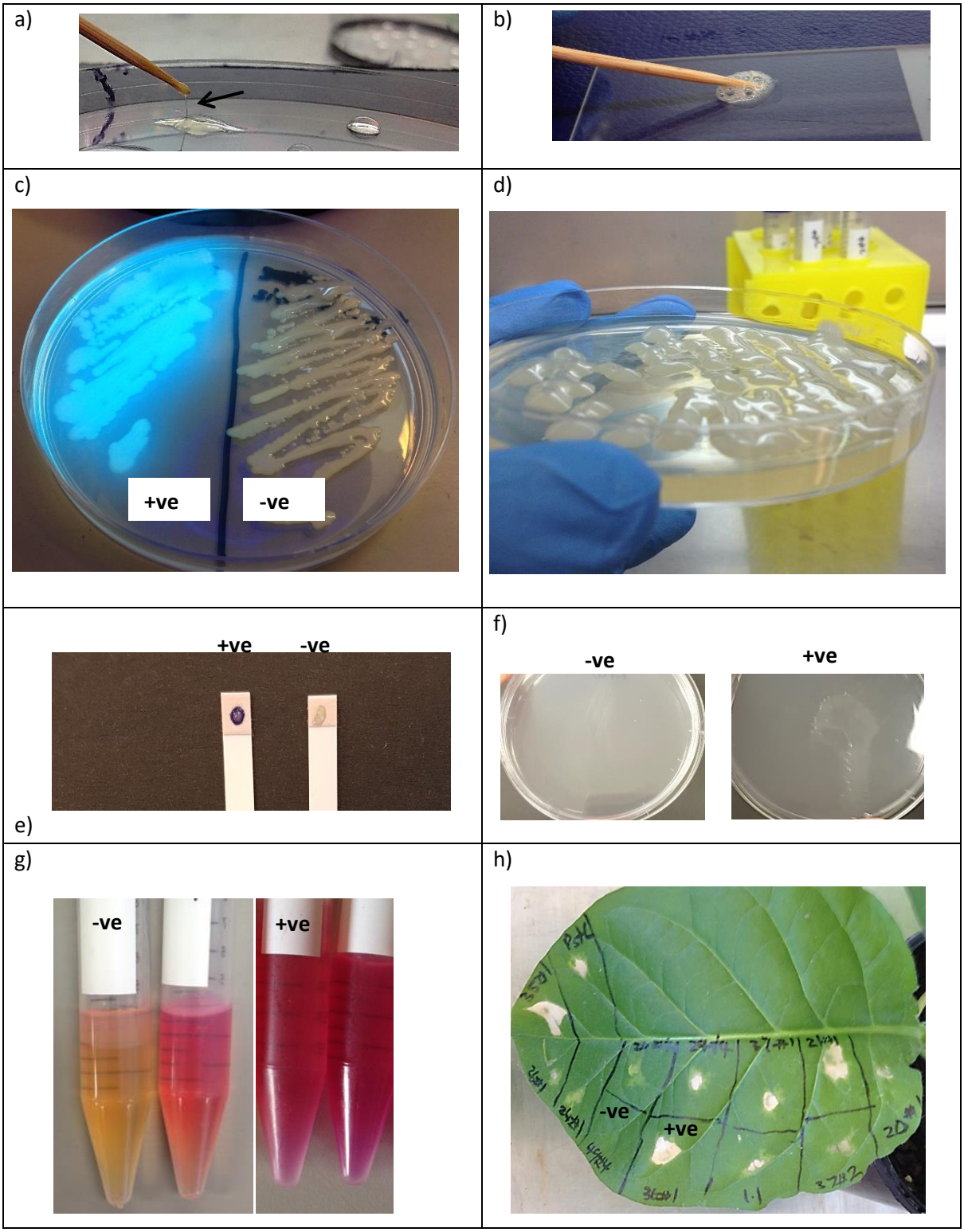

Figure 3-1: Biochemical tests used for the preliminary identification of *Pseudomonas*. Positive result is indicated by '+ve' and negative result indicated by '–ve'. a) mucous string indicating a positive result for the gram test; b) bubbling in hydrogen peroxide indicating a positive result for catalase activity; c) fluorescence of bacterial colonies on Kings B medium; d) white, mucoid and highly domed bacterial growth indicating a positive result for the production of leven; e) oxidase test strips results; f) pectinolytic activity indicated by bacterial growth; g) colour change observed in arginine dihydrolase medium; h) necrosis caused on the leaf by the tobacco hypersensitivity test.

3.3.9 Pathogenicity on tomato

Bacterial suspensions of approximately 1.1 x 10^8 CFU/mL (see section 3.4) were prepared in 10 mmol/L $MgSO_4.5H_2O$ (Pontes *et al.* 2015). Small sterile glass spray bottles were filled with 5 mL of the bacterial suspension. The entire 5 mL was sprayed on young disease free tomato seedlings, at a crop stage of 4-5 true leaves, ensuring thorough leaf and stem coverage. The seedlings were then placed in clear plastic tubes in a glasshouse, these tubes separated the plants from each other to avoid cross contamination during watering. Plants were monitored for 1-2 weeks for the development of black lesions on the leaves (Figure 1-1), typical of bacterial speck disease, this was considered a positive result. The reference isolate *P. syringae* pv. *tomato* (DAR 75965) known to be pathogenic to tomatoes was used as a positive control. BRIP38748 (*P. syringae* pv. *syringae*) known to not be pathogenic to tomatoes was used as a negative control.

A tomato seedling sprayed only with sterile deionized water was also placed in a plastic tube near inoculated seedlings and monitored to ensure there was no disease development. This was done as nursery seedlings were shown to carry *P. syringae* pv. *tomato* whilst remaining symptomless (Cuppels & Elmhirst 1999).

3.3.10 Yeast dextrose carbonate (YDC) agar (suspected Xanthomonas spp. only)

The YDC agar was prepared as two different solutions, which were autoclaved separately. Solution 1 contained 8.0 g dextrose in 200 mL water and solution 2 contained 7.5 g Bacto™ Agar, 8.0 g calcium carbonate and 4.0 g yeast extract in 200 mL water. After autoclaving, the solutions were combined and poured into petri dishes, ensuring the calcium carbonate remained evenly distributed throughout the pouring process. A loop-full of bacterial cells was plated onto the YDC and incubated at 24 °C for 48-72 h. If the bacterial growth was yellow, mucoid, convex and shiny it was considered a positive result (Figure 3-2). Colony growth without these properties was considered a negative result.

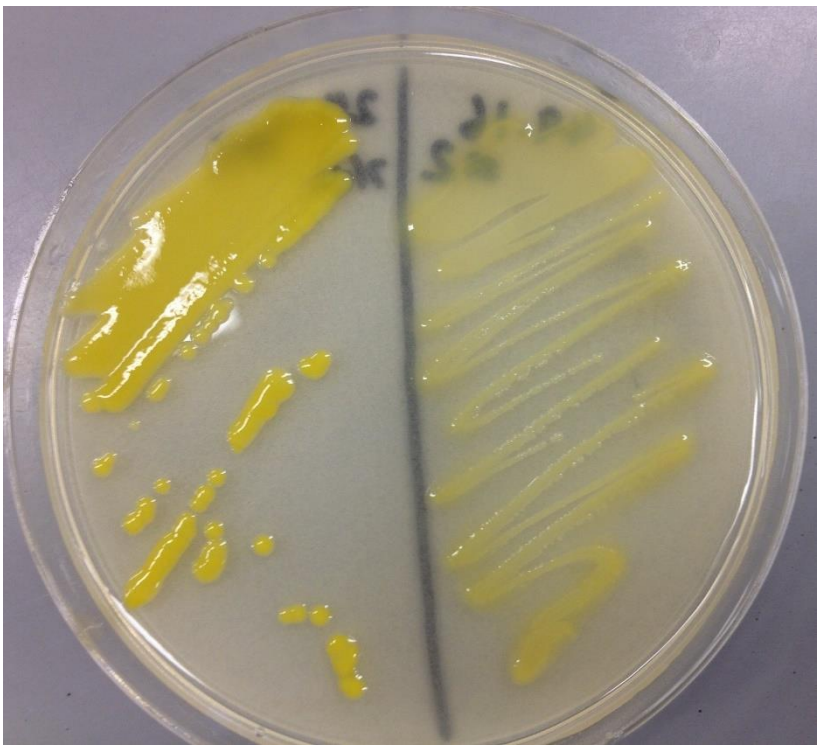

Figure 3-2: *Xanthomonas* growth on YDC agar. Right: yellow, mucoid, convex and shiny growth of reference *X. euvesicatoria* isolate. Left: Bacterial isolate with no convex growth, indicating a negative result.

3.4 Quantification of colony forming units in bacterial suspensions

Adjusting bacterial samples to a standard concentration of CFU before using them in an experiment allows for the optimisation and replicability of experiments. The following methodology was therefore used to produce a standard graph that was used for the duration of this research project to quantify the concentration of CFU used in experiments (Chapters 4, 5 and 6).

The CFU is a unit of measurement used in bacterial methodology to estimate the number of viable cells present in a sample. The OD of a solution containing bacterial cells is proportional to cell density (Widdel 2007). Hence, the OD of a solution can be used to give an estimate of the number of CFU in a sample. The OD can be measured using photometry, which is the measurement of ultra violet, visible and infrared light (Allaby 2008). In this study, a microtiter plate reader was used which measures the absorbance of a sample at a specified wavelength of light. In the case of bacterial suspensions it is more technically correct to call this measurement 'turbidity' rather than 'absorbance' (Widdel 2007). The recommended wavelength to measure the turbidity of bacterial suspensions is at approximately 600 nm; the proportionality between this and the cell density is only true at an OD of ≤ 0.4 (Widdel 2007).

A spectrophotometer rather than a plate reader could also have been used for OD measurements, however, the plate reader allowed for the measurement of smaller volumes and multiple samples in each assessment. Using a plate reader or spectrophotometer to measure OD avoids the need to individually determine the viable cell count of each sample for each experiment through dilution plating of the suspensions on agar growth medium, reducing assessment time from up to 48 h for dilution plating to a few minutes for OD determination.

The following method was used to measure the relationship between the OD and cell density of *Pseudomonas.* The reference isolate *P. syringae* pv. *tomato* (DAR 75965) was plated on GNA and incubated for 48 h at 24 °C. The bacterial growth was then washed off the plates, by pipetting 1-3 mL of sterile deionised water onto the plate and agitating the colonies with a sterile loop to disperse them in the water. This solution was then pipetted off the plate and placed into a sterile tube, this wash step was repeated another 1-2 times. Aliquots of the bacterial suspension were pipetted into wells of the plate with 3 replicates. The OD of the solution was measured in the plate reader with water as a blank. The average OD was then calculated and used in *equation 1* to determine the dilution required to get a set of solutions ranging from OD 0.05 to OD 0.30.

Equation 1

$$Vb\ (\mu L) = \frac{Ca\ (OD) \times Va\ (\mu L)}{Cb\ (OD)}$$

Where *Vb = µL volume of original suspension required*

Cb = OD of original suspension

Ca = target OD of new suspension

Va = µL total volume of new suspension

Given that the OD is not proportional at readings ≤ 0.4 it was sometimes necessary to complete another dilution step to achieve the target OD. Once the set of solutions were ready, a 1:10 serial dilution of each was completed down to 10^{-7}. A 100 µL aliquot of each dilution and sample was spread on GNA plates, 3 replicate plates were completed for each. Plates were incubated for 48 h and the number of colonies growing were counted. Plates with >300 colonies were not counted. The dilution factor that had colony counts of 50-200 were used to calculate the CFU/mL at each OD.

Over the study period two different plate readers were used depending on the location at which experiments were being undertaken, one with a filter at 590 nm and the other at 620 nm. As well as the potential variation at two different wavelengths, different machines can also give slightly different readings, hence standard graphs of OD vs. cell density were completed for each machine (Figure 3-3 and 3-4).

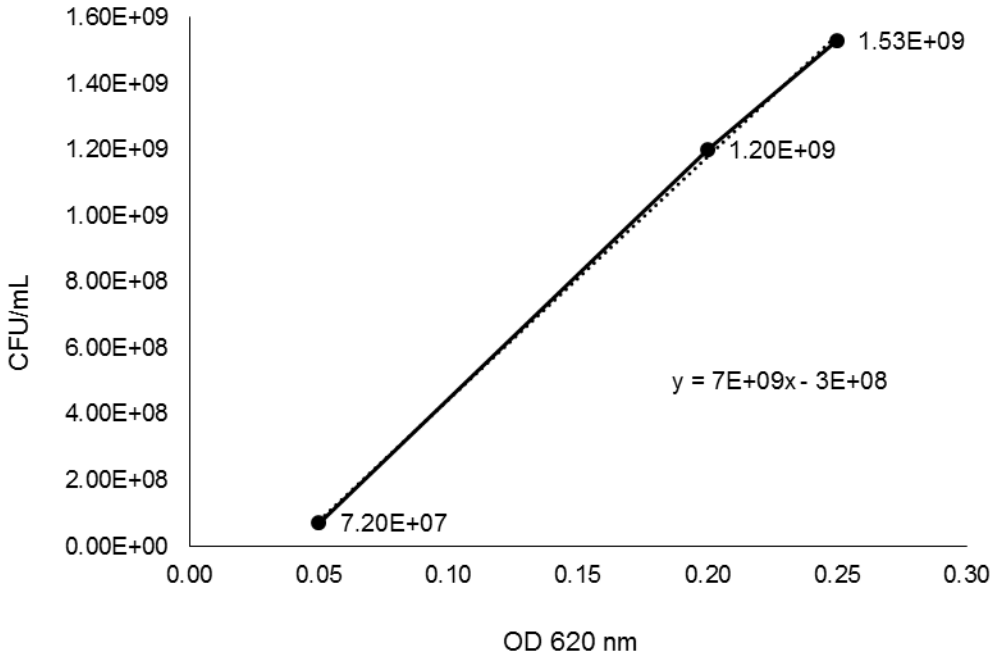

Figure 3-3: Standard graph showing the relationship between optical density (OD) at 620 nm and colony forming units (CFU) for *Pseudomonas syringae* pv. *tomato*. Multiskan FC plate reader. The OD of 100 µL aliquots of bacterial suspensions were measured.

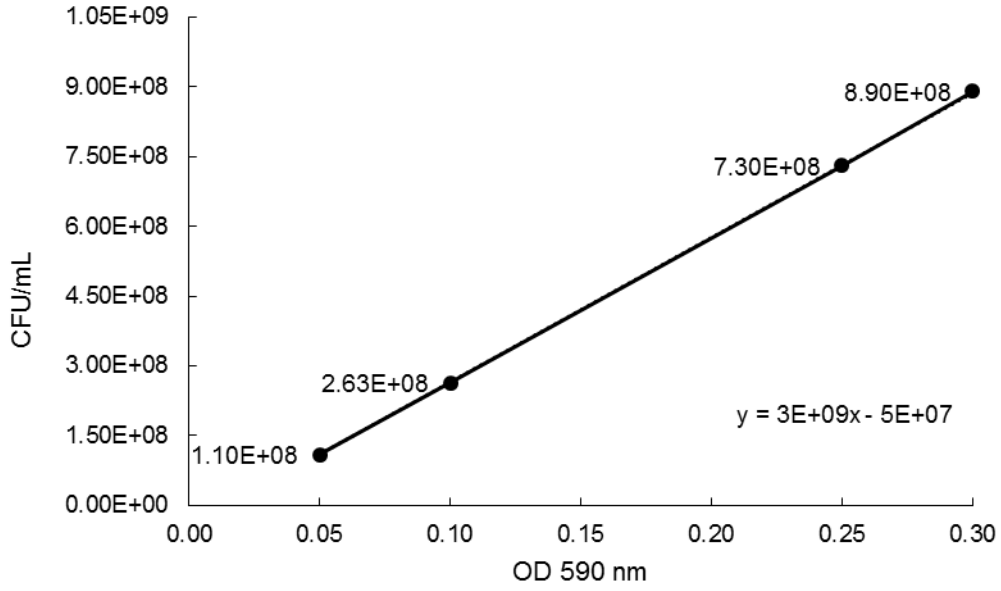

Figure 3-4: Standard graph showing the relationship between optical density (OD) at 590 nm and colony forming units (CFU) for Pseudomonas syringae pv. tomato. Measurements taken on a Biolog Microstation ELX808BLG. The OD of 200 µL aliquots of bacterial suspensions were measured.

3.5 Pathovar determination of *P.syringae* using polymerase chain reaction (PCR) assays.

Biochemical test results were able to identify isolates that were likely to be *P. syringae*. However, to confirm the species and pathovar identity genetic identification was required, PCR assays were used to achieve this.

P. syringae pv. *syringae* causing leaf spotting similar to *P. syringae* pv. *tomato* has been identified on tomatoes (Garibaldi *et al.* 2007; Gullino *et al.* 2009; Jones *et al.* 1981). Mitrev *et al.* (2000), also characterised a *P. syringae* pv. *syringae* isolate from pepper plants. This pathovar has a high tolerance to dryness and sunlight and can be present in large populations (Feil *et al.* 2005). Both *P. syringae* pv. *syringae* and *P. syringae* pv. *tomato* give identical results in the biochemical tests detailed above. It is therefore questioned if *P. syringae* pv. *tomato* has been misdiagnosed in studies relying on cultural identification methods (pers. comm. Cherie Gambley). Genetically characterising the isolates collected in this study was therefore required to determine if isolates were pv. *tomato*.

PCR assays were used to amplify specific gene regions of interest in isolates suspected to be *P. syringae*. Each of the PCRs described below were used throughout this study. Fragments of the following genes were amplified using PCR: *h*ypersensitivity *r*esponse and *p*athogenicity-associated *Z* gene (*hrpZ*$_{Pst}$; section 3.5.1) and coronofacate ligase (*cfl*; section 3.5.2) Multilocus sequence typing (MLST) of four housekeeping genes was also tested (section 3.5.3). The MLST analysis involved the following genes: *gapA, gltA* (also known as *cts*)*, gyrB* and *rpoD*, which encode glyceraldehyde-3-phosphate dehydrogenase*,* citrate synthase*,* DNA gyrase B and sigma factor 70 proteins, respectively.

PCR targeting *hrpZ*$_{Pst}$ and *gltA* genes were completed for all study isolates and *cfl* PCR was completed for most study isolates*.* The PCRs targeting *gapA, gyrB* and *rpoD* were only used for preliminary testing of MLST, whilst the *gltA* PCR was completed for all study isolates. Reference cultures from the ASCT and Plant Pathology Herbarium of Biosecurity QLDDAF were used as controls (section 3.3). Results of these assays are included as additional information in Chapter 5 (section 5.9).

3.5.1 *P. syringae* pv. *tomato* diagnostic PCR targeting the *hrpZ*$_{Pst}$ gene

This PCR diagnostic assay was developed by Zaccardelli *et al*. (2005) and used to screen samples suspected to be *P. syringae* pv. *tomato* for this study. This method amplifies a 532-base pair (bp) fragment of the hrpZ$_{Pst}$ gene, which was demonstrated to be highly specific to *P. syringae* pv. *tomato*, and importantly does not produce a fragment from other pseudomonads. Another important property of this particular PCR assay is that coronatine deficient *P. syringae* pv. *tomato* isolates can also be positively identified (section 3.5.2). Zaccardelli *et al.* (2005) report the method is capable of detecting

the *hrpZ*$_{Pst}$ in pure culture isolates, obtained using isolation and purification techniques detailed in section 3.2, as well as crude field isolates prepared by soaking baterial lesions in sterile distilled water.

The primers used in this study were the same as those published by Zaccardelli *et al.* (2005); the forward primer was MM5F (5'- GAA CGA GCT GAA GGA AGA CA-3') and the reverse was MM5R (5'-CAG CCT GGT TAG TCT GGT TA-3'). The annealing temperature (T_A) of the PCR reaction was optimised by testing *P. syringae* pv. *tomato* (DAR75965) and *P. syringae* pv. *syringae* (DAR77319) reference cultures (Appendix B).

Each 50 µL reaction mixture contained 0.2 µmol/L of each primer, 22 µL sterile deionised water and 25 µL MangoMix™ (Bioline, contains 2.5 mmol/L $MgCl_2$, MangoTaq™ DNA Polymerase, deoxyribonucelotide triphosphates (dNTPs), 5 x Mango*Taq*™ Coloured Reaction Buffer). Bacterial suspensions in water were adjusted to approximately 7.2 x 10^7 CFU/mL, 1 µL of this was added to the reaction mixture. If the MangoMix™ was not available the following reaction mixture was used; 0.2 µmol/L of each primer, 0.2 mmol/L dNTPs, 5 µL 5x Mango*Taq*™ Coloured Reaction Buffer (Bioline), 2.5 mmol/L $MgCl_2$, 0.3 µL MangoTaq™ DNA Polymerase (Bioline). There was no significant difference in the quality of amplified products when MangoMix™ was not used.

DNA amplification was carried out in a Kyratec SuperCycler Thermal Cycler (model SC300) using the following programme: denaturation at 95 °C for 1 min followed by 30 cycles of denaturation at 95 °C for 20 s, T_A at 60 °C for 20 s and extension at 72 °C for 30 s, then a final extension at 72 °C for 5 min. Fragments were visualised using agarose gel electrophoresis and ethidium bromide (EtBr) staining with a 1.5 % w/v agarose gel.

3.5.2 Identifying coronatine production with PCR targeting the *cfl* gene

Coronatine is a phytotoxin that induces plant tissue necrosis, stunting and hypertrophy, it is produced by some *P. syringae* pathovars including pv. *tomato* (Bender *et al.* 1987; Bereswill *et al.* 1994). The chlorotic halos that often form around lesions caused by *P. syringae* pv. *tomato* are also attributed to coronatine production. Targeting coronatine production through the *cfl* gene was previously the standard method for the identification and genetic diversity analysis of *P. syringae* pathovars (Bereswill *et al.* 1994). However, there are naturally occurring *P. syringae* pv. *tomato* isolates that don't produce coronatine (Zaccardelli *et al.* 2005), hence this method cannot detect coronatine deficient *P. syringae* pv. *tomato*. This could result in this pathovar not being identified, therefore, the use of PCR targeting *hrpZ*$_{Pst}$ gene is now more commonly used(section 3.5.1). *P. syringae* pv. *syringae* does not contain all the required genes for coronatine production, instead its virulence is attributed to syringopeptins and syringostatin (Feil *et al.* 2005). Therefore, testing isolates for this *cfl* gene and *hrpZ*$_{Pst}$ can provide information on determining whether an isolate is pv. *tomato* or pv. *syringae*.

The primers used in this study were the same as those published by Bereswill *et al.* (1994); the forward primer was PsNFP1 (5'-GGCGCTCCCTCGCACTT-3') and the reverse was PsNFP2 (5'-GGTATTGGCGGGGGTGC-3'). This method amplifies a 629-bp fragment of the *cfl* gene. Each reaction mixture contained 0.2 µmol/L of each primer, 0.2 mmol/L dNTPs, 10 µL of 5 x Mango*Taq*™ Coloured Reaction Buffer (Bioline), 1.75 mmol/L $MgCl_2$, 0.3 µL of MangoTaq™ DNA Polymerase (Bioline) and 1 µL of approximately 7.2 x 10^7 CFU/mL bacterial suspension, made up to a total volume of 50 µL with sterile deionised water.

The PCR program consisted of denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s, T_A at 68 °C for 20 s and extension at 72 °C for 30 s, then a final extension at 72 °C for 5 min. Fragments were visualised using agarose gel electrophoresis and EtBr staining with a 2 % w/v agarose gel.

3.5.3 Multilocus sequence typing (MLST) of *P. syringae*

The MLST technique that is used to phylogenetically characterise variants of bacterial species (Urwin & Maiden 2003). This technique involves amplifying and sequencing fragments of several housekeeping gene within a species, generally 400-500 bp in size. There are many *P. syringae* pathovars, each varying in the production of toxins, ice nucleation activity and antimicrobial resistance (Hwang *et al.* 2005), these pathovars are grouped into 23 clades within 13 phylogroups (Berge *et al.* 2014).

Primers for 4 housekeeping genes, *gapA, gltA, gryB* and *rpoD*, were used by Hwang *et al.* (2005) to phylogenetically characterise 95 *P. syringae* strains and there is a large database of *P. syringae* pathovar sequences produced by these primers (Plant Associated and Environmental Microbes Database PAMDB; Almeida *et al.* 2010). Therefore, the potential of using MLST in this study to determine isolate identity was explored through sequencing and phylogenetic analysis (Appendix C).

Results of the MLST testing (Appendix C) and detailed analysis published by Berge *et al.* (2014) and Sarkar and Guttman (2004) indicated that *gltA* provided the most consistent separation of *P. syringae* pathovars into phylogroups, with the formation of distinct clades within a phylogenetic tree. Therefore, *gapA, rpoD* and *gryB* were not used for the screening of all study isolates, only *gltA* was applied to all study isolates (section 5.9).

Primers published by Hwang *et al.* (2005) were modified slightly, the forward primer was *gltA174p* (5'-GCC TCB TGC GAG TCG AAG ATC ACC-3') and the reverse primer was *gltA1192p* (5'- CTT GTA VGG RCY GGA GAG CAT TTC-3'). The expected fragment size was approximately 1000 bp.

Each reaction mixture contained 0.2 µmol/L of each primer, 0.2 mmol/L dNTPs, 5 µL 5 x Mango*Taq*™ Coloured Reaction Buffer (Bioline), 2.5 mmol/L $MgCl_2$, 0.3 µL MangoTaq™ DNA Polymerase (Bioline) and 1 µL of bacterial suspension at approximately 7.2 x 10^7 CFU/mL, made up to total volume of 50 µL with sterile deionised water. The PCR program consisted of denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 95°C for 20 s, T_A at 56 °C for 20 s and extension at 72 °C for 45 s, then a final extension at 72 °C for 5 min. Fragments were visualised using agarose gel electrophoresis and EtBr staining with a 2 % w/v agarose gel.

PCR products were submitted to Macrogen Incorporated for purification and sequencing. Consensus sequence generation, multiple sequence alignments and phylogenetic analyses were completed in Geneious (version 10.2.3; Kearse *et al.* 2012).

Chapter 4 - Copper tolerance Methodology

4.1 Chapter Overview

This chapter is made up of a published copper tolerance methodology journal article. The article is included in its entirety. The only changes that have been made is the numbering of tables and figures so that they conform with the numbering system of thesis.

Through analysis of papers for the systematic literature review (Chapter 2), *in vitro* methodology for the determination of copper tolerance was compared to gather the information necessary to develop a method to be used in this study. This comparison highlighted aspects of the methodology that were used inconsistently across the literature as well as factors that were frequently not recorded despite having the potential to significantly affect the results obtained. The major factors affecting copper tolerance results were the media type used and the pH of that media. This chapter/journal article investigates how these factors affect *in vitro* copper tolerance assessments of *P. syringae* pv. *tomato*.

Three *in vivo* experiments were completed to confirm that isolates were copper-tolerant, the results of these experiments is presented in full in the published article. Additional methodological information for these experiments is provided as additional information (section 4.8.1).

The incubation of isolates in copper solutions prior to plating on agar media was investigated as a method for copper tolerance assessment. Results of this testing were not included in the publication, therefore, they are included as additional content in this chapter (section 4.8.2).

Bibliographic Information

Griffin, K, Brown, P & Gambley, C. (2018). Media pH and media type can significantly affect the reliability of *in vitro* copper tolerance assessments of *Pseudomonas syringae* pv. *tomato. Journal of Applied Microbiology*, 125, 216-226. doi: 10.1111/jam.13753

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Declaration of Authorship

Title of Paper: Media pH and media type can significantly affect the reliability of *in vitro* copper tolerance assessments of *Pseudomonas syringae* pv. *tomato*

Status: Published online 23 April 2018

Nature of Candidate Contribution: As the primary author I collected and analysed all data, and wrote all the content.

Nature of Co-authors Contributions: Each of the other authors listed were involved in proof reading and some refinement of sentence structure.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Karina Griffin

Date: 16 July 2018

Media pH and media type can significantly affect the reliability of in vitro copper tolerance assessments of *Pseudomonas syringae* pv. *tomato.*

4.2 Abstract

Aims: There are inconsistencies with *in vitro* copper tolerance screening methodology for *Pseudomonas syringae* pv. *tomato* in the current literature, particularly in relation to the appropriate medium to use, copper tolerance thresholds and reporting medium pH and/or pH adjustment steps. This study investigates the effect of medium and pH on copper tolerance results, including the potential use of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer to stabilise media pH.

Methods and Results: Copper tolerance methodology was investigated through *in vitro* and *in vivo* testing of *P. syringae* pv. *tomato.* Four different media were tested, nutrient agar, Casitone yeast extract glycerol agar, King's B medium and potato dextrose agar. Highly variable copper tolerance profiles were observed for different isolates on the media tested. A pH range of 5.8-7.0 produced consistent copper tolerance data; outside of this range the data was unreliable. Addition of MES to media, buffered the pH to within the acceptable levels.

Conclusions: Copper tolerance thresholds with different media can vary significantly and the lowering effect of copper sulfate on media pH must be considered in media preparation.

Significance and Impact of Study: Methodology presented in the study can be extrapolated to copper tolerance testing for other pathogenic plant bacteria, particularly other pseudomonads.

4.3 Introduction

Pseudomonas syringae pv. *tomato* (Young *et al.* 1978) is a phytopathogenic bacterial species causing bacterial speck on tomatoes (*Solanum lycopersicum* L.). Globally, control of *P. syringae* pv. *tomato* predominantly involves the utilization of copper-based chemical applications. Copper-based chemicals have been the foundation of fungicidal and bactericidal treatments in cropping systems for over 100 years (Merry *et al.* 1983). The availability of alternatives to copper-based products varies between countries and are still not universally used. In the 1980s, *P. syringae* pv. *tomato* isolates exhibiting tolerance to copper-based treatments were reported in a number of American studies, with these works linking the effect to the acquisition of a DNA plasmid (Bender and Cooksey 1986; Mellano and Cooksey 1988a,b). Since this discovery, many reports of copper tolerance in *P. syringae* pv. *tomato*, as well as *Xanthomonas* species infecting tomato and pepper, have been published (Pernezny *et al.* 1995; Shenge, Wydra *et al.* 2008; Griffin *et al.* 2017).

In vitro screening for copper tolerance in bacteria involves monitoring growth using either an agar medium or a broth amended with various concentrations of copper, usually from copper sulphate

pentahydrate (CuSO4.5H2O). Sulphate ions were demonstrated to have no effect on bacterial growth in medium (Zevenhuizen *et al.* 1979), making the use of copper sulphate a prudent choice for copper tolerance screening. While 93% of published studies use an agar-based methodology, surprisingly, there is no standardized medium preparation for this type of testing (Griffin *et al.* 2017). Organic additives, such as peptones and sugars, can affect how bacteria grow on the copper-amended medium. This is a result of the complexation of copper ions by these additives, making them less or more available to interact with the bacteria (Zevenhuizen *et al.* 1979; Menkissoglu and Lindow 1991b; Kyeremeh *et al.* 1998; Hasman *et al.* 2009; Cornish *et al.* 2017). Variations in medium composition that influence copper ion concentration are known to directly affect copper tolerance results (Rathnayake *et al.* 2013). This variation limits direct comparisons of bacterial copper tolerance between studies (Hasman *et al.* 2009).

A limited number of publications are currently available analysing the effect of different media in copper tolerance determination of pathogenic plant bacteria, each producing study-specific recommendations. Zhang *et al.* (2017) reported the use of Castione yeast extract (CYE)- glucose agar produced better results than nutrient agar (NA) for the detection of tolerance in *P. syringae* pv. *phaseolicola*. However, Pernezny *et al.* (2008) reported NA with 0.5% w/v glucose was more suitable than CYE-glucose in a study investigating copper tolerance of xanthomonads. Whereas, Kyeremeh *et al.* (1998) selected potato dextrose agar (PDA) as their medium of choice, with no clear distinction between sensitive and tolerant *Erwinia carotovora* isolates on CYE-glucose agar. Silva and Lopes (1995) observed significant variation in copper tolerance levels *in vitro* for *P. syringae* pv. *tomato* with NA, 535 medium, copper-complexing minimal medium (MMCC; commonly called Casitone yeast extract glycerol agar (CYEG)), and King's B medium (KB). Collectively, these studies indicate that the pathogen system being studied is a major determinant of medium suitability. It may be that certain species grow more vigorously on a specific medium, or the biological mechanisms for reducing copper toxicity within the bacteria affect how they interact with added copper.

In vitro copper tolerance thresholds are generally reported as the minimum inhibitory concentration (MIC) of copper sulphate, which is the concentration of copper at which bacteria can no longer grow. The MIC threshold indicating copper tolerance varies between publications, with approximately 50% of copper tolerance studies of *Xanthomonas* and *Pseudomonas* in tomatoes and pepper using growth at a single concentration of 0.8 mmol/L copper sulphate. Whereas, other studies test at a range of concentrations (Griffin *et al.* 2017). The reason for the selection of 0.8 mmol/L as a single threshold value is not clear, with most studies citing previous work such as Stall *et al.* (1986), Bender and Cooksey (1986), and Ritchie and Dittapongpitch (1991). However, in these publications the rationale for selecting 0.8 mmol/L is not discussed. In Bender and Cooksey (1986), a MIC of 0.4–0.6 mmol/L. was

considered copper sensitive, with a MIC of up to 1.2 mmol/L being tolerant and 1.6–2.0 mmol/L being resistant. This study also correlated the presence of plasmids with *in vitro* results; however, they did not assess *in vivo* levels of control for tolerant vs resistant isolates. Given differences reported between media it is likely that a 0.8 mmol/L threshold may not be suitable for all media.

Furthermore, a study by Hasman *et al.* (2009) showed the pH of a medium can also significantly affect bacterial copper tolerance results. This pH effect is linked to two factors: firstly, medium pH affects growth of bacteria, and a pH outside species-specific optimal ranges can substantially impact their growth. The second factor is that the availability of free copper ions in copper-amended medium can change as the pH rises or drops. It is these free ions that are taken up by the bacteria and metabolized. If theses ions are not available, bacteria will grow and copper tolerance cannot be reliably determined. In an *Escherichia coli* study, Hasman *et al.* (2009) reported that to maintain an appropriate level of free copper ions in the medium the pH should be between pH 5–7. Precipitation of copper hydroxide (Cu(OH)2) occurs at pH >7 and reduced binding of copper to ligands at pH <5 (Hasman *et al.* 2009). Mixon (2012), demonstrated that *in vitro* copper tolerance results for *Xanthomonas* sp. Varied significantly across a pH range of 6.2–7.0. Likewise, growth of fungi on copper-amended medium varies with pH (Starkey 1973; Gadd and Griffiths 1980). Importantly, 74% of *P. syringae* pv. *tomato* and *Xanthomonas* sp. copper tolerance studies did not report the pH of the medium or report an adjustment being made to counter the pH reduction caused by copper sulphate addition (Griffin *et al.* 2017). This is significant as the addition of copper sulphate, even at low concentrations, reduces medium pH due to hydrolysis in solution and the consequential formation of sulphuric acid (H_2SO_4). No detailed studies are available investigating how medium pH affects in vitro copper tolerance assessments for *P. syringae* pv. *tomato*.

A study by Mixon (2012) used 2-(N-morpholino)ethanesulfonic acid (MES) buffer as a medium pH stabilizer for *in vitro* copper tolerance testing. Previously, MES was used to stabilize the pH of nutrient solutions for the testing of metal resistance in plants and for development of so-called 'minimal media' (Dos Santos Utmazian *et al.* 2007; Rathnayake *et al.* 2013). Both MES and 3-N-morpholino-propanesulfonic acid (MOPS) buffers show no binding with copper ions (Mash *et al.* 2003). To our knowledge, Mixon (2012) is the only researcher to report MES use in copper tolerance tests. However, there is potential for further investigation of MES use in copper tolerance screening of plant pathogenic bacteria.

This study aims to define appropriate methodology for the screening of *P. syringae* pv. *tomato* isolates for copper tolerance, in particular, investigation of the effect of medium pH on copper tolerance results. This study examined four agar media, NA, CYEG, KB and PDA, assessing their appropriateness

for copper tolerance determination. The effect of copper sulphate on the pH of these media, and the effect of pH variations on copper tolerance readings were examined. The use of MES to buffer medium pH change caused by the addition of copper sulphate was also investigated.

4.4 Materials and Methods

4.4.1 Bacterial isolate details and preparation

Tomato leaves and/or fruit displaying symptoms of *P. syringae* pv. *tomato* infection were collected from three tomato-producing regions in south-east Queensland, Australia. Species identification followed standard biochemical methods for the determination of *P. syringae* (Lelliott *et al.* 1966; Elkhalfi *et al.* 2013), followed by the detection of the hypersensitivity response and pathogenicity-associated Z gene (hrpZ; Zaccardelli *et al.* 2005) and citrate synthase (gltA) (Hwang *et al.* 2005; Berge *et al.* 2014) using gene-specific PCR amplification and subsequent DNA sequencing. Reaction conditions for the PCRs varied slightly to those originally published (Supplementary Table 4-6; section **Error! Reference source not found.**). Isolates collected for this study are available in the Plant P athology Herbarium of Biosecurity, Queensland Department of Agriculture and Fisheries, Australia.

A selection of 27 *Pseudomonas* isolates were used in this study (Table 4-1) including: 14 *P. syringae* pv. *tomato* isolates collected between 2015 and 2016; three reference isolates from the Agricultural Scientific Collections Trust, New South Wales, Australia (ASCT) and eight isolates collected between 1973 and 1985 from the Plant Pathology Herbarium of Biosecurity. A copper-tolerant isolate A1513R (Andersen *et al.* 1991) of *P. syringae* (pathovar unknown) and copper-sensitive *P. syringae* pv. *tomato* isolate DC3000 (Buell *et al.* 2003) were used as controls for copper tolerance testing.

All isolates were stored in 30% v/v glycerol at -20°C. Bacterial suspensions of approximately 1 x 10^8 CFU per ml prepared in sterile deionized water, from 48-h cultures on glucose nutrient agar (GNA), were used for all experiments.

Table 4-1: Pseudomonas spp. isolates tested in this study

Sample ID	*Pseudomonas* species	Source
BRIP66795	*P. syringae* pv. *tomato*	This study
BRIP66796	*P. syringae* pv. *tomato*	
BRIP66798	*P. syringae* pv. *tomato*	
BRIP66799	*P. syringae* pv. *tomato*	
BRIP66800	*P. syringae* pv. unknown	
BRIP66802	*P. syringae* pv. *tomato*	
BRIP66804	*P. syringae* pv. *tomato*	
BRIP66805	*P. syringae* pv. *tomato*	
BRIP66807	*P. syringae* pv. *tomato*	
BRIP66808	*P. syringae* pv. *tomato*	
BRIP66809	*P. syringae* pv. *tomato*	
BRIP66810	*P. syringae* pv. *tomato*	
BRIP66811	*P. syringae* pv. *tomato*	
STN001	*P. syringae* pv. *tomato*	
BRIP34814	*P. syringae* pv. *tabaci*	Plant Pathology Herbarium of Biosecurity, Queensland Department of Agriculture and Fisheries
BRIP34832	*P. syringae* pv. *striafaciens*	
BRIP34945	*P. viridiflava*	
BRIP38735	*P. syringae* pv. unknown	
BRIP38744	*P. syringae* pv. *tomato*	
BRIP38746	*P. syringae* pv. *tomato*	
BRIP38748	*P. syringae* pv. *syringae*	
BRIP38751	*P. cichorii*	
DAR65987	*P. marginalis*	Agricultural Scientific Collections Trust, New South Wales Australia (ASCT)
DAR77319	*P. syringae* pv. *syringae*	
DAR75965	*P. syringae* pv. *tomato*	
DC3000	*P. syringae* pv. *tomato*	University of Florida, United States of America
A1513R	*P. syringae*	

4.4.2 Effect of copper sulphate on media pH and the buffering pH with MES buffer

The pH of each medium (Table 4-2) was measured with an OrionTM 8102BNUWP ROSS UltraTM pH Electrode (ThermoFisher ScientificTM, Waltham, MA) at three stages during preparation: before and after boiling of the solution to dissolve the agar ingredients, and after autoclaving.

Prior to boiling, pH was adjusted to pH 7.00 +/- 0.2 where required. Molten agar was equilibrated to 65°C in a water bath prior to all pH measurements. After autoclaving, all testing was carried out under sterile conditions.

After autoclaving, the pH of each medium was adjusted to 6.8–7.0. The change in pH following the addition of copper sulphate ($CuSO_4.5H_2O$) was tested at the following concentrations: 0, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 and 5.0 mmol/L. The MES buffer (pH 69), sterilized with a 0.22 µmol/L syringae filter, was added to sterile molten agar to a final concentration of 20 mmol/L prior to adding copper sulphate.

Three replicate subsamples were tested in duplicate for each copper concentration, with or without MES. Fresh batches of media were prepared for the duplicate experiment. Data were analysed in R studio (ver. 3.2.3), using Levene's test for homogeneity of variance (Fox and Weisberg 2011), analysis of variance and Tukey's honestly significance difference (HSD) test (de Mendiburu 2017).

Table 4-2: Recipe details of agar media tested in this study.

Media	Ingredients
Nutrient Agar (NA)	Difco™ mixture: containing 3 g/L beef extract, 5 g/L peptone and 15 g/L agar.
King's B Media (KB)	Recipe King *et al.* (1954): containing 1.5 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 20 g/L Bacto™ Protease Peptone #3, 1.5 g/L dipotassium hydrogen phosphate (K_2HPO_4), 10 mL/L glycerol and 15 g/L Bacto™ Agar.
Casitone Yeast Extract Glycerol Agar (CYEG) [A]	Recipe Araújo *et al.* (2012): containing 1.7 g/L Bacto™ Casitone, 0.352 g/L yeast extract, 0.2% v/v glycerol and 15 g/L Bacto™ Agar.
Potato Dextrose Agar (PDA)	Difco™ mixture: containing 4 g/L potato starch from infusion, 20 g/L dextrose and 15 g/L agar.

[A] In *Pseudomonas* studies CYEG or CYE is generally made with glycerol (e.g. Silva and Lopes 1995; Spotts and Cervantes 1995), however some studies such as Zhang *et al.* (2017) substitute this with glucose.

4.4.3 Effect of pH on MIC of copper sulfate

Molten NA, CYEG, PDA and KB agar containing 20 mmol/L MES buffer (pH 6.9), amended with copper sulfate at 0, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 and 5.0 mmol/L, were adjusted to the following pH prior to pouring; 4.5, 5.5, 5.8, 6.2, 6.5, 6.8 and 7.0 (+/- 0.05).

Once set, plates were spot inoculated with 10 µL aliquots of bacterial suspensions. A 10 µL aliquot of sterile water was used as a control. The *Pseudomonas* isolates used for this experiment were; BRIP66799, BRIP66802, BRIP66810, BRIP38748 and BRIP38735. Plates were incubated at 24 °C for 48 h and then assessed for growth. Three replicate plates were inoculated for each copper concentration and pH, and the experiment was repeated three times with fresh batches of media.

4.4.4 Effect of media on the MIC of copper sulfate

The MIC of copper sulfate for *Pseudomonas* isolates was determined on each of the four media containing 20 mmol/L MES. Concentrations tested were 0, 0.1, 0.2, 0.5, 0.8, 1.0, 1.5, 2.0 and 5.0 mmol/L. The pH of the media was adjusted to within 5.8-7.0, if required, with sterile 0.1 mol/L NaOH solution, prior to pouring.

Plates were spotted with bacterial suspensions, incubated and assessed as described previously. Up to 16 different isolates were tested on each plate, in a 4 x 4 grid pattern. Three replicate plates were spotted for each experiment, and the experiment was replicated three times with fresh batches of

media. The MIC of each isolate on each medium was recorded and isolates were grouped together if they had the same MIC on all media, this created a series of MIC profiles.

4.4.5 Confirmation of copper tolerance *in vivo*

In vivo copper tolerance was tested with isolates DAR75965, BRIP66796 and BRIP66810 as they had different MIC profiles on CYEG. These were 0.8, 1.0 and 1.5 mmol/L respectively.

Tomato cv. Entice were transplanted from seedling trays, at the three to four leaf stage, into 50-mm black plastic square seedling planters in potting mix (Brunnings Brand Potting Mix) with one plant per pot. Plants were grown in open air greenhouse conditions under natural light. Over the testing period, temperature ranged from 15 to 27°C and a relative humidity of 40– 80%. A completely randomized design was used with three plants in a set and three replicates for each treatment. Each set was contained within a cylindrical clear plastic container. These sets were distributed randomly on the greenhouse shelf and rotated randomly each week. Physical barriers were used to ensure no cross-contamination between isolates and treatments.

Treatments were based on commercial application rates, treatments were; an untreated control (UTC), copper hydroxide (CuOH; Champ Dry Prill WG Nufarm 375g ai/kg) at 140 g/100 L, copper oxychloride (CuOxy; Barmac Copper Oxychloride Fungicide 500g ai/kg) at 250 g/100 L, copper sulfate (CuSO; $CuSO_4.5H_2O$ 190 g ai/L) at 280 mL/100L and copper oxide (CuOx; Nordox 75 WG 750 g ai/kg) at 105 g/100 L. A tank mixture of CuOH at 160 g/100 L and manganese/zinc ethylene bis-dithiocarbamates (EBDC; Dithane Rainshield Dow Agrosciences 750 g ai/kg) at 80 g/100 L (CuOH + EBDC) was also used. These rates were equivalent to the commercial formulation ManKocide™ (DuPont). Copper compounds were applied using a hand held plastic spray bottle to the point of run-off. Five applications were made at a 6-8 day intervals, with 2 applications prior to inoculation and 3 after inoculation (Pernezny *et al.* 2008).

Bacterial inoculum was prepared to approximately 1 x 10^8 CFU/mL in 10 mmol/L $MgSO_4.7H_2O$, from 48-h GNA cultures (Pernezny *et al.* 2008; Pontes *et al.* 2015). Inoculum was applied to plants to the point of run-off 20 days after transplanting, ensuring thorough coverage of both the upper and lower leaf surfaces. Overhead sprinklers watered plants every 2-3 days to promote disease development.

Assessments of disease severity were made 15, 22 and 33 days after inoculation (DAI). Assessments included: a count of the number of disease lesions on the foliage of each plant; a visual estimation of the percentage of the leaf area displaying disease symptoms on the third and fourth leaf; and the percentage incidence of leaves displaying disease symptoms on each plant.

4.5 Results

4.5.1 Effect of copper sulfate on media pH and buffering pH with MES buffer

Boiling of the medium solution to dissolve agar ingredients reduced the pH of all four media tested by approximately 0.1 pH units. Autoclaving of the media caused reductions of 0.02–0.1, 0, 0.4–0.8 and 0.1 pH units in NA, KB, PDA and CYEG respectively.

Media pH dropped significantly with the addition of copper sulphate at concentrations of 1 mmol/L and above in NA, PDA and CYEG (Figure 4-1). The pH of KB only had a significant drop with 5 mmol/L copper sulphate.

The addition of MES significantly buffered the pH drop in NA, PDA and CYEG, keeping the pH within 1.00 units of the initial pH at all copper sulphate concentrations (Figure 4-1). The KB medium with and without MES remained within 0.75 units of the initial pH at all copper sulphate concentrations. The MES buffer formed a precipitate with copper sulphate when mixed together; therefore, MES was added to the medium and mixed prior to adding copper sulphate.

The capacity of bacteria to grow on copper-amended NA, PDA, CYEG and KB was not affected by the addition of MES (data not shown).

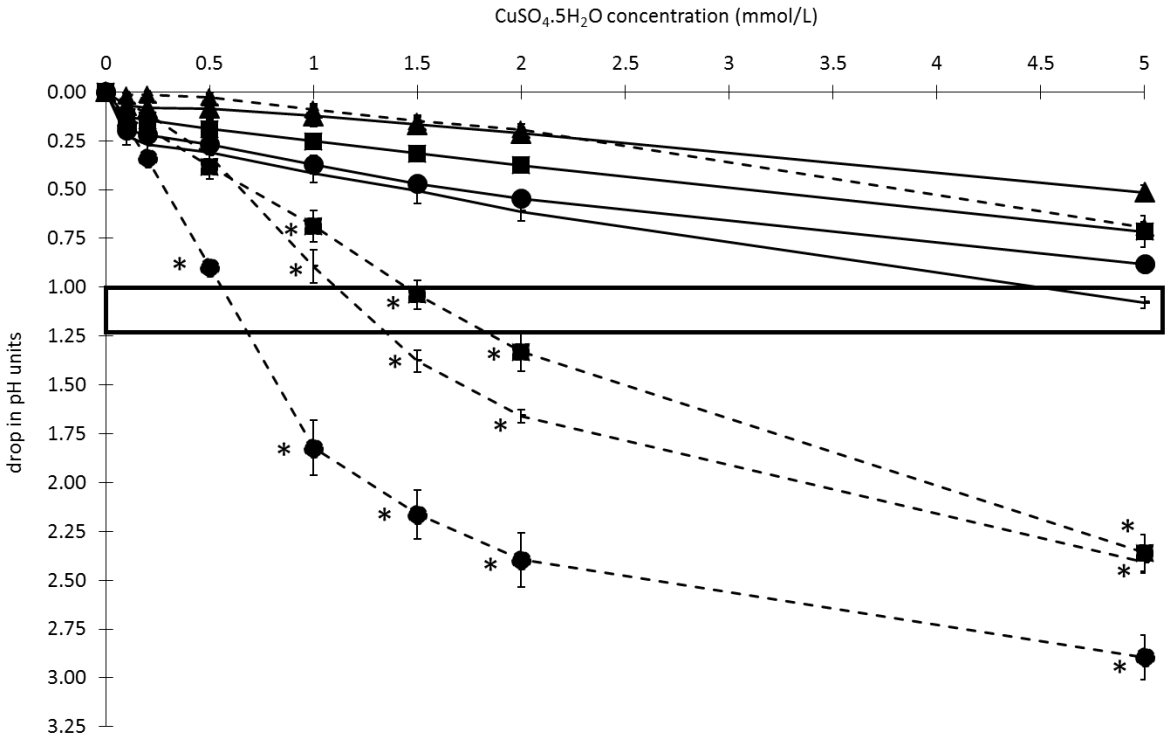

Figure 4-1: Change in media pH units when adding copper sulfate to four different media with and without 20 mmol/L MES buffer at pH 6.9. Boxed area between 1-1.25 pH units represents when the pH starts to negatively affect the reliability of copper tolerance results. Initial pH was between 7 and 6.8 for each media. Error bars represent the standard deviation of replicated data. Three replicate subsamples were tested in duplicate at each copper concentration and with or without MES.

Key: NA = no symbol, KB = ▲, PDA = ■ , CYEG = ●; Dotted lines represent pH units of each media alone; Non-broken lines represent each media with MES added.

*** indicate mean values are significantly lower compared to that media amended with MES as determined by Tukey HSD p<0.05.**

4.5.2 Effect of pH on MIC of copper sulfate

When no copper sulphate was added to media, *P. syringae* isolates BRIP66799, BRIP66802, BRIP66810, BRIP38748 and BRIP38735, grew at pH 5.5–7.0. At pH 4.5, all isolates grew on CYEG with no copper sulphate but they did not grow on NA, KB and PDA, with the exception of BRIP38735 which grew on NA (Table 4-3).

The MIC of the isolates tested did not vary significantly over a pH range of 5.8–7.0 on NA, CYEG, PDA and KB agar. At pH 5.5, the MIC varied for some isolates on KB, NA and CYEG. They had either a higher or lower MIC compared to MIC results obtained within the pH 5.8–7.0 range. Even though isolates grew on CYEG at pH 4.5, MICs tended to be different than at pH 5.8–7.0.

Table 4-3: Effect of changes in pH level on the Minimum Inhibitory Concentration (MIC) of copper sulfate on different media. Isolates tested were BRIP66799, BRIP66802, BRIP66810, BRIP38735 and BRIP38748. Data was replicated three times with fresh batches of medium.

Medium	Isolate ID	MIC (mmol/L)						
		pH 4.5	pH 5.5	pH 5.8	pH 6.2	pH 6.5	pH 6.8	pH 7.0
CYEG	**BRIP66799**	0.0	0.5	1.0	1.0	1.0	1.0	1.0
	BRIP66802	0.2	0.8	1.0	1.0	1.0	1.0	1.0
	BRIP66810	0.0	1.0	1.5	1.5	1.5	1.5	1.5
	BRIP38735	0.2	0.2	0.2	0.2	0.2	0.2	0.2
	BRIP38748	0.0	0.0	0.1	0.1	0.1	0.1	0.1
NA	**BRIP66799**	NG	0.8	2.0	2.0	2.0	2.0	2.0
	BRIP66802	NG	1.0	2.0	2.0	2.0	2.0	2.0
	BRIP66810	NG	1.0	2.0	2.0	2.0	2.0	2.0
	BRIP38735	0.1	0.5	0.5	0.5	0.5	0.5	0.5
	BRIP38748	NG	0.0	0.2	0.2	0.2	0.2	0.2
KB	**BRIP66799**	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP66802	NG	5.0	2.0	2.0	2.0	2.0	2.0
	BRIP66810	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP38735	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP38748	NG	0.2	0.5	0.5	0.5	0.5	0.5
PDA	**BRIP66799**	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP66802	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP66810	NG	2.0	2.0	2.0	2.0	2.0	2.0
	BRIP38735	NG	0.5	0.5	0.5	0.5	0.5	0.5
	BRIP38748	NG	0.0	0.0	0.0	0.0	0.0	0.0

Abbreviation: NG = no growth

4.5.3 Effect of media on the MIC of copper sulfate

Media containing MES was used for these experiments given its capacity to stabilize media pH (Figure 4-1). The MIC of isolates varied between media. Multiple isolates tested in this study grouped into MIC profiles A and C, whereas many isolates had unique profiles (Table 4-4). The MIC values were highest overall on KB medium.

Table 4-4: The Minimum Inhibitory Concentration (MIC) of copper sulfate of *Pseudomonas* spp. isolates with four different media. Isolates are grouped into MIC profiles, which indicate that the MIC values obtained on each media are equivalent. Data was replicated three times with fresh batches of medium.

Sample ID	Minimum Inhibitory Concentration (mmol/L)				MIC Profile
	KB	NA	CYEG	PDA	
BRIP66798	2.0	2.0	1.0	2.0	A
BRIP66799	2.0	2.0	1.0	2.0	
BRIP66800	2.0	2.0	1.0	2.0	
BRIP66802 [A]	2.0	2.0	1.0	2.0	
STN001	2.0	2.0	1.0	2.0	
BRIP66810 [A]	2.0	2.0	1.5	2.0	B
BRIP66795	5.0	2.0	1.0	2.0	C
BRIP66796 [A]	5.0	2.0	1.0	2.0	
BRIP66804	5.0	2.0	1.0	2.0	
BRIP66805	5.0	2.0	1.0	2.0	
BRIP66807	5.0	2.0	1.0	2.0	
BRIP66809	5.0	2.0	1.0	2.0	
BRIP66811	5.0	2.0	1.0	2.0	
BRIP66808	5.0	2.0	1.0	2.0	
BRIP38751	5.0	2.0	0.5	2.0	D
BRIP34814	2.0	0.8	0.8	0.5	E
BRIP34832	2.0	0.8	0.2	0.5	F
BRIP34945	5.0	2.0	2.0	5.0	G
BRIP38735	2.0	0.5	0.2	0.5	H
BRIP38744	2.0	2.0	1.5	5.0	I
BRIP38746	2.0	2.0	1.5	5.0	
BRIP38748	0.5	0.2	0.1	0.0	J
DAR65987	5.0	5.0	1.0	2.0	K
DAR77319	2.0	0.5	0.2	0.5	L
DAR75965 [A]	2.0	1.5	0.8	2.0	M
A1513R	5.0	2.0	1.5	2.0	N
DC3000	1.5	0.5	0.2	0.5	O

[A] Isolates used for *in vivo* testing.

4.5.4 Confirmation of copper tolerance in vivo

There were considerable differences in the overall severity of disease symptoms caused by the isolates tested and the efficacy of copper-based products for disease control was inconsistent (Table 4-5). Significant control was measured at times. However, in general, control with copper compounds was poor. In some cases there were numerical differences between copper- treated plants and the UTC. However, there were large variations in disease severity on plants within the treatment, resulting in high coefficients of variation (CV) and lack of significant differences between means according to Tukey's HSD test. Isolate DAR75965 seemed to be controlled to some degree with copper products by 33 DAI, based on disease severity assessments. Of the three isolates tested, DAR75965 had the lowest MIC (0.8 mmol/L on CYEG) in the in vitro tests.

Isolate BRIP66796 had a significantly lower number of lesions in the copper treatments compared to the UTC. However, these differences were not significant for either disease incidence or severity under low disease pressure. In another experiment, isolate BRIP66796 produced high disease incidence and severity that was not significantly reduced with copper products (Supplementary Table 4-7; section **Error! Reference source not found.**). Disease caused by isolate BRIP66802 (MIC = 1.0 mmol/L on CYEG) w as also shown to not be significantly controlled by copper products in a third in vivo trial (Supplementary Table 4-7; section **Error! Reference source not found.**). Methodological details of these two experiments is included as in Section 4.8.1 as additional information.

Table 4-5: Control of speck disease caused by *Pseudomonas syringae* pv. *tomato* on tomato under greenhouse conditions with copper-based bactericides. Disease assessments: mean number of disease lesions on the foliage of each plant; visual estimation of the mean percentage severity of disease symptoms; and mean percentage incidence of leaves displaying disease symptoms. A completely randomised design was used with 3 plants in a set and 3 replicates, giving 9 plants in total per treatment.

A: Mean number of lesions per plant									
Isolate ID	BRIP66796			DAR75965			BRIP66810		
Assess	15DAI	22DAI	33DAI	15DAI	22DAI	33DAI	15DAI	22DAI	33DAI
UTC	6	5	5	41	40	49	10	15	16
CuOH	2	1*	1*	6	8	5	1	1	2*
CuOH+ Man	2*	1*	1*	6	7	5	3	3.	3*
CuOxy	NT	NT	NT	10	14	16	7	6	7
CuOx	NT	NT	NT	7	8	7	6	5	7
CuSO	NT	NT	NT	16	15	15	10	2	4*
MSError	1**	6	9	805	791	1096	119	102	2
CV	97	121	117	199	186	205	172	191	72
HSD	1	3	4	40	39	46	15	14	2
B: Mean percentage disease severity on the 3-4th leaves									
UTC	1	2	2	13	14	18	4	5	7
CuOH	1	1	1	2	2	2*	1	1*	1
CuOH+ Man	0	0*	1	2	3	3*	1	2	1
CuOxy	NT	NT	NT	5	4	6*	2	2	2
CuOx	NT	NT	NT	2	3	3*	3	2	2
CuSO	NT	NT	NT	12	4	5*	4	1*	1
MSError	1	2	2	103	71	72	23	11	21
CV	119	122	111	163	163	135	191	154	187
HSD	1	2	2	14	12	12	7	5	6
C: Mean percentage incidence of disease									
UTC	31	40	25	70	83	60	39	67	49
CuOH	22	31	10	31	60	33	16	27*	11*
CuOH+ Man	9	22*	10	41	58	33	25	40	23
CuOxy	NT	NT	NT	36	47	35	35	47	27
CuOx	NT	NT	NT	49	66	35	35	38	26
CuSO	NT	NT	NT	49	56	42	29	42	21*
MSError	560	223	236	952	753	483	699	455	381
CV	115	48	102	67	45	55	88	49	75
HSD	28	18	18	43	38	31	37	30	27

Abbreviations: DAI = days after inoculation; MSError = Mean square error; CV = coefficient of variation; HSD = honestly significant difference; NT = not tested, additional data Table S2.

Treatments: Untreated control (UTC), copper hydroxide (CuOH; Champ Dry Prill WG Nufarm 375g ai/kg) at 140 g/100 L, copper oxychloride (CuOxy; Barmac Copper Oxychloride Fungicide 500g ai/kg) at 250 g/100 L, copper sulfate (CuSO; $CuSO_4.5H_2O$ 190 g ai/L) at 280 mL/100L, copper oxide (CuOx; Nordox 75 WG 750 g ai/kg) at 105 g/100 L and tank mixture of CuOH at 160 g/100 L and manganese/zinc ethylene bis-dithiocarbamates (EBDC; Dithane Rainshield Dow Agrosciences 750 g ai/kg) at 80 g/100 L (CuOH + EBDC)

* Indicates mean is significantly ($p<=0.05$) different to the UTC based on Tukey's Honestly Significant Difference test.

** Data square-root transformed to fulfil requirement of equal variance for the Tukey test, means presented are untransformed data.

4.6 Discussion

Results of this study demonstrate that agar medium and its pH can significantly affect the reliability of *in vitro* copper tolerance assessments for *P. syringae*. The addition of copper sulphate significantly reduced the pH of media, affecting the reliability of copper tolerance results. These data support previous findings of variability in copper toxicity caused by low medium pH for *Scytalidium* sp. (Starkey 1973), *Aureobasidium pullulans* (Gadd and Griffiths 1980), *E. coli* (Hasman *et al.* 2009) and *Xanthomona*s sp. (Mixon 2012). Each of these studies report different pH thresholds for specific fungal or bacterial species, demonstrating the importance of species-specific testing prior to selection of an appropriate pH range. Measuring and adjusting medium pH after the addition of copper sulphate prior to copper tolerance testing *in vitro* is essential to ensure the pH is in the required range for the target pathogen.

The use of MES as a buffering agent for pH reduction caused by copper sulphate was highly effective, making it an appropriate addition for the maintenance of pH within an acceptable range for testing copper tolerance of *P. syringae* pv. *tomato* isolates. Addition of MES to media can dramatically decrease the time taken in the preparation of plates as no further adjustment of pH is required after the addition of copper sulphate. A single adjustment to pH 7.0–7.1 can be made after mixing the ingredients in water, prior to autoclaving. Especially useful is that adjustment at this point does not require sterile conditions and raised temperatures, simplifying medium preparation. Given that MES did not affect the growth of *P. syringae* pv. *tomato*, and does not complex copper ions (Mash *et al.* 2003), it is an ideal buffering agent. We recommend that MES be added to any medium used for copper tolerance testing for other plant pathogenic bacteria.

The pH of KB media was fairly stable even after the addition of copper sulphate, so that it can be used without MES or extra pH adjustment. However, the MIC of copper sulphate in KB had a much higher threshold than other media tested. Isolate DC3000, the copper-sensitive reference, had a MIC of 1.5 mmol/L in KB, well above the standard 0.8 mmol/L tolerance indicator reported in most publications. A high MIC in KB was also reported by Silva and Lopes (1995), who found that 250 ppm (1.0 mmol/L) copper sulphate in KB medium was equivalent to CYEG with 50 ppm (0.2 mmol/L) copper sulphate. The presence of dipotassium hydrogen phosphate (K_2HPO_4) in KB likely produces a buffering effect in the medium (Wishart *et al.* 2017), hence the observed stabilization of pH. Goto *et al.* (1993) demonstrated phosphate buffer increased observed copper tolerance. Therefore, the phosphate-buffering capacity of K_2HPO_4 may lead one to conclude that isolates are quite resistant to copper when in fact they are not. In addition, other components in KB, including peptones and sugars, may complex copper ions, making them unavailable (Zevenhuizen *et al.* 1979; Menkissoglu and Lindow 1991b;

Kyeremeh *et al.* 1998; Hasman *et al.* 2009; Cornish *et al.* 2017). It is therefore recommended to avoid the use of KB for copper tolerance testing, given the high tolerance thresholds measured.

The significant variability in MIC results obtained on the different media tested in this study and previous publications (Silva and Lopes 1995; Kyeremeh *et al.* 1998; Pernezny *et al.* 2008) highlight the importance of medium selection for copper tolerance testing. An MIC of 0.8 mmol/L on one medium is not equivalent to 0.8 mmol/L on another medium, making comparisons between studies difficult and potentially misleading the interpretation of results. Given these differences and the observation that many pseudomonad isolates had unique MIC profiles in this study, a standardization of methodology is required, both in the type of medium used and its preparation. Adopting standard methodology would allow for meaningful comparisons of copper tolerance between tomato production areas.

The pathogen system being investigated is a major determinant of the suitability of different media for copper tolerance determination. However, it is logical to select a medium with a low copper ion-complexing capacity to ensure that these ions are available to interact with the bacteria or fungi being tested. The effect on their growth can then be quantified with some certainty. Two examples of low copper ion-complexing mediums are the MES-buffered minimal medium formulated by Rathnayake *et al.* (2013) containing over 10 ingredients, and CYEG agar (Zevenhuizen *et al.* 1979; Silva and Lopes 1995) containing only four ingredients. Replacing a proportion of the peptones in a recipe with ammonium phosphate has also been suggested to increase the availability of free copper ions in the medium (Cornish *et al.* 2017). Simple medium recipes are more convenient to prepare and results of this study suggest CYEG medium may be particularly appropriate for copper tolerance testing of *P. syringae* pv. *tomato* isolates. This medium has also been used for other *P. syringae* pathovars (Menkissoglu and Lindow 1991b; Spotts and Cervantes 1995) and has potential for use with other phytopathogenic bacteria that grow on CYEG.

In vivo data collected in this study demonstrated the inconsistency of copper products for the control of disease caused by *P. syringae* pv. *tomato* isolates with MIC levels ≥0.8 mmol/L copper sulphate on CYEG. There was a significant reduction in the severity of disease caused by isolate DAR75965 (MIC = 0.8 mmol/L) with copper products. However, these differences were not statistically significant for percentage incidence and the number of lesions. An MIC value of 08 mmol/L in CYEG may represent the copper tolerance threshold that corresponds to a lack of control of bacterial speck. Furthermore, the detection of *copA* gene fragments in isolates BRIP66796, BRIP66802, BRIP66810 and DAR75965, which were homologous to copper-tolerant reference sequences in GenBank (NCBI), suggest study isolates carry genetically mediated copper tolerance (unpublished data). Results for the copper-

sensitive reference isolate, DC3000, suggest that a MIC value of ≤0.2 mmol/L on CYEG, be considered copper sensitive. This MIC threshold of CYEG is in agreement with the *P. syringae* pv. *tomato* copper sensitivity threshold of 50 ppm (0.2 mmol/L) copper sulphate, determined *in vivo* by Silva and Lopes (1995). Further investigation and *in vivo* studies using a wider variety of sensitive and tolerant *P. syringae* pv. *tomato* with variable MIC values would be useful to confirm tolerance thresholds.

Commercial rates of copper-based products used *in vivo* for the control of disease caused by *P. syringae* pv. *tomato*, had a significantly higher concentration of copper (213–615 mmol/L) than those used for *in vitro* testing (0.1–5 mmol/L). As with *in vitro* copper tolerance testing, *in vivo* experiments are affected by the availability of free copper ions on plant surfaces. On plants, copper availability from sprays is dependent on climatic conditions, the copper formulation, leaf surface pH, the presence of free water and the release of compounds from plant tissues (Menkissoglu and Lindow 1991a; Scheck and Pscheidt 1998). These factors reduce copper availability to a much larger degree than in media used for *in vitro* testing. It is, therefore, not surprising that a larger amount of copper is required when applied *in vivo* to detect copper tolerance.

This study demonstrates that minor variations in methodology can significantly affect in vitro copper tolerance results for *P. syringae* pv. *tomato*. The MIC of copper sulphate obtained for various pseudomonads varied significantly between the four media tested. We suggest avoiding KB for copper tolerance testing of *P. syringae* pv. *tomato* given the potential phosphate buffering and high MIC levels observed in the copper-sensitive control. While NA, PDA or CYEG are considered appropriate media for tolerance testing, CYEG is our preferred medium, given its low copper-complexing capacity and previously published use for other *P. syringae* pathovars. The lowering effect of copper sulphate on medium pH must be considered in the preparation of agar medium, due to the poor reliability of tolerance results outside a pH range of 5.8–7.0. The addition of MES to NA, PDA or CYEG medium prior to adding copper sulphate, significantly buffered pH changes to within acceptable levels and can significantly reduce agar plate preparation time. Data collected highlight how important standardization of methods is for producing comparable results regionally and internationally. The data presented should be considered in any laboratory that is completing copper tolerance testing in other pathogenic plant bacteria, particularly other pseudomonads.

4.7 Published supplementary tables

Table 4-6: Reaction details for PCR amplification of *h*ypersensitivity *r*esponse and *p*athogenicity-associated *Z* gene (*hrpZ*) and citrate synthase *(gltA)*. DNA amplification was carried out in a Kyratec SuperCycler Thermal Cycler (model SC300).

Gene target	*hrpZ*	*gltA*
Reaction ingredients (total volume 50 µL)	• 1µL of bacterial suspension in sterile water (approx. 7.2 x 10^7 CFU/mL; OD_{620nm} = 0.03-0.05) • 0.2 µmol/L of each primer • 25 µL MangoMix™ (Bioline BIO-25033, contains 2.5 mmol/L $MgCl_2$, MangoTaq™ DNA Polymerase, dNTPs, 5x coloured reaction buffer). • 22 µL sterile deionised water	• 1µL of bacterial suspension in sterile water (approx. 7.2 x 10^7 CFU/mL; OD_{620nm} = 0.03-0.05) • 0.2 µmol/L of each primer • 0.2 mmol/L dNTPs • 5 µL 5x Mango*Taq*™ Coloured Reaction Buffer (Bioline) • 2.5 mmol/L $MgCl_2$, 0.3 µL MangoTaq™ DNA Polymerase (Bioline) • 38.2 µL sterile deionised water
Amplification program	Denaturation at 95 °C for 1 min 30 cycles: • Denaturation 95 °C for 20 s • Annealing 60 °C for 20 s • Extension 72 °C for 30 s Final extension at 72 °C for 5 min	Denaturation at 95 °C for 1 min 30 cycles: • Denaturation 95 °C for 20 s • Annealing 56 °C for 20 s • Extension 72 °C for 45 s Final extension at 72 °C for 5 min
Expected fragment size (bp)	532	1018

Table 4-7: Additional *in vivo* testing of *Pseudomonas syringae* pv. *tomato* control on tomato under greenhouse conditions with copper-based bactericides. Disease assessments include: the mean number of disease lesions on the foliage of each plant; a visual estimation of the mean percentage severity of disease symptoms; and the mean percentage incidence of leaves displaying disease symptoms.

Isolate ID BRIP66796									
	Mean number of lesions per plant			Mean percentage disease severity on the 3-4th leaves			Mean percentage incidence of disease		
Assess	5DAI	13DAI	30DAI	5DAI	13DAI	30DAI	5DAI	13DAI	30DAI
UTC	16	23	23	5	9	11	74	70	58
CuOH H	17	29	26	6	10	14	65	79	66
CuOxy H	20	33	32	7	11	15	79	78	71
CuSO H	23	51*	45	8	14	22	85	84	82*
MSError	153	484	318	33	79	140	327	233	274
CV	65	65	57	89	79	76	24	20	24
HSD	16	28	23	7	11	15	23	20	21

Isolate ID BRIP66802						
	Mean percentage disease severity on the 3-4th leaves			Mean percentage incidence of disease		
Assess	7DAI	17DAI	25DAI	7DAI	17DAI	25DAI
UTC	31	39	41	55	48	51
CuOH L	40	57	61	75	65	61
CuOH H	7	19	27	61	53	57
CuOxy L	27	38	42	68	67	74
CuOxy H	8	10	14	53	61	58
CuSO L	17	34	39	66	72	67
CuSO H	18	23	30	57	63	62
MSError	522	713	840	430	330	270
CV	108	85	80	33	30	27
HSD	41	48	52	37	33	30

Abbreviations: DAI = days after inoculation; MSError = Mean square error; CV = coefficient of variation; HSD = honestly significant difference.

Treatments: Untreated control (UTC), copper hydroxide (CuOH; Champ Dry Prill WG Nufarm 375g ai/kg) at 105 (L) and 140 (H) g/100 L, copper oxychloride (CuOxy; Barmac Copper Oxychloride Fungicide 500g ai/kg) at 220 (L) and 250 (H) g/100 L and copper sulfate (CuSO; $CuSO_4.5H_2O$ 190 g ai/L) at 220 (L) 280 (H) mL/100L.

* Indicates mean is significantly ($p<=0.05$) different to the UTC based on Tukey's Honestly Significant Difference test.

4.8 Additional Information

These sections were no included in the publication.

Three *in vivo* experiments were completed to confirm that isolates were copper-tolerant, the results of these experiments are presented in the publication (Table 4-5 and Table 4-7). Additional methodological information for these experiments is provided as additional information (section 4.8.1).

The incubation of isolates in copper solutions prior to plating on agar media was investigated as a method for copper tolerance assessment. Results of this testing were not included in the publication, therefore, they are included as additional content in this chapter (section 4.8.2).

4.8.1 *In vivo* experimental data on the efficacy of copper based products for the control of disease caused by *P. syringae* pv. *tomato*

4.8.1.1 Methods

Tomatoes were transplanted from seedling trays, at the 3-4 leaf stage, into plastic pots with commercial potting mix (Brunnings Potting Mix) with one plant per pot. Plants were grow in open air greenhouse conditions under natural light, and over the testing periods temperatures ranged from 15-30 °C and 40-80% relative humidity. A randomised experimental design was used with 3 plants in a set and 3 replicates for each treatment, giving 9 plants in total for each treatment. Treatments were separated by physical barriers to avoid cross-contamination during treatment application, inoculation and watering.

Treatments were based on commercial application rates. These included; an UTC, CuOH at 105 and 140 g/100 L, CuOxy at 220 and 250 g/100 L, and CuSO at 220 and 280 mL/100 L. These copper mixtures were applied using a hand held plastic spray bottle to the point of run-off, and a total of five applications were made.

Bacterial inoculum was prepared to approximately 1 x 10^8 CFU/mL in 10 mmol/L $MgSO_4.7H_2O$, from 48 h GNA cultures (Pernezny *et al.* 2008; Pontes *et al.* 2015). Inoculum was applied to plants to the point of run-off, ensuring thorough coverage of both upper and lower leaf surfaces. Overhead sprinklers watered plants every 2-3 days to promote disease development. The application, inoculation and assessment timing varied slightly between experiments (Table 4-8). These differences were not considered to significantly affect the experimental outcome.

Table 4-8: Experimental conditions for the *in vivo* testing of copper tolerance with *P. syringae* pv. *tomato* isolates BRIP66796 and BRIP66802.

Experiment Number	1	2
Tomato cultivar	Entice	Pinnacle
Isolate ID	BRIP66796	BRIP66802
Treatment and Application Rate	UTC CuOH @ 140 g/100 L CuOxy @ 250 g/100 L CuSO @ 280 mL/100L	UTC CuOH @ 105 and 140 g/100 L CuOxy @ 220 and 250 g/100 L CuSO @220 and 280 mL/100L
First Application (DAT)	4	11
Application interval (days)	7	7
Inoculation (DAT)	19	22
Last application (DAT)	32	39
Assessment timings (DAI)	5, 13, 30	7, 17, 25

Abbreviations: DAT = Days after transplanting, DAI = Days after inoculation, UTC = untreated control, CuOH = copper hydroxide, CuOxy = copper oxychloride, CuSO = copper sulfate, CuOx = copper oxide and CuOH + EBDC = copper hydroxide plus manganese/zinc ethylene bis-dithiocarbamate tank mix.

Assessments of disease were made on each plant, these included: a count of the number of disease lesions on the foliage; the percentage severity of infection as a visual estimation of the percentage of the leaf area displaying disease symptoms on the 3rd and 4th leaf; and the percentage incidence of leaves displaying disease symptoms. Experiment 1 was completed in August 2016 with isolate BRIP66796 and experiment 2 was completed in November 2016 with isolate BRIP66802. Experiment 3 was completed in August 2017 with isolates BRIP66796, BRIP66810 and DAR75965, data for this experiment is presented within the published journal article as supplementary material (Table 4-5).

Data was analysed in R studio, using Levene's Test for homogeneity of variance, ANOVA and Tukey's HSD test.

4.8.1.2 Results

Copper products CuOH, CuOxy and CuSO applied at commercial rates did not provide significant control of bacterial disease symptoms, caused by *P. syringae* pv. *tomato* isolates BRIP66796 and BRIP66802, when compared to the UTC (Table 4-7). With CuSO at the high rate, there was a significantly higher mean number of lesions compared to the UTC on plants inoculated with BRIP66796.

4.8.1.3 Conclusion

The failure of copper-based products to effectively control disease symptoms caused by *P. syringae* pv. *tomato*, suggests that isolates BRIP66796 and BRIP66802 are copper-tolerant, hence other isolates with equivalent MIC levels are also likely to be tolerant.

4.8.2 Copper tolerance assessments using copper solutions

The systematic review of literature (Chapter 2) found that 93% of copper tolerance publications used copper-amended agar media to screen *P. syringae* pv. *tomato* and *Xanthomonas* spp. isolates for *in vitro* tolerance. The remaining publications used broth based methodology or incubated of bacteria in copper solutions prior to plating on agar media to screen for copper tolerance (Adaskaveg & Hine 1985; do Carmo *et al.* 2001; Marco & Stall 1983; Martin *et al.* 2004). The potential of using copper solutions to assess copper tolerance was explored.

4.8.2.1 Methods

Methods of Pernezny *et al.* (2008), do Carmo *et al.* (2001), Marco and Stall (1983) and Adaskaveg and Hine (1985) were adapted in these experiments. Three isolates were selected for testing based on their MIC profiles observed with copper-amended agar medium (Table 4-4). These were BRIP38748 (MIC = 0.2 mmol/L on CYEG), BRIP66802 (MIC = 1 mmol/L on CYEG, 2mmol/L on KB) and BRIP66796 (MIC = 1 mmol/L on CYEG, 5 mmol/L on KB). Bacterial suspensions of approximately 2 x 10^8 CFU/mL were prepared from 24-36 h cultures on GNA in sterile deionised water.

Copper solutions of different commercial copper formulations were prepared in deionised water and sterilised via autoclaving. Concentrations used were based on commercial application rates specified on product labels. These where: CuOH at 105 g/100 L (low rate) and 140 g/100L (high rate), CuOxy at 220 g/100L (low rate) and 250 g/100L (high rate) and CuSO at 220 mL/100L (low rate) and 280 mL/100L (high rate). Sterile deionised was used as the control.

The following was added to a sterile 50 mL conical flask, under aseptic conditions; 10 mL of the copper solution and 10 mL of bacterial suspension. These mixtures were incubated at room temperature with gentle shaking (60 rpm) for 2 h. The mixtures were then gravity filtered through sterile Watman No. 2 filter paper before preparing a serial dilution down to 10^{-4}. Spread plates were prepared with 100 µL of each sample on GNA, three replicate plates were spread per sample. Plates were incubated for 72 h at 24 °C before counting the number of colonies present, plates containing more than 300 colonies were not counted. Five experiments were completed, with freshly prepared copper and bacterial solutions prepared each time.

4.8.2.2 Results and Discussion

Copper solutions

The response of BRIP38748, BRIP66802 and BRIP66796 to incubation in copper solutions varied considerably between experiments (Table 4-9), with colony growth observed on plates containing isolates incubated in copper solutions in some experiments but not others. There were also instances where colonies in control treatments failed to grow on GNA. The methods used were almost identical to those used by Pernezny *et al.* (2008) and similar to those used by Marco and Stall (1983), do Carmo *et al.* (2001) and Adaskaveg and Hine (1985). However, given the poor replicability of data collected in these experiments the use of copper solutions for determining copper tolerance was not pursued further for the purposes of this thesis study.

Table 4-9: Results of *in vitro* copper tolerance testing of isolates BRIP38748, BRIP66802 and BRIP66796 using copper solutions. Data presented as the number of colony forming units per mL (CFU/mL) present on GNA plates and the percentage of growth relative to the non-inoculated control (%control).

Isolate	BRIP66802		BRIP66796		BRIP38748	
	CFU/mL	%control	CFU/mL	%control	CFU/mL	%control
Experiment 1						
Control	4.1×10^{7}		NG		1.5×10^{6}	
CuOH L	5.7×10^{5}	0.2	5.0×10^{2}	-	0.0	-
CuOH H	1.0×10^{5}	0.2	5.5×10^{3}	-	0.0	-
CuOxy L	8.5×10^{4}	6.9	2.4×10^{6}	-	3.0×10^{2}	2.0×10^{-2}
CuOxy H	2.8×10^{6}	1.4	2.0×10^{6}	-	1.0×10^{2}	6.5×10^{-3}
CuSO L	ND	-	0.0	-	0.0	-
CuSO H	ND	-	3.9×10^{4}	-	0.0	-
Experiment 2						
Control	3.7×10^{7}		1.6×10^{7}		1.7×10^{8}	
CuOH L	1.7×10^{3}	4.5×10^{-3}	0.0	-	0.0	-
CuOH H	3.0×10^{3}	8.2×10^{-3}	0.0	-	0.0	-
CuOxy L	1.6×10^{2}	4.3×10^{-4}	1.0×10^{5}	0.6	0.0	-
CuOxy H	1.9×10^{2}	5.2×10^{-4}	6.8×10^{6}	43.9	0.0	-
CuSO L	5.7×10^{1}	1.6×10^{-4}	1.5×10^{6}	9.7	0.0	-
CuSO H	3.0	8.2×10^{-6}	5.0×10^{5}	3.2	0.0	-
Experiment 3						
Control	2.4×10^{8}		2.1×10^{8}		NG	
CuOH L	0.0	-	0.0	-	-	-
CuOH H	0.0	-	0.0	-	-	-
CuOxy L	0.0	-	2.2×10^{5}	1.0×10^{-1}	-	-
CuOxy H	2.7×10^{5}	1.2×10^{-1}	2.9×10^{4}	1.4×10^{-2}	-	-
CuSO L	4.5×10^{3}	1.9×10^{-3}	1.2×10^{5}	5.8×10^{-2}	-	-
CuSO H	8.2×10^{4}	3.5×10^{-2}	1.5×10^{5}	6.9×10^{-2}	-	-
Experiment 4						
Control	2.60×10^{7}		2.2×10^{8}		NG	
CuOH L	0.0	-	0.0	-	-	-
CuOH H	0.0	-	0.0	-	-	-
CuOxy L	0.0	-	1.2×10^{4}	5.4×10^{-3}	-	-
CuOxy H	0.0	-	1.1×10^{4}	4.9×10^{-3}	-	-
CuSO L	0.0	-	3.6×10^{4}	1.7×10^{-2}	-	-
CuSO H	4.33×10^{3}	1.7×10^{-2}	1.1×10^{4}	5.2×10^{-3}	-	-
Experiment 5						
Control	1.7×10^{5}		9.8×10^{6}		NG	
CuOH L	0.0	-	0.0	-	-	-
CuOH H	0.0	-	0.0	-	-	-
CuOxy L	4.3×10^{2}	0.3	2.4×10^{4}	2.4×10^{-1}	-	-
CuOxy H	1.7×10^{4}	10.4	1.4×10^{3}	1.5×10^{-2}	-	-
CuSO L	0.0	-	0.0	-	-	-
CuSO H	0.0	-	0.0	-	-	-

Abbreviations: CuOH = copper hydroxide low (L) and high (H) rates, CuOxy = copper oxychloride low (L) and high (H) rates, CuSO = copper sulfate low (L) and high (H) rates, NG = No growth

Chapter 5 - Copper tolerance in Australian *P. syringae* pv. *tomato* isolates

5.1 Chapter Overview

The main body of this chapter presents the results of copper tolerance testing completed on all Australian *P. syringae* pv. *tomato* isolates collected for this thesis study.

Study isolates were collected from a range of commercial outdoor tomato crops between 2015 and 2017. *P. syringae* isolates provided by the Plant Pathology Herbarium of Biosecurity QLD DAF, collected from 1970-80, were also screened to determine whether copper tolerance was present in the past. The Bundaberg and the Granite Belt regions of QLD were the main areas sampled, with a small number of isolates from the Gatton region of QLD, New England region of NSW and Echuca VIC.

This chapter also contains data investigating the relative stability of copper tolerance in the *P. syringae* pv. *tomato* isolates through *in vivo* testing (section 5.8). The general genetic characteristics of the study isolates that were used to confirm that isolates were *P. syringae* pv. *tomato* is also included (section 5.9).

Systematic study of copper tolerance in *Pseudomonas syringae* pv. *tomato* in Eastern Australia

5.2 Abstract

Pseudomonas syringae pv. *tomato* is a damaging bacterial pathogen that causes bacterial speck disease in tomato crops. Over the last 30 years, there has been an increasing number of reports globally of copper tolerance or resistance in bacterial species including *P. syringae* pv. *tomato*. This study investigated the prevalence of copper tolerance in *P. syringae* pv. *tomato* isolates in Eastern Australia. Overall 100% of isolates collected between 2015 and 2017 were determined to be copper-tolerant and herbarium isolates from the 1970s also tested positive for tolerance. This is the first systematic report of copper tolerance in *P. syringae* pv. *tomato* isolates from commercial tomato properties in Queensland, Australia. This study also discusses strategies for mediating copper tolerance development. Globally, the management of disease caused by *P. syringae* pv. *tomato* relies heavily on copper based products, with a limited number of alternative products, often of unproven efficacy, available. The presence of widespread copper tolerance, therefore, has serious implications for commercial tomato producers.

5.3 Introduction

Diseases caused by pathogenic bacteria belonging to *P. syringae* cause significant damage in many cropping systems, and in annual crops such as vegetables, these diseases are suggested to be increasing in frequency (Lamichhane *et al.*, 2015). Many factors could be contributing to this increase including; changes in temperature and rainfall, increased international trade of seed, inadequate screening for seed contamination, and intensification of production systems including increased planting density and reduced or inadequate crop rotation (Lamichhane *et al.*, 2015). Globally, bacterial diseases of tomato (*Solanum lycopersicum* L.) are amongst the most widespread and difficult to control pests that impact commercial crops. The pathogenic bacteria *P. syringae* pv. *tomato* (Young *et al*., 1978) causing bacterial speck disease is one of these. Dark lesions on the foliage and sometimes the fruit are the primary symptoms, which can lead to a reduction in crop yield and quality (Miller & Jones, 2014). Like many plant bacterial pathogens, *P. syringae* pv. *tomato* is dispersed through water or dew and proliferates rapidly under humid and wet conditions (Miller & Jones, 2014). This pathogen has an optimum growth range from 18 to 24°C and can survive on seeds and plant debris, making it highly persistent (Miller & Jones, 2014).

There are limited pesticide control options available for bacterial diseases in tomato, with copper based bactericides currently one of the few registered products globally. Whilst there are a few alternatives products available in some countries, such as acibenzolar-s-methyl (e.g. Huang *et al.,*

2012) and *Bacillus subtilis* (e.g. Fousia *et al.,* 2015), these are yet to be registered for bacterial disease control in Australia. This puts significant pressure on primary producers as they must rely on copper based products, which are commonly applied at least once a week for almost the entire crop cycle. Copper products are broad spectrum pesticides, hence are used in most agricultural crops for the control of a range of fungal and bacterial pathogens. However, their effectiveness for bacterial disease control in tomatoes can be inconsistent and often suboptimal (Griffin *et al.*, 2017). High copper use is also a problem for the environment, with accumulation in the soil affecting both natural systems and future crops (Van-Zwieten *et al.,* 2004).

Perhaps unsurprisingly copper tolerance or resistance is reported globally in *P. syringae* pv. *tomato* and *Xanthomonas* spp. from tomato crops (Griffin *et al.*, 2017; Pernezny *et al.*, 1995; Shenge, Wydra *et al.*, 2008). Tomato producers in Queensland (QLD) and other eastern states of Australia have noticed a reduction in the relative efficacy of copper products for bacterial disease control, however, the lack of effective alternatives means that they continue to use these products at shorter spray intervals and increased application rates. In Australia, copper tolerance in bacterial pathogens of tomato and pepper are reported (Hall *et al.*, 2011; Martin *et al.*, 2004; Tesoriero *et al.*, 1997). However, to date, no tolerance data for *P. syringae* pv. *tomato* isolates from commercial properties in QLD are published, despite the fact that QLD produces close to 70% of Australia's total tonnage of fresh market tomatoes with a gross value of AUD$122 billion (ABS, 2017). A majority of these tomatoes are field grown, rather than in protected cropping systems, these field crops are particularly susceptible to infection with *P. syringae* pv. *tomato* and *Xanthomonas* spp. In tomato growing regions of Australia, including parts of South East and Northern QLD, New South Wales (NSW) and Victoria (VIC), warm and often wet conditions mean that either *P. syringae* pv. *tomato* or *Xanthomonas* spp. is usually present in the crops.

This research study aimed to determine the prevalence of copper tolerance in *P. syringae* pv. *tomato* isolated from commercial tomato growing regions of QLD and other eastern Australian states. This involved *in vitro* copper tolerance screening of *P. syringae* pv. *tomato* collected from 2015 to 2017 and herbarium isolates of *P. syringae* pv. *tomato* from the 1970s.

5.4 Materials and Methods

5.4.1 Bacterial isolation and identification

Tomato leaves and/or fruit displaying symptoms of *P. syringae* pv. *tomato* infection were collected from commercial properties in three geographically and climatically distinct tomato producing regions in South East QLD, Australia. Leaf samples from the Australian states of VIC and NSW were also obtained. Species identification followed standard biochemical, pathogenicity and molecular methods

for the determination of *P. syringae* pv. *tomato* (Chapter 3). *P. marginalis* (DAR65987)*, P. syringae* pv. *syringae* (DAR77319) and *P. syringae* pv. *tomato* (DAR75965) isolates from the Agricultural Scientific Collections Trust, New South Wales Australia (ASCT) were used as reference standards for isolate identification. Herbarium *P. syringae* pv. *tomato* specimens BRIP38746 and BRIP38744, collected in 1973, were provided by the Plant Pathology Herbarium of Biosecurity QLD Department of Agriculture. A copper-tolerant *P. syringae* (A1513R; Andersen *et al.,* 1991) and copper sensitive *P. syringae* pv. *tomato* (DC3000; Buell *et al.,* 2003) were provided by the University of Florida as reference isolates for copper tolerance testing.

5.4.2 In vitro screening of *P. syringae* pv. *tomato* isolates for copper tolerance

The method described in Chapter 4 was used to screen bacterial isolates for copper tolerance and is briefly described here. Casitone yeast extract glycerol (CYEG) agar, containing 1.7 g/L Bacto™ Casitone, 0.352 g/L Bacto™ yeast extract, 0.2% v/v glycerol and 15 g/L Bacto™ Agar, was made up and adjusted to pH 7.2 prior to autoclaving. Agar media was cooled to 65 °C in a water bath before adding filter-sterilized 1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 6.9) to a final concentration of 20 mmol/L. A 0.1 M stock solution of copper sulfate pentahydrate ($CuSO_4.5H_2O$) was added to portions of the molten media to give concentrations of 0, 0.1, 0.2, 0.5, 0.8, 1, 1.5, 2 and 5 mmol/L. The pH of the copper amended media was adjusted to pH 5.8-7.0, as required.

Bacterial isolates were cultured on glucose nutrient agar for 48 h at 24°C and then prepared in sterile deionised water to approximately 7.2 x 10^7 CFU/mL. A 10 µL aliquot of the bacterial suspension was pipetted onto three replicate plates of each concentration of copper and up to 16 isolates were tested on each plate in a 4 x 4 grid pattern. Plates were incubated for 48 h at 24 °C before recording the minimum inhibitory concentration (MIC) of copper sulfate for each isolate. Results were replicated at least three times on different days using freshly made batches of agar medium each time and plates were inoculated within 1 day of their preparation.

5.5 Results

5.5.1 Bacterial isolation and identification

A total of 101 plant samples were taken from 2015-17 and *P. syringae* pv. *tomato* was successfully isolated from 27 of these (Table 5-1). If multiple tomato cultivars or crops with different planting times were being grown on the same property, a sample of each was taken. *Xanthomonas* spp. and unknown fungal species were detected in bacterial speck like lesions on plant samples where *P. syringae* pv. *tomato* was not found. Samples were obtained from six regions of Eastern Australia, these are illustrated in Figure 5-1. Note that the region where herbarium specimens BRIP38746 and BRIP38744 no longer grows tomatoes commericially, hence no samples could be collected here from 2015-17.

Table 5-1: Sample details of *Pseudomonas syringae* pv. *tomato* isolates used in this study including the region, property, crop stage, season and year of collection, the tomato type and cultivar.

Isolate ID	Region[a]	Property Number	Crop Stage (BBCH)	Collection time	Tomato type	Cultivar
BRIP66795	A	1	61	Spr 2015	Gourmet	unknown
BRIP66796	A	4	89	Spr 2015	Gourmet	Leon
BRIP66797	A	4	89	Spr 2015	Roma	unknown
BRIP66798	A	2	82	Spr 2015	Gourmet	Ninja
BRIP66799	A	2	82	Spr 2015	Gourmet	Stewart
BRIP66800	A	6	69	Spr 2015	Gourmet	Stewart
BRIP66801	A	6	65	Spr 2015	Gourmet	Stewart
BRIP66802	A	6	64	Spr 2015	Gourmet	Stewart
BRIP66803	B	17	69	Sum 2015-16	Bush	Coltrane
BRIP66804	B	17	71	Sum 2015-16	Cherry	Cherry Bite
BRIP66805	B	17	81	Sum 2015-16	Gourmet	Montenegro
BRIP66806	B	17	83	Sum 2015-16	Ox Heart	Rugantino
BRIP66807	B	10	71	Sum 2015-16	Roma	Romeo
BRIP66808	C	15	82	Sum 2015-16	Bush	Entice
BRIP66809	C	15	82	Sum 2015-16	Gourmet	Ninja
BRIP66810	C	18	85	Sum 2015-16	Roma	Romeo
BRIP66811	C	15	73	Spr 2015	Bush	Entice
BRIP66812	A	2	72	Spr 2016	Gourmet	Ninja
BRIP66813	E	13	61	Sum 2016-17	Bush	H3402 mix
BRIP66814	D	12	81	Sum 2016-17	Gourmet	Rifle
BRIP66815	D	12	81	Sum 2016-17	Gourmet	Silviana
BRIP66816	D	12	81	Sum 2016-17	Roma	Romeo
BRIP66817	D	12	81	Sum 2016-17	Gourmet	Leon
BRIP66818	C	19	89	Sum 2016-17	Gourmet	unknown
BRIP66819	C	14	71	Sum 2016-17	Gourmet	unknown
BRIP66820	C	20	88	Sum 2016-17	Roma	Romeo
BRIP66821	C	23	81	Sum 2016-17	Roma	Romeo
BRIP38746[b]	F	24	N/A	Sum 1973-74	Gourmet	unknown
BRIP38744[b]	F	25	N/A	Sum 1973-74	Gourmet	unknown
DC3000[c]	N/A	N/A	N/A	N/A		N/A
A1513R[c]	N/A	N/A	N/A	N/A		N/A

Abbreviations: BBCH = Biologische Bundesanstalt, Bundessortenamt und CHemische Industrie, standard rating scale based on plant stages of phenological development. Spr = Spring. Sum = Summer. N/A = not available/not applicable.

[a] Regions: A= Bundaberg QLD, B= Gatton QLD, C=Granite Belt QLD, D= New England NSW, E= Echuca VIC, F = Brisbane-Sunshine Coast QLD.

[b] *P. syringae* pv. *tomato* isolates provided by the Plant Pathology Herbarium of Biosecurity Queensland Department of Agriculture and Fisheries.

[c] Copper sensitive and tolerant *P. syringae* reference isolates provided by the University of Florida, DC3000 is sensitive (Buell *et al.*, 2003) and A1513R is tolerant (Andersen *et al.*, 1991). The pathovar type of A1513R has not been confirmed (Andersen *et al.*, 1991).

Figure 5-1: Map of Australia showing regions of where *P. syringae* pv. *tomato isolates* were collected from tomato crops of the Eastern states. Regions: A= Bundaberg QLD, B= Gatton QLD, C=Granite Belt QLD, D= New England NSW, E= Echuca VIC, F = Brisbane-Sunshine Coast QLD.

The incidence of infection caused by *P. syringae* pv. *tomato* was lower in region A (Bundaberg, QLD) during the 2016-17 growing season, hence fewer isolates were collected that year. A majority of the symptomatic samples from 2016-17 were infected with *Xanthomonas* spp*.* All properties sampled from 2015-17 applied the first copper spray within 7 days of planting and then used an application interval of 5-10 days, depending on local conditions such as rainfall and relative disease pressure. Lower intervals were used when rainfall was more frequent and disease pressure was moderate to high. The primary copper formulation applied to crops was copper hydroxide.

All *P. syringae* pv. *tomato* isolates collected between 2015 -17 were determined to be copper-tolerant (Table 5-2). The highest MIC recorded was 1.5 mmol/L of copper sulfate and was recorded in only one

isolate (BRIP66810) from 2015-17. This MIC was equivalent to the copper-tolerant reference A1513R. The two *P. syringae* pv. *tomato* herbarium isolates from the summer of 1973-74 (BRIP38746 and BRIP38744) also had this high tolerance level. All other isolates from 2015-17 had a MIC of 0.8-1.0 mmol/L, which are considered copper-tolerant based on criteria in Chapter 4 (Griffin *et al.* 2018). The copper-sensitive reference isolate DC3000 had a MIC of 0.2 mmol/L and no other isolates were determined to be copper-sensitive.

There was a minor difference in the copper tolerance level of some isolates collected from the same property with different cultivars, however, the overall difference was only 0.2 mmol/L. This was also the case for *P. syringae* pv. *tomato* isolates collected from plants at different crop stages.

Table 5-2: The minimum inhibitory concentration (MIC) of copper sulfate pentahydrate in Casitone Yeast Extract Glycerol Agar for Pseudomonas syringae pv. tomato isolates. Results replicated at least 3 times using freshly made batches of medium.

Isolate ID	Property Number	MIC (mmol/L)
BRIP66795	1	1.0
BRIP66796; BRIP66797	4	1.0
BRIP66798	2	0.8
BRIP66799	2	1.0
BRIP66802; BRIP66801	6	1.0
BRIP66800	6	0.8
BRIP66803; BRIP66804; BRIP66805; BRIP66806	17	1.0
BRIP66807	10	1.0
BRIP66808; BRIP66809; BRIP66811	15	1.0
BRIP66810	18	1.5
BRIP66812	2	1.0
BRIP66813	13	1.0
BRIP66814	12	0.8
BRIP66815; BRIP66816; BRIP66817	12	1.0
BRIP66818	19	0.8
BRIP66819	14	1.0
BRIP66820	20	1.0
BRIP66821	23	0.8
DAR75965	N/A	0.8
BRIP38746	24	1.5
BRIP38744	25	1.5
DC3000	N/A	0.2^{Sen}
A1513R[b]	N/A	1.5

[Sen] Copper sensitive MIC value

[b] The pathovar of this *P. syringae* isolate is unknown (Andersen *et al.*, 1991).

5.6 Discussion

This is the first systematic study investigating copper tolerance in *P. syringae* pv. *tomato* in Eastern Australia. Globally, few studies screening *P. syringae* pv. *tomato* isolates for copper tolerance have been published (Chapter 2), and to date, Australian tomato producers in the state of Queensland only had anecdotal evidence of copper tolerance in their crops. This study has confirmed that tolerance is present and highlights the need for growers to adapt their management strategies to prevent the build-up of highly copper-tolerant *P. syringae* pv. *tomato* populations.

All except one isolate (BRIP66810, MIC = 1.5 mmol/L) collected from QLD in 2015-17 had a MIC of 0.8-1.0 mmol/L copper sulfate on CYEG medium. Based on the criteria in Chapter 4 and the detection of *cop* genes (Chapter 6) homologous to those previously described (Bender & Cooksey 1987; Bender & Cooksey 1986; Mills *et al.* 1993; Mills *et al*. 1994), these MIC values indicate all the isolates are tolerant to copper. *P. syringae* pv. *tomato* from NSW and VIC were also copper-tolerant, consistent with reports by Tesoriero *et al.* (1997) and Hall *et al*. (2011). There were no consistent trends between the copper tolerance levels of *P. syringae* pv. *tomato* between different geographical areas, tomato-types,

cultivars or properties. None of the *P. syringae* pv. *tomato* isolates collected for this study were determined to be copper-sensitive. A high incidence of copper tolerance has also been reported in other studies that have screened at least 20 *P. syringae* pv. *tomato* isolates (Bender & Cooksey, 1986; Pernezny *et al.*, 1995; Silva & Lopes, 1995; Tesoriero *et al.*, 1997). The incidence of tolerance ranges from 85% reported by Pernezny *et al.* (1995) who screened 80 isolates, to 60%, reported by Bender and Cooksey (1986) and Shenge, Wydra *et al.* (2008) who screened 20 and 56 isolates, respectively. The uniform incidence of copper tolerance in the current study, therefore, reflects either an increase in prevalence over time or a higher than average incidence in the region studied.

A high prevalence of tolerance is attributed to the long term and intensive use of copper products in agricultural systems (Hall *et al*. 2011; Tesoriero *et al.* 1997). Most Australian growers apply copper products within 7 days of transplanting and continue treatment every 7-10 days for the duration of the cropping cycle. On some properties, the application interval is reduced to 3-5 days when conditions are conducive to infection and/or there is a high incidence of disease in the young crop. In the field cropping systems studied, the crop cycle from transplanting to the end of harvest is 12-14 weeks, therefore the total number of copper sprays applied to a crop could be anywhere between 8 and 32. Recommended application intervals for copper products are universal, so many tomato producing regions across the world use a high amount of copper on their crops (Byrne *et al.* 2005; Pontes *et al.* 2016). The continued use of copper on rotation crops also means that a high level of copper remains in the system, including copper accumulation in soils which can negatively impact the environment and other crop types (Chibuike & Obiora 2014; Van-Zwieten *et al*. 2004; Wightwick *et al*. 2010).

Herbarium specimens were examined to assess changes in copper tolerance in QLD over time. Herbarium isolates BRIP38746 and BRIP 38744, collected in 1973-74, had a MIC of 1.5 mmol/L. This high level of tolerance was observed in only one of the 2015-17 field isolates, BRIP66810. The herbarium isolates were collected from properties in the Brisbane and Sunshine Coast regions of QLD, no commercial properties exist in this region any more. The reason for the higher level of copper tolerance in these three isolates BRIP66810, BRIP38746, and BRIP38744 is unknown. The bacterial species is known to be seed transmitted so it is likely there were multiple introductions of *P. syringae* pv. *tomato* into Australia from different regions where there are populations with this higher tolerance (Cuppels & Elmhirst, 1999; Lamichhane *et al.*, 2015; Miller & Jones, 2014). Alternatively, there could have been an evolution of Australian bacterial populations from local selection pressure, which resulted in increased copper tolerance.

There were minimal differences in the level of tolerance measured in *P. syringae* pv. *tomato* isolates collected at an early crop stage, prior to the commencement of harvest, compared to isolates from crops at the end of harvest. As would be expected, there were no significant differences between copper tolerance levels in isolates collected from different tomato cultivars on the same property. The absence of differences suggests tolerance levels remain stable at least over a single crop-cycle. Martin *et al.* (2004) reported that the percentage of copper-tolerant *Xanthomonas campestris* pv. *vesicatoria* on pepper increased after 21 copper sprays. Hence, potential changes in copper tolerance levels in consecutive crops over a number of seasons warrants further investigation. It is postulated that MIC levels are affected by the genetic determinants of copper tolerance, such as the coding sequences for the *cop* genes (Bondarczuk & Piotrowska-Seget, 2013; Mellano & Cooksey, 1988a & b; Mills *et al.*, 1993; Puig & Thiele, 2002).

Results of this study, along with those previously reported (Hall *et al.*, 2011; Tesoriero *et al.*, 1997), indicate that Australian growers should assume that copper-based disease control programs are likely to be, at best, only partially successful in the management of bacterial speck disease in tomato crops. However, the observation that most *P. syringae* pv. *tomato* collected had a lower tolerance level compared to the highly tolerant control, suggests there is potential for producers to implement management strategies to mediate the development of high levels of tolerance. There are currently no alternatives to copper products registered in Australia for bacterial speck disease control in tomato. While some *P. syringae* pv. *tomato* resistant tomato cultivars are available commercially in Australia, these are generally used for processing rather than fresh market consumption and are not suited to growing conditions in all regions. However, there could be opportunities to reduce the total amount of copper being applied over the cropping period. In walnut crops affected by bacterial blight (caused by X. *arboricola* pv. *juglandis*), Ninot *et al.* (2002) demonstrated that omitting the last four of seven standard copper applications on the crop over three years significantly reduced copper accumulation in the soil whilst maintaining commercially acceptable disease control. In tomato crops significant reductions in fruit quality and yields caused by bacterial disease are attributed to infection early in the growing cycle (Dougherty 1978; Yunis *et al.* 1980). Hence, copper use could be minimised later in the crop cycle just prior to the commencement of harvest. Experimental investigation of this is therefore warranted.

Employing other chemical groups besides copper for fungal disease control could also reduce the total amount of copper used within the cropping system. On farm hygiene practices such as removing infected plant debris prior to replanting, using seed that has been treated to remove bacterial pathogens, crop rotation and equipment cleaning are also essential components in effective management strategies (Cuppels & Elmhirst, 1999; Lamichhane *et al.*, 2015; Preston, 2000). While

there are alternatives to copper products under assessment for bacterial disease control, it is unclear when these will become available in Australia. Using copper in a tank mixture with ethylene bis-dithiocarbamate (EBDC) is widely accepted as an effective alternative for controlling disease caused by *P. syringae* even when tolerant populations are present (Huang *et al.*, 2012; Scheck & Pscheidt, 1998). However, the current registration status of products containing EBDC are under review and they may become unavailable (APVMA, 2015).

5.7 Conclusion

Copper tolerance was determined to be widespread in Australian *P. syringae* pv. *tomato* collected between 2015 and 2017, as well as in herbarium specimens from 1973-74. Copper tolerance in agriculturally significant bacterial species such as *P. syringae* pv. *tomato* will continue to have a significant effect on commercial enterprises. For now continued copper use is inevitable, however, it is important that producers are aware of how tolerance develops and have the information available to actively implement more effective management strategies when they become available.

5.8 Stability of copper tolerance in *P. syringae* pv. *tomato*

5.8.1 Introduction

Resistance is defined as "a heritable, statistically defined decrease in the sensitivity to a chemical in a pest population" and this is relative to how a susceptible population is effected by a chemical (Dennehy & Dunley, 1993). Species that are tolerant or resistant to pesticides carry genetic or epigenetic modifications which enable their survival (R4PNetwork, 2016; van den Bosch *et al.*, 2011). In *P. syringae* pv. *tomato*, *cop* genes located in plasmid DNA are suggested to mediate copper tolerance (Bondarczuk & Piotrowska-Seget, 2013; Mellano & Cooksey, 1988a & b; Mills *et al.*, 1993; Puig & Thiele, 2002). Pesticide resistance or tolerance can be categorised as either stable or unstable (Dennehy & Dunley, 1993). Stable refers to when resistance increases with pesticide use and then does not decline once the use of the pesticide is discontinued, whilst with unstable resistance the frequency of resistance decreases when the pesticide is not used. These could also be considered in terms of the biological fitness of *P. syringae* pv. *tomato* carrying plasmids mediating copper tolerance. In other species the fitness response varies considerably, with some reporting plasmid mediated pesticide resistant bacteria or fungi are less biologically fit compared to their sensitive counterparts (Araújo *et al.*, 2012; Kleitman *et al.*, 2005; Lilley & Bailey, 1997), whilst other resistant species have equivalent or greater fitness than those that are sensitive (Sanoamuang & Gaunt, 1995; Sundin & Bender, 1994). To our knowledge, the stability or biological fitness of copper tolerance in *P. syringae* pv. *tomato* is largely unknown. With the intensive use of copper products on tomato crops, it is hypothesized that tolerance levels in *P. syringae* pv. *tomato* would increase as more copper is applied and decrease once copper is removed.

This research study aimed to determine the stability of tolerance levels when selection pressure from copper use was removed. Three greenhouse and one field experiment was conducted with selected copper-tolerant isolates to investigate the stability of tolerance levels over a single crop cycle.

5.8.2 Methods

The *P. syringae* pv. *tomato* isolates tested were BRIP66796, BRIP66802, DAR75965 and BRIP66810; both BRIP66796 and BRIP66802 had an equivalent MIC of 1.0 mmol/L on CYEG, DAR75965 and BRIP66810 had MICs of 0.8 and 1.5 mmol/L, respectively (Table 4-4). Three greenhouse experiments were completed (Table 5-3).

Table 5-3: Details of greenhouse experiments to determine copper tolerance stability in *Pseudomonas syringae* pv. *tomato* including isolate ID, treatment details, treatment application, inoculation and sample timing.

Experiment Number	1	2	3
Tomato cultivar	Entice	Pinnacle	Entice
Isolate ID	BRIP66796	BRIP66802	BRIP66796; DAR75965; BRIP66810
Treatment [a]	UTC CuOH H CuOxy H CuSO H	UTC CuOH L CuOH H CuOxy L CuOxy L CuSO L CuSO H	UTC CuOH H CuOxy H CuSO H CuOx CuOH + EBDC
First Application (DAT)	4	11	7
Application interval (days)	7	7	6-8
Inoculation (DAT)	19	22	20
Last application (DAT)	32	39	35
Sample timing (DAI)	27	25	33

Abbreviations: DAT = Days after transplanting, DAI = Days after inoculation, UTC = untreated control, CuOH = copper hydroxide, CuOxy = copper oxychloride, CuSO = copper sulfate, CuOx = copper oxide and CuOH + EBDC = copper hydroxide plus manganese/zinc ethylene bis-dithiocarbamate tank mix. [a] Treatment application rates (g/100 L): CuOH at 105 (L) and 140 (H); CuOxy at 220 (L) and 250 (H); CuSO at 220 (L) and 280 (H); CuOx at 105; and CuOH + EBDC at 160 and 80 respectively.

Tomatoes were transplanted from seedling trays, at the 3-4 leaf stage, into plastic pots with commercial potting mix (Brunnings Potting Mix) with one plant per pot. Plants were grow in open air greenhouse conditions under natural light, over the testing periods temperatures ranged from 15-30°C and 40-80% relative humidity. Treatments were separated by physical barriers to avoid cross-contamination during treatment applications, inoculation and watering. A completely randomized design was used with three plants in a set and three replicates for each treatment. These sets were distributed randomly on the greenhouse shelf and rotated randomly each week. Physical barriers were used to ensure no cross-contamination between isolates and treatments.

Treatments were based on commercial application rates, these included; an untreated control UTC, CuOH at 105 and 140 g/100 L, CuOxy at 220 and 250 g/100 L, CuSO at 220 and 280 mL/100L and CuOx at 105 g/100 L. A tank mixture of CuOH at 160 g/100 L and EBDC at 80 g/100 L was also used, rates applied were equivalent to the commercial formulation ManKocide™ (DuPont 2012). Copper compounds were applied using a hand held plastic spray bottle to the point of run-off, a total of five applications were made.

Bacterial inoculum was prepared to approximately 1 x 10^8 CFU/mL in 10 mmol/L $MgSO_4.7H_2O$, from 48 h GNA cultures (Pernezny *et al.*, 2008; Pontes *et al.*, 2015). Inoculum was applied to plants to the point of run-off, ensuring thorough coverage of both upper and lower leaf surfaces. Overhead sprinklers watered plants every 2-3 days to promote disease development. The application, inoculation and sample timing varied slightly between experiments (Table 5-3). At the end of the experimental period the percentage incidence of infection was determined visually for each treatment, this was a percentage of leaves displaying symptoms of *P. syringae* pv. *tomato* infection. Infected plant material was collected from each treatment for the re-isolation of *P. syringae* pv. *tomato*. The re-isolated bacteria were confirmed to be *P. syringae* pv. *tomato* using diagnostic methods described in Chapter 3 and tested for copper tolerance using the *in vitro* screening method described in Chapter 4, each isolate was given a unique identifier.

A field experiment was undertaken in which no copper compounds were applied. The experiment carried out on an agricultural research station in Bundaberg QLD in spring 2016. Tomatoes at the 3-4 leaf stage were transplanted at 40 cm spacing, into mounded rows (1.5 m spacing) covered in plastic with subsurface trickle irrigation, as per commercial cropping practice of the region. Plants were irrigated and fertilised following commercial crop practices for the duration of the experiment. Isolate BRIP66796 was used in this experiment. A bacterial suspension of BRIP66796 was prepared using the same method detailed for the greenhouse experiments, this suspension was sprayed over the entire crop area ensuring thorough leaf coverage. Plants were inoculated 21 days after transplanting (DAT) and leaf samples were taken 26 days after inoculation (DAI).

5.8.3 Results

In the greenhouse experiments, disease symptoms, including dark lesions on the foliage, were present on all plants inoculated with the *P. syringae* pv. *tomato* irrespective of whether copper products were applied or not. The mean percentage incidence of infection on plants was between 11 and 82% leaves infected. For three of the four isolates tested (BRIP66796, BRIP66802 and DAR75965), the level of copper tolerance remained stable regardless of the copper formulation used and whether the plants were treated or not treated with copper products (Table 5-4). By contrast, the fourth isolate, BRIP66810 the MIC was lowers in the re-isolated bacteria (unique identifier 122.1) from plants where no copper was applied but remained stable when re-isolated from plants treated with copper. This re-isolated *P. syringae* pv. *tomato* from plants not treated with copper had a MIC of 0.2 mmol/L, which is equivalent to the copper-sensitive isolate DC3000, and represents a 1.3 mmol/L reduction in MIC from that of the original inoculum isolate, BRIP66810. All five isolates derived from the infected plants treated with the copper products exhibited MIC values of 1.5 mmol/L, the same as the original BRIP66810 (Table 5-4).

Table 5-4: Results of copper tolerance stability testing under greenhouse conditions, including the Minimum Inhibitory Concentration (MIC)* of inoculum and re-isolated *Pseudomonas syringae* pv. *tomato*, treatments and disease incidence at sampling. The unique identifiers represent re-isolated *P. syringae* pv. *tomato* from each treatment. Results replicated at least three times using freshly made batches of medium.

Inoculum Isolate ID	MIC of inoculum (mmol/L)	Unique identifier	Treatment [a]	MIC of re-isolated Pst (mmol/L)	Mean percentage incidence/plant
BRIP66796	1.0	78.1 & 113.1[b]	UTC	1.0	58 & 25
		79.1 &114.1[b]	CuOH H	1.0	66 & 10
		80.1	CuOxy H	1.0	71
		81.1	CuSO H	1.0	82
BRIP66802	1.0	87.1	UTC	1.0	51
		88.1	CuOH L	1.0	61
		89.3	CuOH H	1.0	57
		90.1	CuOxy L	1.0	74
		91.2	CuOxy H	1.0	58
		92.1	CuSO L	1.0	67
		93.2	CuSO H	1.0	62
DAR75965	0.8	116.2	UTC	0.8	60
		117.1	CuOH H	0.8	33
		119.1	CuSO H	0.8	42
		121.1	CuOH + EBDC	0.8	33
BRIP66810	1.5	122.1	UTC	0.2	49
		123.1	CuOH H	1.5	11
		124.2	CuOxy H	1.5	27
		125.1	CuSO H	1.5	21
		126.2	CuOx	1.5	26
		127.1	CuOH + EBDC	1.5	23

* Minimum inhibitory concentration of $CuSO_4.5H_2O$ in CYEG agar amended with MES buffer (pH 6.9).
Abbreviations: Pst = *Pseudomonas syringae* pv. *tomato*, UTC = untreated control, CuOH = copper hydroxide, CuOxy = copper oxychloride, CuSO = copper sulfate, CuOx = copper oxide and CuOH + EBDC = copper hydroxide plus manganese/zinc ethylene bis-dithiocarbamate tank mix.
[a] Treatment application rates (g/100 L): CuOH at 105 (L) and 140 (H); CuOxy at 220 (L) and 250 (H); CuSO at 220 (L) and 280 (H); CuOx at 105; and CuOH + EBDC at 160 and 80 respectively.
[b] Two unique identifiers are given for isolate BRIP66796, as this isolate was tested in both experiment 1 and 3.

Under field conditions, disease symptoms, including dark lesions on the foliage, were present on plants inoculated with the *P. syringae* pv. *tomato.* The level of copper tolerance in re-isolated *P. syringae* pv. *tomato* (unique identifier 82.1) from plants inoculated with isolate BRIP66796, remained stable at a MIC of 1.0 mmol/L when plants were not treated with copper products. This mirrored results obtained for isolate BRIP66796 in the greenhouse experiments.

5.8.4 Discussion

Greenhouse experiments showed that copper tolerance levels of isolates BRIP66796, BRIP66802 and DAR75965 remained the same whether copper products were applied or not, suggesting copper tolerance is stable in these isolates. By contrast, the level of copper tolerance in isolate BRIP66810 decreased in untreated control plants, with MIC values equivalent to the copper-sensitive control in re-isolated *P. syringae* pv. *tomato*. When plants inoculated with isolate BRIP66810 were sprayed with

copper products, the copper tolerance level of re-isolated *P. syringae* pv. *tomato* was equivalent to the inoculum. These observations for isolate BRIP66810, which had the highest MIC of all isolates, suggest there could be a biological fitness cost for *P. syringae* pv. *tomato* with higher levels of copper tolerance, hence removing selection pressure from the use of copper may favour the redevelopment of copper-sensitivity. This could be linked to the carrying of plasmid mediated pesticide tolerance, which has been reported to negatively affect biological fitness in some species (Araújo *et al.,* 2012; Kleitman *et al.*, 2005; Lilley & Bailey, 1997). Although, Sundin and Bender (1994) suggest plasmid mediated resistance to copper and streptomycin persists in *P. syringae* pv. *syringae*.

Genetic differences related to the *cop* genes may explain the stability in copper tolerance identified in some but not all isolates in the current study. Understanding the stability or biological fitness of copper tolerance in *P. syringae* pv. *tomato*, would provide valuable insights into how copper-based disease control programs could be modified to mediate or possibly reduce the prevalence copper-tolerant *P. syringae* pv. *tomato.* Hence, bacterial speck disease could be controlled more effectively in tomato crops.

This is the first investigation of the stability of copper tolerance in *P. syringae* pv. *tomato*. The findings of this study suggest periodic removal of copper based products from spray programs as a potential strategy for the management of copper tolerance in *P. syringae* pv. *tomato* in commercial cropping systems. For example rotation of copper with the recently registered *B. subtilis* product mentioned in Chapter 1. However, given the preliminary nature of results in this study, further replication under both greenhouse and field conditions with more isolates is required to clarify this relationship, along with additional biological fitness testing similar to Araújo *et al.* (2012). A greater understanding of evolution of copper tolerance within cropping systems could underpin the development of more effective and sustainable management programs.

5.9 Genetic characterisation of *P. syringae* pathovars

The following presents the genetic characteristics of the isolates collected from the field and obtained from herbarium collections that were used in this study. Methodology for these experiments is defined in section 3.5. These results confirm the species and pathovar designation of *P. syringae* pv. *tomato*, for field isolates. Isolates that did not conform with expected results are also discussed in section 5.9.3.

5.9.1 Results and discussion of PCR assays targeting *hrpZ$_{Pst}$* and *gltA*

All suspected *P. syringae* study isolates collected between 2015 and 2017 produced amplification products with the *hrpZ$_{Pst}$* primer set (section 3.5.1) suggesting they were *P. syringae* pv. *tomato*. As expected, the herbarium and ASCT isolates BRIP34832, BRIP34945, BRIP38748, BRIP38751, BRIP38831, BRIP34814, DAR77319 and DAR65987 (Table 3-3), did not produce amplification products with *hrpZ$_{Pst}$* confirming they were not pv. *tomato*. Herbarium isolates BRIP38735 and BRIP38826 did not produce an amplification product with *hrpZ$_{Pst}$* suggesting their designation as pv. *tomato* may not be correct (Appendix E).

Further confirmation of the pathovar identity of study isolates was obtained by sequence analysis of *gltA* fragments (section 3.5.3). Phylogenetic analysis of *gltA* nucleotide (nt) sequences was completed in Geneious using the reference standards described by Berge *et al.* (2014) (Appendix D). Analysis included multiple sequence alignment with CLUSTALW (Aiyar 1999) and tree building with the Neighbour-Joining method (Saitou & Nei 1987) using the HKY (Hasegawa-Kishino-Yano substitution model; (Hasegawa *et al.* 1985) and Geneious default settings.

All isolates, excluding BRIP66800, were determined to be in phylogroup 1a, which contains the following pathovars: *tomato, antirrhini, apii, avii, berberidis, lachrymans, maculicola, morsprunorum, persicae* and *spinacae* (Table S8; Berge *et al.* 2014). Sequence data for g*ltA* placed BRIP66800 in phylogroup 2 with BRIP38826 and BRIP38735 (Appendix D). Zaccardelli *et al.* (2005) demonstrated that *hrpZ$_{Pst}$* was highly specific to *P. syringae* pv. *tomato,* and there are no reports of any of the other pathovars of phylogroup 1a isolated from tomato. Therefore, the pathovar designation of study isolates was pv. *tomato*. To confirm this, 54 *gltA* reference sequences of *P. syringae* pv. *tomato* were extracted from the PAMDB. These reference sequences (Table 5-5) were compared to the *gltA* sequences of study isolates, using CLUSTALW for multiple sequence alignment in Geneious. The gltA sequences of isolates from the Plant Pathology Herbarium of Biosecurity QLD DAF and ASCT references were also included in this analysis.

Table 5-5: PAMDB reference nt sequences of *gltA* from *Pseudomonas syringae* pv. *tomato* used in the analysis of isolates collected in this study. Isolate identification (ID) numbers are listed, including the PAMDB schema the isolates were from.

Isolate ID			
DCT6D1 [A]	19 [B]	410 [B]	PT32 [B]
TF1 [A]	20 [B]	16 [B]	PT30 [B]
PT23 [A]	A9 [B]	838-16 [B]	21 [B]
DC89-4H [A]	407 [B]	836-2 [B]	PT21 [B]
DC84-1 [A]	PST26L [B]	838-6 [B]	Max1 [B]
1318 [A]	22 [B]	838-9 [B]	24 [B]
487 [A]	Max14 [B]	PT29 [B]	JL1031 [B]
DC3000 [A]	ICMP3435 [B]	PT28 [B]	OH314 [B]
KN10 [A]	ICMP3455 [B]	23 [B]	ICMP3449 [B]
2170 [A]	NCPPB1108 [B]	316	PT2 [B]
	838-4 [B]	ICMP9305 [B]	ICMP3443 [B]
	T1 [B]	PT26 [B]	18 [B]
	B181 [B]	PT13 [B]	315 [B]

[A] Hwang MLST schema; [B] Yan MLST schema

All study isolate sequences were trimmed to 888 bp, with the exception of BRIP66811 (882 bp) and DAR65987 (876 bp) which were missing a few bp at the start of their sequences. All isolates collected in this study had 100% nt identity to each other, with the exception of BRIP66800. Isolate BRIP66800 was collected from the same property as BRIP66801 and BRIP66802 with the only difference being crop stage (Table 3-1). Isolate BRIP66800 had 93.9% nt identity to BRIP66801, BRIP66802 and all other suspected *P. syringae* pv. *tomato* isolates collected for this study (Table 3-1). Pathovar characterisation of BRIP66800 is explored further in Appendix E.

Alignment of PAMDB sequences found that the Hwang and Yan MLST schema would not align with each other. The Yan MLST schema aligned with the first half of *gltA* sequences of study isolates (393 bp), whereas the Hwang MLST schema (529 bp) aligned with the second half. This suggests that different primer sets were used for data collection for these two MLST schema.

All study isolates suspected to be *P. syringae* pv. *tomato* collected between 2015-2016, except BRIP66800, shared 99-100% nt sequence identity to *P. syringae* pv. *tomato* of the Hwang MLST schema (Appendix D). However, there was one isolate of the Hwang schema, 2170, which only shared 94% nt identity with the other isolates of this schema and the study isolates. This isolate, 2170, shared the highest sequence similarities with the *P. syringae* pv. *syringae* isolate DAR77319 (98%), BRIP38826 (100%), BRIP38735 (99%) and BRIP66800 (99%). BRIP66800 had 94% nt identity with to all *P. syringae* pv. *tomato*, except 2170, of the Hwang MLST schema.

All study isolates suspected to be *P. syringae* pv. *tomato* collected between 2015-2016, except BRIP66800, shared 99-100 % nt sequence identity to *P. syringae* pv. *tomato* of the Yan MLST schema (Appendix D). BRIP66800 had 94% nt identity with to all *P. syringae* pv. *tomato* of the Yan MLST

schema. Given the high sequence similarities of *P. syringae* pv. *tomato gltA* sequences in the Yan and Hwang MLST, it is proposed that isolate 2170 may not be pathovar tomato.

As expected there were a greater number of sequence differences between *P. syringae* pv*. tomato* and other pathovars or subspecies from the herbarium specimens used in this study. The nt identity of these other Pseudomonads to *P. syringae* pv*. tomato* ranged from 86-94% (Appendix D).

Results of this comparison support the designation of study isolates as *P. syringae* pv. *tomato*, with the exception of BRIP66800.

5.9.2 Results and discussion of PCR targeting the *cfl* for coronatine production

All study isolates determined to be *P. syringae* pv. *tomato* collected between 2015 and 2017, except BRIP66807, were determined to be carrying the *cfl* gene (section 3.5.2). BRIP66807 was suspected to be a coronatine deficient *P. syringae* pv. *tomato*.

The herbarium and ASCT isolates BRIP34832, BRIP34945, BRIP38748, BRIP38751, BRIP38831, BRIP34814, BRIP38826, BRIP38735, DAR77319 and DAR65987, did not produce amplification products with the PsNFP1/P2 primer sets. As noted previously, *P. syringae* pv. *syringae* does not contain all the required genes for coronatine production. The failure of this PCR assay to amplify DNA in BRIP38826 and BRIP38735 provides further evidence that their designation as pv. *tomato* may not be correct.

The successful amplification of the *cfl* and hrpZ$_{Pst}$ gene in BRIP66800 suggest this isolate is *P. syringae* pv. *tomato*. However, *gltA* sequence data for g*ltA* placed BRIP66800 in phylogroup 2 with BRIP38826 and BRIP38735 (section 5.9.1). The pathovar identity of BRIP66800, BRIP38826 and BRIP38735 was therefore explored further.

5.9.3 Potential *P. syringae* pv. *syringae* isolates from tomato

BRIP38735 and BRIP38826 were provided as *P. syringae* pv. *tomato* reference isolates from the Plant Pathology Herbarium of Biosecurity QLDDAF. The biochemical tests for these isolates produced a profile consistent with *P. syringae*, however, they gave negative results in the *hrpZ$_{Pst}$* and *cfl* PCR tests suggesting that they may not be pv. *tomato* and may have been misdiagnosed. In th*e sequence ana*lysis of *gltA* fragments (section 5.9.1) each *P. syringae* pv. *tomato* isolate tested was consistently positioned within phylogroup 1a closest to *P. syringae* pv. *tomato* reference isolates, whilst BRIP38735 and BRIP38826 were not. Isolate BRIP66800 was also not grouped with phylogroup 1a and shared high sequence similarity to BRIP38735 and BRIP38826.

MLST analysis revealed that both BRIP38735 and BRIP38826 were generally always positioned within phylogroup 2 (Appendix C, Table C-3) and *gltA* sequence data for BRIP66800 suggested it was also in phylogroup 2a (Appendix D). This phylogroup contains 12 pathovars including pv. *syringae* (Table S8;

Berge *et al.* 2014). As noted previously, *P. syringae* pv. *syringae* causing leaf spotting similar to *P. syringae* pv. *tomato* have been identified on tomatoes (Garibaldi *et al.* 2007; Gilardi *et al.* 2010; Gullino *et al.* 2009; Jones *et al.* 1981). Hence, BRIP66800, BRIP38735 and BRIP38826 isolated from tomato plants, may be *P. syringae* pv. *syringae,* or another pathovar within phylogroup 2a.

Gene sequences of *gltA* were extracted from the National Centre for Biotechnology Information (NCBI) GenBank database and compared to gene sequences from BRIP38735, BRIP38826 and BRIP66800 using multiple sequence analysis techniques (Appendix E). This extra analysis was unable to provide an accurate designation of these isolates to a particular pathovar. However, it is hypothesised that these isolates are *P. syringae* pv. *syringae*. Further characterisation of BRIP38735 and BRIP3882 is warranted for future studies, to investigate if they represent a unique *P. syringae* pathovar or are simply genetic variants of an existing phylogroup 2a pathovar. Further characterisation of BRIP66800 is also required to investigate if it represents a genetic variant of *P. syringae* pv. *tomato*.

Chapter 6 - Genetic basis of copper tolerance in Australian *P. syringae* pv. *tomato*

6.1 Chapter overview

This chapter is made up of a journal article that has been published in the Australasian Plant Pathology journal. The genetic basis of copper tolerance in Australian *P. syringae* pv. *tomato* isolates is investigated.

Given the high incidence of copper tolerance identified in study isolates (Chapter 5), it was hypothesised that isolates were also carrying Cop proteins. Therefore, the genetic bases of copper tolerance in Australian *P. syringae* pv. *tomato* was investigated through PCR assays and next generation sequencing (NGS) analysis.

Additional information on the optimisation of the PCR assay conditions are included in Appendix F.

Bibliographic Information

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Nature of Co-authors Contributions: Each of the other authors listed were involved in proof reading, genome sequence analysis and refinement of sentence structure.

Candidate's Declaration

I declare that the publication above meets the requirements to be included in the thesis as outlined in the Research Higher Degree Theses Policy and Procedure.

Karina Griffin

Date: 16 July 2018

Genetic basis of copper tolerance in Australian *Pseudomonas syringae* pv. *tomato*

6.2 Abstract

The genetic basis of copper-tolerance in Australian *P. syringae* pv. *tomato* was investigated through PCR assays and genome analysis. Seven PCR assays were tested targeting copper metabolising (*cop*) genes, this included previously published assays as well as three new assays. These assays varied in their ability to detect *cop* genes in copper tolerant isolates and no one set of primers tested amplified all isolates, however, there is potential for these to be developed further for diagnostic purposes. The genomes of three copper tolerant isolates were sequenced using the Illumina platform. The genome assemblies of these isolates identified putative Cop and CopR/CusS operons homologous to those previously characterised in *P.syringae* pv. *tomato* as mediators of copper-tolerance. Analysis also suggests that the Cop and CopR/CusS operons may be located on either plasmid or chromosomal DNA, depending on the isolate studied. An additional CopAB complex was identified in the genomic assemblies of the three *P.syringae* pv. *tomato* isolates, and was homologous to chromosomal CopA and CopB in a copper sensitive *P.syringae* pv. *tomato* reference genome. Other potential copper metabolising genes were also identified. This is the first genomic analysis of copper tolerant *P.syringae* pv. *tomato* isolated outside of America, with PCR assays and genetic analysis revealing that the genetics of copper-tolerance in *P.syringae* pv. *tomato* is complex and diverse.

6.3 Introduction

The cytotoxicity of copper has been exploited in agricultural systems for bacterial and fungal pathogen control for over 100 years (Merry *et al.* 1983). However, since the mid-1980s there have been increasing reports of copper-tolerance in a wide range of bacterial and fungal species important to the agricultural environment (Adaskaveg & Hine 1985; Altimira *et al.* 2012; Cervantes & Gutierrez-Corona 1994; Cooksey 1994; Tang *et al.* 2013). Tolerance to copper has been exhibited by *P.syringae* pv. *tomato*, a pathogen causing bacterial speck disease on tomatoes (*Solanum lycopersicum* L.), often complicating effective disease control (Griffin *et al.* 2017). A large range of alternative products for bacterial disease control in tomatoes have been published (e.g. Briceno-Montero and Miller, 2004; da Silva *et al.* 2014; Ji and Wilson 2003), with a small subset of these now in commercial use (Griffin *et al.* 2017). However, the effectiveness of these products is often variable and commercial availability limited, hence copper remains the primary control option for many growers. Past research reports that copper-tolerance of *P.syringae* pv. *tomato* is mediated by a large conjugative plasmid carrying copper metabolising (*cop*) genes (Bender & Cooksey 1986, 1987). These *cop* genes are known to encode proteins that upregulate the transport and efflux of copper ions from the cell preventing the

normal cytotoxic profile of copper, which is exploited in the treatment of disease caused by bacterial pathogens (Cooksey 1994; Mellano & Cooksey 1988a, 1988b; Puig *et al.* 2002).

Australian tomato producers have noticed a reduction in the relative efficacy of copper products for bacterial disease control, however, the lack of effective alternatives means that they continue to use these products at shorter spray intervals and increased application rates. In Australia, copper tolerance in bacterial pathogens of tomato and pepper are reported (Hall *et al.*, 2011; Martin *et al.*, 2004; Tesoriero *et al.*, 1997). However, to date, no systematic studies presenting tolerance data of *P.syringae* pv. *tomato* isolates are published that reflect current tolerance levels. Additionally, there are no published studies available investigating whether the genetic profile of Australian *P.syringae* pv. *tomato* varies when compared to isolates from other countries.

Like many microorganisms, bacteria require copper in low concentrations for use in a number of essential metabolic processes (Brown *et al.* 1992; Cervantes & Gutierrez-Corona 1994; Harrison *et al.* 1999). Bacteria have copper-containing proteins, called cuproenzymes, which utilise copper as a catalytic redox cofactor for cell growth, as well as mediating electron transfer for protection against oxidative stress (Argüello *et al.* 2013; Puig & Thiele 2002). Importantly, despite these essential systems utilising catalytic levels of copper ions, copper can quickly become cytotoxic to bacterial cells (Cervantes & Gutierrez-Corona 1994). Cabral (1991) demonstrated that 5-10 µmol/L Cu^{2+} increased cellular respiration, whilst 20-25 µM Cu^{2+} completely inhibited respiration in *P. syringae,* hence 20-25 µmol/L likely represents the copper toxicity threshold. Increased respiration is attributed to normal cellular function, with a range of 1-10 µmol/L Cu^{2+} universally considered the optimal range for most microorganisms (Cervantes & Gutierrez-Corona 1994). Cytotoxicity caused by copper is a result of free radical formation *via* Fenton-like reactions, and/or through oxidation of sulfhydryl-groups (Dupont *et al.* 2011). These reactive intermediaries can cause DNA damage, degrade lipids, and disrupt normal protein function leading to cell death.

Normal cellular requirements for copper mean that there are a number of copper-chelating proteins naturally expressed in bacteria which are chromosomally controlled. For example proteins encoded by the *cueAR* operon in *P. putida* (Adaikkalam and Swarup 2002). Homologs to plasmid *cop* genes, associated with copper-tolerance, are also found in chromosomal DNA of *P.syringae* pv. *tomato* (Lim & Cooksey 1993). These homologs have been reported in both copper-tolerant and sensitive pseudomonads including *P.syringae* pv. *tomato, P. cichorii* and *P. fluorescens* (Cooksey *et al.* 1990; Lim *et al.* 1993). These are suggested to be the basis of the evolutionary development of the plasmid encoded *cop* genes mediating copper-tolerance in pseudomonads (Cervantes & Gutierrez-Corona 1994). Under laboratory conditions, Lim and Cooksey (1993) demonstrated that changes in the

expression and/or regulation of these chromosomal Cop homologs could induce copper-tolerance in normally sensitive cell isolates. Suggesting copper-tolerance could also be caused by chromosomal *cop*-like genes that are overexpressed in the cell (Cooksey 1994). Vargas *et al.* (1995) proposed to have isolated *Pseudomonas* spp. with chromosomally encoded copper-tolerance genes; Lee *et al.* (1994) reported them in *Xanthomonas campestris* pv. *juglandis*; and a chromosomal gene cluster attributed to copper-tolerance was identified by Basim *et al.* (2005). No chromosomally induced copper-tolerance has been reported in field isolates of *P.syringae* pv. *tomato* (Lim & Cooksey 1993). However, the extent to which this has been studied in *P.syringae* pv. *tomato* and other pseudomonads is limited.

The plasmid encoded *cop* genes of *P.syringae* pv. *tomato* have strong homology to *cop* genes of other pseudomonads including *P. cichorii, P. putida*, a *P. syringae* from impatiens and *P. syringae* pv. *syringae*, as well as other bacterial species including *Xanthomonas* spp., and non-phytopathogenic species, such as *Synechocystis* spp. *Cupriavidus metallidurans* (Bondarczuk & Piotrowska-Seget 2013; Cooksey 1990; Cooksey & Azad 1992; Cooksey *et al.* 1990). Additional copper-tolerance mechanisms encoded by plasmid DNA in *P. syringae* continue to be discovered, such as the *cusCBA* operon and *copG* in *Pss* (Gutiérrez-Barranquero *et al.* 2017) and *czc/cuABC* in *P. syringae* pv. *actinidiae* (Colombi *et al*. 2017). However, the mechanisms behind the functional proteins they encode has yet to be determined. Colombi *et al.* (2017) also identified integrative conjugative elements (ICEs) contributing to copper-tolerance in *P. syringae* pv. *actinidiae*. Importantly, despite a general consensus that plasmid *cop* genes are essential for copper-tolerance in *P.syringae* pv. *tomato*, the gene and protein characterisation work undertaken to form these conclusions is solely based on isolates collected in America (Cha & Cooksey 1991; Cooksey 1990; Cooksey & Azad 1992; Lim & Cooksey 1993; Mellano & Cooksey 1988a, 1988b; Zhang *et al.* 2006).

There are six Cop-proteins characterised in *P.syringae* pv. *tomato* which are encoded on two operons, each controlled by their own promotor, the copper resistance (Cop) operon carrying *copA, -B, -C, -D* and the regulatory CopR/S-operon (Mellano and Cooksey 1988a; Mills *et al.* 1993; Mills *et al.* 1994). CopA and -C are proposed to function in transporting copper across the plasma membrane, and CopB and -D are transporter proteins in the outer membrane, and cytoplasmic membrane, respectively (Bondarczuk & Piotrowska-Seget 2013; Cha & Cooksey 1991). Both CopA, and -C have copper binding sites; with CopA a multi-copper oxidase enzyme, binding 11 copper atoms, while CopC binds only one copper atom (Cha & Cooksey 1991). CopC is suggested to have a dual function, both in chaperoning copper for normal cellular processes and interacting with CopA and -B for copper detoxification (Arnesano *et al.* 2002; Bondarczuk & Piotrowska-Seget 2013; Puig *et al.* 2002). The copper transport function of CopC together with CopD upregulates copper uptake into the cell (Arnesano *et al.* 2002; Bondarczuk & Piotrowska-Seget 2013). If copper uptake is increased in the absence of functional CopA

and -B, bacteria are known to become hypersensitive to copper (Cha & Cooksey 1993). Studies, therefore, suggest CopA and –B are the primary determinants of copper detoxification given the high number of copper binding sites in CopA, and the ability of CopB to transport copper across the outer membrane out of the cell (Bondarczuk & Piotrowska-Seget 2013; Puig *et al.* 2002). Mellano and Cooksey (1988a) demonstrated that expression of all four of these Cop sequences is required for full resistance, however, if only CopA and -B are present, a low level of resistance is still apparent. The presence of the *copA* gene sequence is considered to be indicative of copper-tolerance in many bacterial species and has been used as a target for Polymerase Chain Reaction (PCR) to determine if an isolate is copper tolerant (Lejon *et al.* 2007; Wei *et al.* 2009).

The final two Cop proteins, CopR and -S, are linked to copper signalling and the regulation of the *cop* promotor involved in Cop operon gene expression (Mills *et al.* 1993; Mills *et al.* 1994). The CopS signalling protein, located in the cytoplasmic membrane, is proposed to sense copper overloading in the periplasmic space, resulting in the induction of plasmid *cop* gene expression by CopR; together, forming a two-component regulatory system (Mills *et al.* 1993; Mills *et al.* 1994; Puig *et al.* 2002).

Current knowledge indicates the presence of all six Cop-proteins, ideally within operons, seems to be the best indicator of copper-tolerance in *P.syringae* pv. *tomato*. This hypothesis was tested during this study through the investigation of the potential genetic determinants of copper-tolerance in *P.syringae* pv. *tomato* isolates collected in Australia. This was explored through sequence analysis of DNA fragments amplified by PCR primers targeting *cop-A, -B, -C* and *–D* in a range of field isolates of *P.syringae* pv. *tomato* which were also evaluated for their levels of copper tolerance *in vitro*. Based on these results, three *P.syringae* pv. *tomato* isolates were selected for genome analysis. Genome assemblies were annotated with Prokka genome annotation software and compared to reference sequences to identify putative Cop open reading frames (ORFs) and cop promoter regions. Sequence information from Australian *P.syringae* pv. *tomato* was compared against reference sequences to investigate similarities and differences

6.4 Methods

6.4.1 Sample details and reference strains

P.syringae pv. *tomato* isolates collected from diseased field tomato plants (Table 6-1) and two reference isolates, the copper sensitive DC3000 (Buell *et al.* 2003) and copper tolerant A1513R (Andersen *et al.* 1991), were used in the experimental analysis. Species identification of Australian *P.syringae* pv. *tomato* followed standard biochemical methods for the determination of *P. syringae* (Lelliott *et al.* 1966; Elkhalfi *et al.* 2013) and a tomato pathogenicity test, followed by the detection of the *P.syringae* pv. *tomato* specific hypersensitivity response and pathogenicity associated Z gene

(*hrpZ*; Zaccardelli *et al.* 2005) and sequencing of citrate synthase (*gltA*) (Hwang *et al.* 2005; Berge *et al.* 2014). Most of the Australian *P.syringae* pv. *tomato* isolates tested in this study were previously characterised by Griffin *et al.* (2018), including species identification and copper tolerance status. Additional Australian *P.syringae* pv. *tomato* isolates were also collected and tested in this study, the copper tolerance status of these was determined using the methods of Griffin *et al.* (2018).

Bacterial isolates were stored at -80 °C in 30% (v/v) glycerol solutions, prior to use they were cultured on glucose nutrient agar for 48 h at 24 °C. Herbarium specimens collected between 1973 and 1985 were provided by the Plant Pathology Herbarium of Biosecurity, QLD Department of Agriculture and Fisheries, and a reference *P.syringae* pv. *tomato* specimen from the Agricultural Scientific Collections Trust, New South Wales Australia, were also used (Table 6-1).

Eight GenBank (NCBI, 2018) sequences from *P.syringae* pv. *tomato* and *P.syringae* pv. *syringae* isolates were selected to use as references for the PCR sequence and genome analysis (Table 6-1). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank BioProject PRJNA526967, accessions SNVE00000000 (BRIP66810), SNVF00000000 (BRIP66796) and SNVG00000000 (BRIP38746).

Table 6-1: Details of *Pseudomonas* isolates used in this study, including the isolate ID, *Pseudomonas* species and GenBank accessions. Reference sequences from GenBank included in the analysis are also included. Genome assemblies were completed for isolates in bold text.

Isolate ID	Pseudomonas species	GenBank Accession (cop gene)	Source
BRIP66803	Pst[b]	MH003876 (p-A[d])	This study
BRIP66812	Pst	MH003871 (p-A)	
BRIP66813	Pst	MH003870 (p-A)	
BRIP66816	Pst	MH003869 (p-A)	
BRIP66818	Pst	MH003868 (p-A)	
BRIP66819	Pst	MH003867 (p-A)	
BRIP66820	Pst	MH003866 (p-A)	
BRIP66795	Pst	MH003880 (p-A)	Griffin et al. 2018
BRIP66796[a]	Pst	MH003879 (p-A) MH890505 (A,B,C,D,R,CusS) MH890506 (AB complex) SNVF00000000	
BRIP66798	Pst	MH003878 (p-A)	
BRIP66802	Pst	MH003877 (p-A)	
BRIP66807	Pst	MH003875 (p-A)	
BRIP66808	Pst	MH003874 (p-A)	
BRIP66809	Pst	MH003873 (p-A)	
BRIP66810[a]	Pst	MH003872 (p-A) MH003883 (p-B) MH003884 (p-C) MH003885 (p-D) MH890507 (A,B,C,D,R,CusS) MH890508 (AB complex) SNVE00000000	
STN001	Pst	MH003863 (p-A)	
DAR75965	Pst	MH003865 (p-A)	ASCT[e]
BRIP38746[a]	Pst	MH003881 (p-A) MH890502 (A,B,C,D) MH890503 (AB complex) MH890504 (R/CusS) SNVG00000000	PPHB[f]
BRIP38744	Pst	MH003882 (p-A)	
BRIP34945	P. viridiflava	-	
BRIP38735	P. syringae pv. unknown	-	
BRIP38748	Pss[c]	-	
BRIP34814	P. syringae pv. tabaci	-	
BRIP38826	P. syringae pv. unknown	-	
BRIP34832	P. syringae pv. striafaciens	-	
BRIP38751	P. cichorii	-	
DC3000	Pst	AE016853[g]	Buell et al. (2003)
A1513R	P. syringae pv. unknown	MH102380 (p-A)	Andersen et al. (1991)
GenBank References			
PT23	Pst	M19930	Mellano and Cooksey (1988a)
PT23	Pst	L05176	Mellano and Cooksey (1988a); Mills et al. (1993)
PT14	Pst	JQ418536	Summers and Wireman (2012)
B728a	Pss	CP000075	Feil et al. (2005)
UMAF0170	Pss	KY362372	Gutiérrez-Barranquero et al. (2017)
7B44	Pss	KY362373	
B13-200	Pst	CP019871	Xu and Toussaint (2017)

[a] Whole genome shotgun project deposited at DDBJ/ENA/GenBank BioProject PRJNA526967; [b] *Pst* = *P. syringae* pv. *tomato*; [c] *Pss* = *P. syringae* pv. *syringae*; [d] p = partial sequence derived from PCR assay; [e]ASCT: = Agricultural Scientific Collections Trust, New South Wales Australia; [f]PPHB = Plant Pathology Herbarium of Biosecurity, Queensland Department of Agriculture and Fisheries; [g] Whole genome of this isolate also used as a GenBank reference in sequence analysis

6.4.2 PCR assay for the detection of cop genes

The PCR assays were done using slightly modified versions of the four previously published primer sets targeting copA, -B, -C and -D (Nakajima et al. 2002) (Table 6-2). Additionally, reference sequences M19930, JQ418536, CP000075, KY362372 and KY362373 from the GenBank database (NCBI; Table 6-1) were aligned to design three primer sets which targeted different regions of the copA gene (Table 6-2). BLAST searches of these copA primer sequences show they are not specific for P.syringae pv. tomato. These primer sets were used in PCR to test all study isolates.

Table 6-2: Details of primers used in Polymerase Chain Reaction including sequence information, targeted gene region and expected fragment size.

Primer name[a]	Source	Sequence (5' to 3')	Targeted gene region	Expected size (nt)
CopAF	Nakajima et al. (2002)[b]	ATGGAATCAAGAACTTCTCGACGT	Entire copA	1802
CopAR		CTCCTCTACCCGAACTTCGCGGAAC		
CopBF		ACTGTTTTGAATAGACTCCAC	Entire copB	979
CopBR		AACCACATGCGCACGCCCAGGACTAA		
CopCF		CGCATGTTGTTGAACCGCACAAGT	Entire copC	380
CopCR		CTTGACCTTAAACGTCACGCT		
CopDF		AACATGGAAGATCCGCTCAGCATC	Entire copD	922
CopDR		TCCATCTCAGGGGACAGTGT		
PCopAF1	This study	[c]TTATGGTAAACATCACCGGCA	Core copA	1258
PCopAR1		CTCATGGCCTGCATGTC		
CopAF3'		CGTACTTCGACATCCGTATTCC	3' end copA	770
CopAR3'		GGGTGAGTCATCATCGTGTC		
CopAF5'		CGTGTTGAGCGGTACGGAGT	5' end copA	645
CopAR5'		CAAGCCGGTCCAGTTCATATT		

[a]read directions: F = forward primer, R = reverse primer
[b]The restriction endonuclease sites included in the primers of Nakajima *et al.* (2002) were removed from the primers used for this study
[c]Underlined nucleotides were additional nucleotides included at the 5'end of the primer to increase the annealing temperature of the reaction. These nucleotides have no match to the DNA target

Bacterial isolates were suspended in sterile deionised water to approximately 7 x 107 CFU/mL, 1 µL of this preparation was added to each reaction mixture (total reaction volume 50 µL). Amplification reaction mixtures and conditions were optimised for each primer set and reactions were carried out in a Kyratec SuperCycler Thermal Cycler (model SC300). The primer combinations CopAF/AR, CopBF/BR, CopCF/CR and CopDF/DR were used in reaction mixtures which contained 0.2 µmol/L of each primer, 10 µL 5x MangoTaq Coloured Reaction Buffer (Bioline), 2.5 mM MgCl2, 0.2 mM dNTPs and 0.3 µL MangoTaq DNA polymerase (Bioline). Reaction conditions were denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s then annealing at 60 °C for 20 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. Reaction mixtures and cycling parameters for PCopAF1/R1, CopAF3'/R3' and CopAF5'/R5' were the same as above, except used an

annealing temperature of 55 °C. Amplified DNA fragments were purified and sequenced by Macrogen Incorporated.

6.4.3 Sequence analysis of PCR amplified DNA

Sequence analysis of PCR amplified DNA fragments was completed using Geneious software v 11.1.2 (Kearse *et al.* 2012). Consensus nucleotide (nt) sequences of PCR products were assembled and then aligned with reference sequences using the CLUSTALW algorithm v2.1 (Aiyar 1999), IUB cost matrix. The DNA sequences were translated and resulting amino acid (aa) sequences were also aligned using the CLUSTALW algorithm, BLOSUM cost matrix. Alignments were used to determine nt and aa sequence similarities.

6.4.4 Genome library preparation

Genomic DNA libraries were prepared for isolates BRIP66796, BRIP66810 and BRIP38746. Isolate BRIP38746 was collected in 1973 and had an MIC of 1.5 mmol/L copper sulfate on CYEG medium (Griffin *et al.* 2018; Chapter 4). Isolate BRIP66810 collected in 2015 also had an MIC of 1.5 mmol/L. Isolate BRIP66796 was chosen as a representative sample of Australian *P.syringae* pv. *tomato* collected in 2015 with an MIC of 1.0 mmol/L.

DNA from bacterial cultures grown for 48 hr on King's B medium were prepared as per Wilson (2001) with the following modifications; 100% isopropanol was substituted for the phenol/chloroform/isoamyl alcohol and an additional extraction with 100 mg/mL RNaseA in TE buffer was completed prior to ethanol washes. Illumina fragment libraries were constructed as per the NEBNext® Ultra™ DNA Library Prep Kit for Illumina with the following modifications. DNA in 90 µl TE buffer (pH 8.0) was sheared for 7 min in a sonicating water bath in ultra-thin walled tubes at 4 °C. End-repair reactions (total reaction volume = 50 µL) contained 5 µL T4 DNA ligase buffer (New England Biolabs, NEB), 0.4 mM dNTPs, 1 µL T4 DNA polymerase (NEB), 0.2 µL Klenow DNA polymerase (NEB) and 1 µL T4 polynucleotide kinase (NEB) and were incubated for 30 min at 20 °C. Non-templated 3'-single-dA extension was performed for 30 min at 37 °C and the reaction mixture (total reaction volume = 50 µL) contained 5 µL Klenow buffer (NEB), 0.2 mM dATP and 1 µL Klenow exo- (3' to 5'; NEB). Adapter ligation mixtures (total reaction volume = 50 µL) contained 1.5 µL of T4 DNA ligase buffer, 0.03 µM NEBNext Adaptor and 0.5 µL T4 DNA ligase. Ligations were incubated for 15 min at 20 °C before adding 3µL of USER enzyme to the mixture, followed by incubation for 15 min at 37 °C. All reaction clean-ups used solid phase reversible immobilisation (SPRI) as per methods of Fisher (2011).

Index primers were added to each DNA preparation using PCR, the mixture (total reaction volume = 50 µL) contained 15 µL of Phusion MasterMix (NEB), 0.5 µM index primer and 0.5 µM Universal PCR Primer (NEB). Reaction conditions were denaturation at 98 °C for 30 s, followed by 20 cycles of

denaturation at 98 °C for 10 s then annealing and extension at 65 °C for 75 s, with a final extension at 65 °C for 5 min. Fragments of *ca*. 500 nt were size selected by gel electrophoresis and electro-eluted into TE buffer and quantified using the Quant-iT dsDNA HS assay (Thermo Fisher Scientific).

Genome preparations were sequenced by Macrogen Inc. using the Illumina Miseq platform with 150 nt paired-ends.

6.4.5 Genomic library assembly

Sequence reads were provided from Macrogen Inc. as trimmed data, in which adapter sequences were removed with Scythe (v0.994) and Sickle, and reads shorter than 36 nt were removed. These reads were then assembled with three different genomic library assembly algorithm packages to compare contig outputs. For BRIP66796 the packages were plasmidSPAdes v3.12.0 (Antipov *et al.* 2016), Geneious *de novo* Assembly v 11.1.2 (Kearse *et al.* 2012) and Velvet v1.2.09 (Zerbino & Birney 2008; Zerbino *et al.* 2009). For the remaining two isolates, BRIP66810 and BRIP38746, this was reduced to just PlasmidSPAdes and Geneious *de novo* assembly. Prior to the Geneious *de novo* assembly, reads were paired (expected distance 348 nt) and duplicate reads were removed (Kmer = 75; BBTools Dedupe v 37.64; Bushnell 2014). Geneious assembly used the medium-low sensitivity settings with no gaps allowed and contigs using less than 1000 sequences for assembly were not used in further analysis. PlasmidSPAdes and Velvet assemblies were run on un-paired data using default settings. Velvet was completed as a short-paired analysis. Contigs less than 2000 nt were removed before annotating with Prokka genome annotation software v1.12 (Seemann 2014) and any *cop* genes identified were mapped to the original reads to check coverage using Geneious (medium-low sensitivity, no gaps allowed).

6.4.6 Genome assembly sequence analysis

Putative copper metabolising genes identified with Prokka genome annotation software were extracted, translated and compared to reference sequences using CLUSTALW multiple sequence analysis. Differences between Cop A, -B, -C, -D, -R and CopS/CusS ORFs were also analysed phylogenetically in Geneious using the UPGMA tree build method (Sneath and Sokal, 1973) and the Jukes-Cantor genetic distance model (Jukes and Cantor 1969). The following Genbank reference sequences were used in this comparison; M19930 (CopA, -B, -C and -D), L05176 (CopR and -S), JQ418536 (CopA, -B, -C and -D), AE016853 (CopAB complex), KY362372 (CopA, -B, -C, -D, -R and -S) and CP019871 (CopA, -B, -C, –D and CopABcomplex). Further details of these reference sequences are provided in Table 6-1. CP019871 carried two ORFs of CopA, -B, -C and -D, both sets were used in the phylogenetic analysis. Additional coding regions used in the phylogenetic comparison were the DNA-binding response regulator of CP019871, the two component heavy metal response transcriptional

regulator of JQ418536 and two heavy metal sensor histidine kinases of JQ418536. These additional coding regions were included due to their location immediately downstream of the *copA-D* region and sharing high sequence similarities with *copR* and *copS* of the reference *P.syringae* pv. *tomato* strain PT23 (M19930 and L05176). Putative Cop ORFs identified in genome assemblies were also compared to the *cop* PCR sequences.

The region upstream of the putative CopA ORF was screened for the plasmid-borne cop promoter (Pcop) and the chromosomal *cop* homolog promoter (PcopH) containing cop-box, a copR binding region (Mills *et al.* 1994). A MegaBLAST analysis (Morgulis *et al.* 2008) of the putative copper metabolising genes and putative plasmid components identified with PlasmidSPAdes was completed to verify identification. Any plasmid components identified with PlasmidSPAdes that carried Cop ORFs were also mapped to any putative plasmid DNA determined through the Geneious *de novo* assembly.

6.5 Results

6.5.1 Sample details and reference strains

All isolates used in this study (Table 6-1) which were collected from commercial properties in Eastern Australia fulfilled all biochemical tests for their designation as *P.syringae* pv. *tomato*. All isolates caused tomato seedlings to develop bacterial speck disease, hence were determined to be pathogenic. The gene sequences of *hrpZ* and *gltA* were also consistent with their designation as *P.syringae* pv. *tomato*.

6.5.2 PCR assay for the detection of cop genes

The primer sets of Nakajima *et al.* (2002), targeting c*opA, -B, -C* and -*D*, were used to screen Australian isolates of *P.syringae* pv. *tomato* for copper genes. The primers only amplified DNA fragments of the expected size from a single isolate, BRIP66810. All other Australian isolates tested produced no amplification products with any of the primer sets. To evaluate this further, three new sets of primers were designed to *P.syringae* pv. *tomato* and *P.syringae* pv. *syringae cop* gene sequences available on GenBank and was limited to evaluation of *copA*. The three primer sets CopAF5'/R5', PCopAF1/R1 and CopAF3'/R3' and were designed to amplify the 5'end, core and 3'end of the copA gene, respectively.

Detection of the *copA* gene with these three new primer sets varied between isolates (Table 6-3). All three primers amplified DNA fragments of the expected size from isolate BRIP66810. CopAF5'/R5', targeting the 5' end of *copA*, did not produce an amplification product in any other *P.syringae* pv. *tomato* isolate tested. Conversely, CopAF3'/R3', targeting the 3' end of *copA*, amplified DNA fragments of the expected size in all copper tolerant *P.syringae* pv. *tomato* isolates, except BRIP38744 and BRIP38746. PCopAF1/R1, designed to amplify the core region of *copA,* successfully amplified DNA in BRIP66810, BRIP38744 and BRIP38746 but not in other Australian *P.syringae* pv. *tomato*. A1513R was

successfully amplified with PCopAF1/R1 and CopAF3'/R3'. None of the other *P. syringae* pathovars tested were amplified by the any of the primers tested, irrespective of their level of copper-tolerance or sensitivity. No PCR amplification was observed in DC3000, the copper sensitive reference, for any of the primer sets. In summary, no one primer set amplified *copA* reliably from all copper tolerant *P.syringae* pv. *tomato* isolates.

Table 6-3: Summary of Polymerase Chain Reaction results targeting the *copA* gene in Australian *Pseudomonas syringae* pv. *tomato* and reference isolates A1513R and DC3000. Including isolate ID, minimum inhibitory concentration (MIC) and the fragment of *copA* detected by PCR.

Isolate ID	MIC[a] (mmol/L)	Fragment of copA detected by PCR
DC3000	0.2 SEN	None
BRIP38748	0.2 SEN	None
BRIP38735	0.2 SEN	None
BRIP38826	0.2 SEN	None
BRIP38751	0.5 SEN	None
BRIP66798	0.8	3'-end [c]
BRIP66818	0.8	3'-end
DAR75965	0.8	3'-end
BRIP34814	0.8	None
BRIP34832	0.8	None
BRIP66795	1.0	3'-end
BRIP66796 [b]	1.0	3'-end
BRIP66802	1.0	3'-end
BRIP66803	1.0	3'-end
BRIP66807	1.0	3'-end
BRIP66808	1.0	3'-end
BRIP66809	1.0	3'-end
STN001	1.0	3'-end
BRIP66812	1.0	3'-end
BRIP66813	1.0	3'-end
BRIP66816	1.0	3'-end
BRIP66819	1.0	3'-end
BRIP66820	1.0	3'-end
BRIP38746	1.5	Core [d]
BRIP38744	1.5	Core
A1513R	1.5	Core 3'-end
BRIP66810	1.5	Entire [e] Core 3'-end 5'-end [f]
BRIP34945	2.0	None

[a] MIC = Minimum inhibitory concentration of $CuSO_4.5H_2O$ in Casitone Yeast Extract Glycerol medium agar containing 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.9 (Griffin *et al.* 2018).
SEN = copper-sensitive isolate.
[b] Genome scaffolds were prepared for isolates in bold text.
[c] 770nt from the 3'-end of *copA* as amplified by the primer set CopAF3'/R3'.
[d] core region of 1258 nt of *copA* as amplified by the primer set PCopAF1/R1.
[e] entire 1802 nt of *copA* as amplified by the primer set CopAF/AR.
[f] 645 nt from the 5'-end of *copA* as amplified by the primer set CopAF5'/R

6.5.3 Sequence analysis of PCR amplified DNA

To further evaluate the copper-tolerance genes amplified by PCR, DNA fragments were sequenced and compared to references (M19930, JQ418536, KY362372, KY362373 and CP000075) using CLUSTALW multiple sequence analysis (Table 6-4). The *copA, -B, -C* and *–D* sequences of isolate BRIP66810, had 100% nt and aa sequence identity to their corresponding genes in the two *P.syringae* pv. *tomato* plasmid reference sequences, M19930 and JQ418536. By contrast, the entire *copA* nucleotide sequence of this isolate was 95 to 100% identical with the *Pss copA* reference sequences (KY362372, KY362373 and CP000075).

Sequences generated by PCR from the 3'-end of *copA* showed that all Australian *P.syringae* pv. *tomato* isolates, except BRIP66810, were 100% identical to each other at both the nt and aa levels (Table 6-4). Furthermore, these isolates shared only 62-64% nt and 65-67% aa identity with BRIP66810, *P.syringae* pv. *tomato* references (M19930 and JQ418536) and *Pss* references (KY362372, KY362373 and CP000075).

Sequences of four Australian *P.syringae* pv. *tomato* isolates were amplified with the *copA* core primers. Of these, isolates BRIP38746 and BRIP38744 had identical sequences to each other and shared 70% nt and 77% aa identity with BRIP66810 and M19930 (Table 6-4). The fourth isolate amplified in this PCR, A1513R, did not share close homology with any isolate analysed.

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Table 6-4: Distance matrix of Australian *Pseudomonas syringae* pv. *tomato* isolates and reference sequences for the 3'-end region of *copA*, equivalent to 748 nucleotides (249 amino acids) in M19930. Area above black line = % nucleotide sequence identity and the area below the black line = % amino acid sequence identity. The minimum inhibitory concentration (MIC) of copper sulfate (mmol/L) on Casitone Yeast Extract Glycerol medium is included for study isolates.

	B798	B818	D65	B795	B796	B802	B803	B807	B808	B809	STN001	B812	B813	B816	B819	B820	A15	B810	M	JQ	CP75	KY72	KY73	MIC
B798		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	61	63	63	63	62	63	62	0.8
B818	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	61	63	63	63	62	64	62	0.8
D65	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	61	63	63	63	62	64	62	0.8
B795	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	60	63	63	63	62	63	62	1.0
B796	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	61	63	63	63	62	64	62	1.0
B802	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	61	63	63	63	62	63	62	1.0
B803	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	61	63	63	63	62	64	62	1.0
B807	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	61	63	63	63	62	64	62	1.0
B808	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	61	63	63	63	62	64	62	1.0
B809	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	61	63	63	63	62	63	62	1.0
STN001	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	61	63	63	63	62	64	62	1.0
B812	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	61	63	63	63	62	64	62	1.0
B813	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	61	63	63	63	62	64	62	1.0
B816	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	61	63	63	63	62	64	62	1.0
B819	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	61	63	63	63	62	64	62	1.0
B820	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		61	63	63	63	62	64	62	1.0
A1513R	65	65	65	64	65	64	65	65	65	64	65	65	65	65	65	65		66	66	66	67	66	67	1.5
B810	67	67	67	66	67	66	67	67	67	66	67	67	67	67	67	67	69		100	100	94	99	94	1.5
M	67	67	67	66	67	66	67	67	67	66	67	67	67	67	67	67	69	100		100	94	99	94	-
JQ	67	67	67	66	67	66	67	67	67	66	67	67	67	67	67	67	69	100	100		94	99	94	-
CP75	66	67	67	65	67	66	67	67	67	65	67	67	67	67	67	67	70	94	94	94		93	100	-
KY72	67	67	67	66	67	66	67	67	67	66	67	67	67	67	67	67	69	99	99	99	93		93	-
KY73	66	67	67	65	67	66	67	67	67	65	67	67	67	67	67	67	70	94	94	94	100	93		-

Sample ID Abbreviations: B798 = BRIP66798; B818 = BRIP66818; D65 = DAR75965; B802 = BRIP66802; B803 = BRIP66803; B807 = BRIP66807; B808 = BRIP66808; B809 = BRIP66809; STN = STN001; B810 = BRIP66810; B812 = BRIP66812; B813 = BRIP66813; B816 = BRIP66816; B819 = BRIP66819; B820 = BRIP66820; A15 = A1513R; M = M19930; JQ = JQ418536; CP = CP000075; KY72 = KY362372; KY73 = KY362373

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6.5.4 Genomic library assembly

Whole genome draft assemblies were derived for the three Australian *P.syringae* pv. *tomato* isolates, BRIP38746, BRIP66796 and BRIP66810, and analysed for the presence of Cop ORFs. The mean fragment coverage of the Cop regions identified with Prokka for each isolate was an average of 50, with a minimum recorded mean of 34. The putative Cop ORFs identified with Prokka from the three different *de novo* sequencing assemblies, Velvet, plasmidSPAdes and Geneious, were 100% identical to each other for BRIP66796. For the remaining two isolates, BRIP66810 and BRIP38746, the *cop* genes identified with plasmidSPAdes and Geneious were 100% identical to each other.

6.5.5 Genome assembly sequence analysis

Genomic annotations with Prokka identified putative ORFs for Cop or Cop-like proteins including CopA, -B, -C, -D, -R, -Z and CusS. Additional putative CopA coding regions were identified through Prokka, however, these had very low sequence homology to references (<40%) hence were excluded from further analysis. Additional putative CopG ORFs were identified through sequence comparisons with reference *P.syringae* pv. *tomato*. A *cop* gene region containing *copA, -B, -C* and *-D* was identified in all three isolates, this region was designated as a putative Cop operon. A putative CopR/CusS operon was present on the same contig as the Cop operon in all three isolates. The CopR/CusS operon was immediately downstream of the Cop operon in BRIP66796 and BRIP66810. The CopR/CusS operon in BRIP38746 was 10,454 nt downstream of the putative Cop operon. Immediately upstream of the CopA ORF, BRIP66796 contained a region with 99% nt sequence similarity to the chromosomal cop promoter PcopH and BRIP66810 contained a region with 100% nt sequence similarity to the plasmid-borne cop promoter Pcop. By comparison, the upstream sequence of CopA in BRIP38746 was lacking in either promoter region.

An additional CopA coding region, 1,770 nt and 589 aa in length, was also identified in all three isolates on different contigs to the putative Cop and CopR/CusS operons. This CopA was located next to an additional CopB, 885 nt and 294 aa in length. This CopAB complex shared 99-100% sequence identity with the CopAB complex on the chromosome of AE016853 (*P.syringae* pv. *tomato*) and CP019871 (*P.syringae* pv. *tomato*).

Using plasmidSPAdes, putative plasmid components were found in the whole genome scaffolds of all three Australian *P.syringae* pv. *tomato* isolates. Selected sequences were further evaluated using MegaBLAST to identify similarities with known plasmid sequences on GenBank. Twenty two plasmid components over 2,000 nt were identified in BRIP66796, with three greater than 10,000 nt in length. No Cop coding regions were found in any of these components. Thirty one plasmid components over 2,000 nt were identified in BRIP66810, with nine greater than 10,000 nt in length. One component

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(11,589 nt) carried the putative Cop and CopR/CusS operons, and was highly similar to plasmid DNA references including JQ418536 (*P.syringae* pv. *tomato*) and KY362372 (*P.syringae* pv. *syringae*). Eleven plasmid components over 2,000 nt were identified in BRIP38746, with four greater than 10,000 nt in length. The Geneious *de novo* assembly identified one circular contig 30,575 nt in length, which matched one of the plasmid components identified using plasmidSPAdes. This component carried the putative Cop and CopR/CusS operons. MegaBLAST analysis of this putative plasmid revealed that it had high similarity to a number of plasmid sequences including KY362369 (*Pss*) and CP024713 (*P. syringae* pv. *actinidiae*). The CopAB complex was not present on any of the putative plasmid components of BRIP38746, BRIP66810 and BRIP66796.

Phylogenetic analysis of putative the Cop and CopR/CusS operons of BRIP38746, BRIP66810 and BRIP66796 showed that the Cop ORFs of each isolate clustered in separate groups (Figure 6-1). Those from BRIP66810 always grouped with plasmid Cop ORFs of the *P.syringae* pv. *tomato* reference strain PT23 (M19930 and L01576), JQ418536 and KY362372. BRIP66810 Cop ORFs were generally identical or had minor sequence differences to these references. The BRIP38746 Cop ORFs clustered most closely with the BRIP66810 group but on a separate branch with the exception of CusS which showed a slightly closer relationship with BRIP66810. The BRIP66796 Cop ORFs were always grouped with CP019871 chromosomal Cop ORFs, with branching indicating these had significant sequence differences to BRIP38746, BRIP66810 and other references (M19930, L01576, JQ418536 and KY362372). The CusS coding region of BRIP66796 was in a separate clade to all other sequences. MegaBLAST analysis of the putative Cop operon of BRIP66796, revealed it had high similarity to chromosomal DNA of a number of other pseudomonads.

Other potential copper metabolising genes including a multicopper oxidase (*mmcO;* Uniprot I6WZK7), copper-sensing transcriptional repressor (*csoR*, UniProt O32222) and transcriptional regulatory protein (*cusR*, UnioProt P0ACZ8) were identified in all three Australian *P.syringae* pv. *tomato* isolates analysed. Coding regions for cation efflux system proteins CusA (Uniprot P38054) and CusB (Uniprot P77239) were also identified in BRIP38746 and BRIP66796, plus a CusF (UniProt 77214) in BRIP66796.

The CopA nt and aa sequences derived from BRIP38746 and BRIP66796 by PCR were 100% identical to the corresponding regions on the Cop operon derived from whole genome sequencing. Similarly, the *copA, -B, -C* and *–D* nt and aa sequences derived from BRIP66810 by PCR were 100% identical to that derived through whole genome sequencing. Nucleotide sequences of *copA, -B, -C* and *-D* on the Cop operon of BRIP66810 confirmed the presence of binding sites for all primers tested, hence validating PCR results. PCR results were also validated in BRIP38746 and BRIP66796, with excessive mismatches in most of the primer binding sites of *copA, -B, -C and –D* confirming amplification failures.

The only functional primer binding sites in *copA* of BRIP38746 were PCCopAF1/R1. The *copA* of BRIP66796, CopAF3'/R3' primer binding sites were present with minor mismatches.

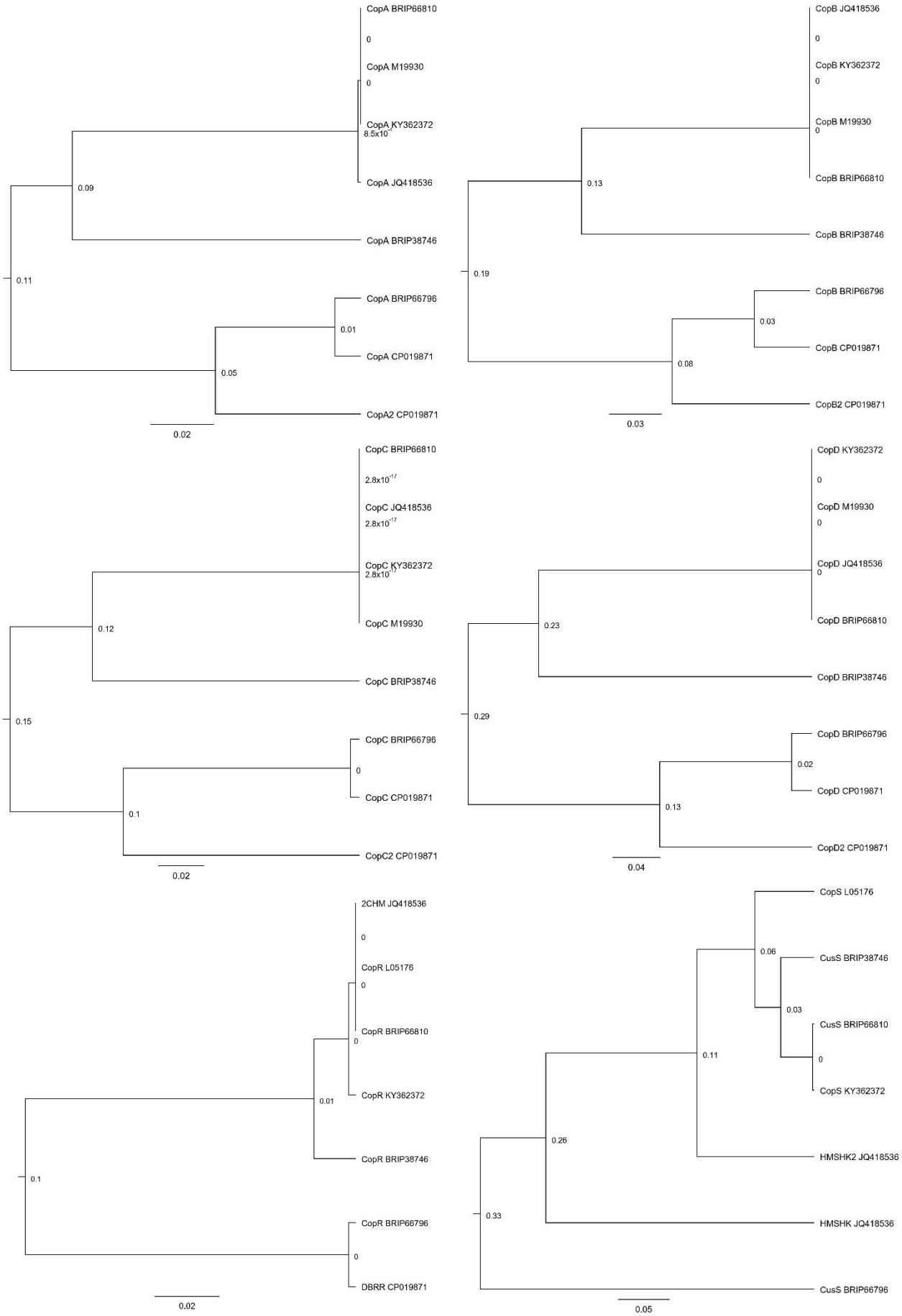
CopA BRIP66810
0
CopA M19930
0
CopA KY362372
8.5x10-17
CopA JQ418536
0.09
CopA BRIP38746
0.11
CopA BRIP66796
0.01
CopA CP019871
0.05
CopA2 CP019871
0.02
CopB JQ418536
0
CopB KY362372
0
CopB M19930
0
CopB BRIP66810
0.13
CopB BRIP38746
0.19
CopB BRIP66796
0.03
CopB CP019871
0.08
CopB2 CP019871
0.03
CopC BRIP66810
2.8x10-17
CopC JQ418536
2.8x10-17
CopC KY362372
2.8x10-17
CopC M19930
0.12
CopC BRIP38746
0.15
CopC BRIP66796
0
CopC CP019871
0.1
CopC2 CP019871
0.02
CopD KY362372
0
CopD M19930
0
CopD JQ418536
0
CopD BRIP66810
0.23
CopD BRIP38746
0.29
CopD BRIP66796
0.02
CopD CP019871
0.13
CopD2 CP019871
0.04
2CHM JQ418536
0
CopR L05176
0
CopR BRIP66810
0
CopR KY362372
0.01
CopR BRIP38746
0.1
CopR BRIP66796
0
DBRR CP019871
0.02
CopS L05176
0.06
CusS BRIP38746
0.03
CusS BRIP66810
0
CopS KY362372
0.11
0.26
HMSHK2 JQ418536
0.33
HMSHK JQ418536
CusS BRIP66796
0.05

Figure 6-1: UPGMA trees of Cop ORFs in three Australian *Pst* compared to GenBank reference sequences M19930 (CopA, -B, -C and -D), L05176 (CopR and -S), JQ418536 (CopA, -B, -C and -D, 2CHM, HMSHK and HMSHK2), KY362372 (CopA, -B, -C, -D, -R and -S) and CP019871 (CopA, -B, -C and –D, DBRR). Protein sequences were aligned with CLUSTALW prior to Geneious tree building using the UPGMA method and Jukes-Cantor distance model. Values on each node represent node height. Abbreviations: 2CHM = two component heavy metal response transcriptional regulator; DBRR = DNA-binding response regulator; HMSHK = heavy metal sensor histidine kinase. Note: CP019871 carries two ORFs for CopA, -B, -C and –D; and two HMSHK are present downstream of Cop operon in JQ418536.

6.6 Discussion

The genetic basis of copper-tolerance in a number of Australian P.syringae pv. tomato isolates was evaluated using PCR assays and genomic analysis of three selected isolates. Three different copper-tolerance determinants were found among the Australian P.syringae pv. tomato isolates and although they shared similarities to previously the described Cop and CopRS operons, there were some notable differences.

Cop gene nt and aa sequences of BRIP66810, BRIP66796 and BRIP38746 derived from PCR assays were identical to those derived through genome assembly, validating the results of PCR. Initially PCR assays used primers designed by Nakajima *et al.* (2002) which were specific to plasmid pP23D of a P.syringae pv. tomato known to be copper tolerant. However, these primers only amplified cop genes from a single Australian isolate (BRIP66810), hence additional primers were designed in this study. These primers were also variable in their ability to detect cop genes and no one set of primers tested amplified all isolates. The most successful primer set was copAF3'/R3' targeting the 3' end of copA, which successfully amplified DNA in most copper tolerant isolates and there was no amplification of the copper sensitive reference. The mixed success with PCR, as compared to the data obtained through whole genome sequencing, for the detection of cop genes in this study suggests the latter approach is superior for derivation of the genetic components involved in copper-tolerance. Sequencing of a larger range of copper sensitive and tolerant P.syringae pv. tomato is needed to develop a reliable molecular diagnostic assay for testing field isolates.

The whole genome sequences of three isolates revealed the genetics of copper-tolerance in P.syringae pv. tomato is complex and varied. Genomic analysis revealed that BRIP66810, BRIP66796 and BRIP38746 carried a number of copper metabolising genes. Coding regions similar to previously characterised Cop-proteins were considered the most likely candidates to be involved in copper-tolerance (Bender and Cooksey 1986, 1987; Cooksey 1994; Mellano and Cooksey 1988a, b). These included a putative Cop operon containing CopA, -B, -C, -D and a putative CopR/CusS operon in all three isolates. CusS rather than CopS was identified in the analysis and was located immediately downstream of putative CopR, this configuration is similar to the two component regulatory system

reported to regulate the expression of the cop genes (Mills *et al.* 1993, 1994; Puig *et al.* 2002). The CusS of BRIP66810 was identical to CopS described by Mills *et al.* (1993) and CusS of BRIP38746 was highly similar to CopS. This suggests CusS and CopS may be the same proteins in the context of P.syringae pv. tomato.

Genome analysis revealed that the putative Cop-proteins of BRIP66796 were less similar to BRIP66810, BRIP38746 and other plasmid references. The high similarity of BRIP66796 with chromosomal cop genes of CP019871 and the absence of plasmid components carrying cop genes suggests this isolate has chromosomally mediated copper-tolerance. A region highly similar to the chromosomal PcopH promoter described by Mills *et al.* (1994) was also identified in BRIP66796 and is also present in CP019871. The copper-tolerance status of CP019871 is unknown limiting further comparisons. Alternatively, the Cop and CopR/CusS Operons of BRIP66796 could be encoded in ICEs similar to those identified by Colombi *et al.* (2017) in P.syringae pv. actinidiae. The literature suggests that chromosomal cop-like genes could be involved in copper-tolerance in P.syringae pv. tomato (Cooksey 1994; Lim and Cooksey 1993) and there are reports of this in other bacterial species (Lee *et al.* 1994). Recent research on xanthomonads affecting solanaceous crops in Australia also reports putative chromosomally encoded copper-tolerance (Roach 2018).

In agreement with previous research on copper-tolerance systems of P.syringae pv. tomato, the putative Cop and CopR/CusS operons of the two remaining Australian P.syringae pv. tomato isolates studied, BRIP66810 and BRIP38746, are proposed to be on plasmid DNA. The putative plasmid encoded Cop and CopR/CusS operons of BRIP66810 were almost identical to those described in previous studies (Bender and Cooksey 1986, 1987; Mellano and Cooksey 1988a; Mills *et al.* 1993). Although BRIP38746, had a number of nt and aa differences and variations in gene size when compared to the references from these studies, the level of similarity was still high. A region 100% identical to the Pcop promoter region (Mills *et al.* 1994) was identified in BRIP66810, whereas no promoter region was detected in BRIP38746. However, the putative plasmid of BRIP38746 did carry a putative CopR/CusS operon and therefore may carry a unique, as yet unidentified, promoter region for the operon.

Overall, this study suggests that Australian P.syringae pv. tomato can carry either chromosomal or plasmid mediated copper-tolerance. Additionally, it appears that chromosomal copper-tolerance may be linked to a lower level of tolerance, a MIC of 1.0 mmol/L (BRIP66796), and plasmid mediated tolerance to a higher level of tolerance, 1.5 mmol/L (BRIP38746 and BRIP66810). As this study was limited to only three isolates, further analysis is required to clarify the importance of gene location in copper tolerant P.syringae pv. tomato phenotypes. Both BRIP66810 and BRIP38746 had the same level

of copper-tolerance *in vitro*, which was higher than most Australian P.syringae pv. tomato (Griffin *et al.* 2018). These two isolates were collected over 40 years apart with BRIP66810 isolated in 2015 and BRIP38746 in 1973, hence the genetic variation in the putative Cop proteins observed was expected. The high similarity between Cop-proteins in BRIP66810 and American references (Bender and Cooksey 1986, 1987; Mellano and Cooksey 1988a; Mills *et al.* 1993) suggests a recent transfer of genetic information between countries. Most of Australia's commercial tomato seed is imported and often not screened or treated for P.syringae pv. tomato. Genetic differences observed in BRIP66796 when compared to American references may be attributed to localised evolution in Australia.

A CopAB complex was identified in all three isolates, which had significant sequence differences compared to the CopA and CopB present on the putative Cop operon. The CopAB complex was almost identical to CopA and CopB of the copper sensitive P.syringae pv. tomato reference isolate (AE016853; Buell *et al.* 2003). This copper sensitive P.syringae pv. tomato does not contain CopC, CopD or the regulatory proteins CopR and CopS, suggesting this CopAB complex has a role in copper homeostasis (Buell *et al.* 2003). However, the presence of only CopA and -B has also been suggested to provide low level copper-tolerance in P.syringae pv. tomato (Mellano and Cooksey 1988a). The identification of coding regions for other copper metabolising proteins suggests that copper-tolerance may not only be attributed to proteins encoded by the standard Cop and CopR/S operons defined by Mellano and Cooksey (1988a) and Mills *et al.* (1993, 1994). Gutiérrez-Barranquero *et al.* (2013) demonstrated CopG that it played a role in copper-tolerance in some *P.syringae* pv. *syringae* and P.syringae pv. tomato isolates. Coding regions for CopG were detected in BRIP38746 and BRIP66796.

Genetic analysis undertaken in this study reveals that the genetics of copper-tolerance in P.syringae pv. tomato is complex and diverse with a number of areas warranting further investigation. In particular functional analysis of the putative Cop operon, CopR/CusS operon and the CopAB complex to determine their cellular location, expression levels and function in copper detoxification and/or homeostasis. Analysis also revealed potential chromosomally encoded cop genes and additional copper metabolising proteins, such as CopG, which may have a role in copper-tolerance of P.syringae pv. tomato. Copper-tolerance in agriculturally significant bacterial species such as P.syringae pv. tomato will continue to have a significant effect on commercial enterprises and for now continued copper use is inevitable due to limited availability of effective alternatives. Understanding the genetic determinants of copper-tolerance has the potential to inform more effective pesticide resistance management strategies to improve overall disease control.

Chapter 7 - General Discussion, Future Work and Conclusions

7.1 Discussion

This thesis study has systematically explored copper tolerance in *P. syringae* pv. *tomato* from commercial properties in Eastern Australia. With widespread, long term use of copper-based pesticides in cropping systems there is significant selection pressure for pesticide tolerance or resistance to develop. Tomato producers in QLD and other states of Australia have reported a reduction in the relative efficacy of copper products for bacterial disease control, and this is presumed to be due to heavy usage of copper based products. The lack of effective alternatives to copper for the control of foliar bacterial pathogens has contributed to the continued use of these products. Shorter spray intervals and increased application rates in response to reduced product efficacy may also be contributing to further selection pressure for copper tolerance in bacterial populations. This study has experimentally confirmed tolerance is present in *P. syringae* pv. *tomato* and highlights the need for alternative disease management strategies to better manage copper-tolerant *P. syringae* pv. *tomato* populations.

Copper tolerance in bacterial pathogens causing disease on tomato and pepper is present across the world and is a major constraint in cropping systems (Chapter 2). All Australian *P. syringae* pv. *tomato* isolates collected in this study were determined to be copper-tolerant, and no sensitive isolates were identified (Chapter 5). Herbarium isolates collected in the 1970s were also copper-tolerant, suggesting this tolerance is not a recent development in Australia. Through genetic analysis, this tolerance was attributed to the presence of *cop* genes on a Cop operon similar to those previously characterised in American *P. syringae* pv. *tomato* isolates (Chapter 6).

The high prevalence of tolerance is largely attributed to the long term and intensive use of copper products in agricultural systems (Hall *et al.* 2011; Tesoriero *et al.* 1997). Most Australian growers apply copper products within 7 days of transplanting and continue treatment every 7-10 days for the duration of the cropping cycle. On some properties this application interval is reduced to 3-5 days when conditions are conducive for bacterial infection and/or there is a high incidence of disease in young crops. In Australia, the tomato crop cycle from transplanting to the end of harvest is 12-14 weeks, therefore, the total number of copper sprays applied to a crop could be anywhere between 8 and 32. Recommended application intervals for copper products are universal, so many tomato producing regions across the world also use a high amount of copper on their crops (Byrne *et al.* 2005; Pontes, N. *et al.* 2016). The continued use of copper on other crops grown in rotation with tomatoes results in a high level of copper in the production environment. This can lead to copper accumulation in soils which can negatively impact the environment and cause phytotoxicity in crops (Chibuike &

Obiora 2014; Van-Zwieten *et al.* 2004; Wightwick *et al.* 2010). This study presents the first systematic report of copper tolerance in *P. syringae* pv. *tomato* isolates from commercial tomato properties in Eastern Australia, particularly in QLD (Chapter 5). Results of this study, along with those previously reported (Hall *et al.*, 2011; Tesoriero *et al.*, 1997), indicate that Australian growers should assume that copper-based disease control programs are likely to be, at best, only partially successful in management of bacterial speck disease in tomato crops.

Currently there are minimal alternatives to copper registered for disease control in tomato and pepper. Almost 100 studies investigating copper alternatives for bacterial disease control were identified in the systematic review (Chapter 2), with a large number of these based on biological products such as essential oils, non-pathogenic bacteria, plant or compost extracts, systematic acquired resistance inducers and bacteriophages. Employing alternatives to copper in disease management programs could not only address the constraints caused by copper tolerance, but also provide a more environmentally sustainable approach to crop production.

Unlike copper products, which are broad spectrum pesticides that have similar biological effects on both *P. syringae* pv. *tomato* and *Xanthomonas* spp., many biological products are often more specific in their toxicity. Of the published studies on bacterial disease control in tomato and pepper, a large majority measured the efficacy of biological products for the control of disease caused by *Xanthomonas* spp. A biological product that effectively controls *Xanthomonas* spp. may not control disease caused by *P. syringae* pv. *tomato*. This becomes a problem in commercial systems where the exact bacterial species causing disease is often not diagnosed and these pathogens can occur together (Chapter 1). The efficacy of biological products can also be highly dependent on environmental conditions, crop nutrition status, soil type and other products applied to control non-bacterial pests (e.g. Hannusch & Boland 1996; Wong *et al.* 2002). Therefore, their integration into management programs may not be effective if the biological product is not compatible with other elements of the pest and disease management programs. Given the potential crop quality and yield reductions *P. syringae* pv. *tomato* can cause and the paucity of efficacy studies for copper alternatives on this pathogen, there is an immediate need for further research in this area. This would provide tomato producers with greater confidence when choosing an alternative product to copper for bacterial disease management.

A majority of published copper tolerance studies in tomato have screened *Xanthomonas* spp. isolates rather than *P. syringae* pv. *tomato* (Chapter 2), with only few published studies available that have surveyed the relative prevalence of both of these bacterial species on crops from different regions (Pernezny *et al.* 1995; Shenge, Mabagala*, et al.* 2008). Through the collection of isolates for this study

(Chapters 4 and 5), it was observed that there was a lower prevalence of *P. syringae* pv. *tomato* in the Bundaberg region in the 2016-17 spring/summer growing period compared to the same period in 2015-16. Whereas, isolates collected from the Granite Belt region were exclusively *P. syringae* pv. *tomato*. These differences along with the minimal availability of published studies suggest there is significant scope for a more detailed surveys of the relative prevalence of *P. syringae* pv. *tomato* and *Xanthomonas* spp. on crops from different regions. Understanding what species is most likely to be present in a particular area would inform more effective management strategies, especially as new copper alternatives come into the market which may not control *P. syringae* pv. *tomato* and *Xanthomonas* spp. to the same degree.

Protocols for the identification of bacterial species present in diseased crops should also incorporate assessment of copper tolerance in the isolates, as this information is important for decisions on appropriate management strategies. Various methodologies for copper tolerance screening have been published, with difference between protocols in media composition, copper tolerance thresholds and media pH (Chapter 2 & 4). Assessment of different media determined that CYEG was most suitable for copper tolerance testing of *P. syringae* pv. *tomato* (Chapter 4). This decision was based on the media's low capacity to complex copper ions (Zevenhuizen *et al.* 1979) and lower tolerance thresholds compared to other media tested. Adjustment of media pH was determined to be a critical step in the screening protocol to obtain consistent copper tolerance results. Of previously published *P. syringae* pv. *tomato* and *Xanthomonas* spp. copper tolerance studies analysed in the systematic review, 74% did not report the pH of the medium and/or report an adjustment being made to counter the pH reduction caused by the addition of copper sulfate (Chapter 2). The interpretation of copper tolerance assessments in these studies is therefore questionable, and comparisons between studies must be considered unreliable due to likely media pH differences. The methodology testing completed for this study (Chapter 4) demonstrated that MES was an effective stabiliser of media pH, and that the use of MES not only increased the reliability of tolerance results, but significantly reduced plate preparation time. Previously, the use of MES as a pH buffering agent for *in vitro* copper tolerance testing had only been reported once (Mixon 2012). It is recommended that the data presented in this study of copper tolerance methodology should be considered in any laboratory that is completing copper tolerance testing in other pathogenic plant bacteria, particularly other pseudomonads.

Despite recognised benefits of *in vivo* testing, only 4 of the 23 *in vitro* copper tolerance screening studies identified in the systematic review (Chapter 2) confirmed copper tolerance *in vivo*. The rationale behind the choice of copper tolerance thresholds used in publications was therefore unclear. *In vivo* testing demonstrated that disease caused by *P. syringa*e pv. *tomato* isolates with an MIC of ≥ 0.8 mmol/L copper sulfate on CYEG medium, were inconsistently controlled with commercial rates of

copper products (Chapter 4). Isolates determined to be copper-tolerant *in vitro*, also carried *copA* genes similar to those previously characterised as determinants for copper tolerance (Bender & Cooksey 1986, 1987) (Chapter 6). The only copper-sensitive *P. syringae* pv. *tomato* isolate available for this study was imported from the USA. No copper sensitive *P. syringae* pv. *tomato* were identified in this study either in samples collected between 2015-17 or in herbarium isolates from the 1970s. This limited the potential for completing further *in vivo* testing as there were strict biosecurity restrictions on the imported isolate, limiting its use to *in vitro* experiments only. Therefore, further *in vivo* testing with more copper sensitive *P. syringae* pv. *tomato* and isolates with different MIC levels is warranted to determine the most appropriate *in vitro* threshold for copper tolerance assessments.

The choice of practices to incorporate in an integrated disease management strategy to mediate tolerance development requires identification of the degree of stability or the biological fitness of copper tolerance in *P. syringae* pv. *tomato*. Experimental data in Chapter 5 suggested that copper tolerance may not be stable in all isolates when copper selection pressure is removed *in vivo*. Tolerance in isolate BRIP66810 was unstable and this isolate had almost identical *cop* genes to copper-tolerant American references (Chapter 6). The high similarity of these genes to the references (Mellano & Cooksey 1988a,b; Summers & Wireman 2012) and plasmid analysis, suggested that the *cop* genes of BRIP66810 are encoded on plasmid DNA. In contrast, BRIP66976 which maintained its copper tolerance status, was carrying also *cop* genes on a Cop operon, however, genome analysis suggested that this was encoded on chromosomal DNA. Additionally, this operon had high homology to chromosomal *cop* genes in another *P. syringae* pv. *tomato* reference (Xu & Toussaint 2017). Differences observed in the copper tolerance stability of BRIP66810 and BRIP66976 may therefore be linked to plasmid mediated pesticide tolerance, which has been reported to negatively affect biological fitness in other species (Araújo *et al.* 2012; Kleitman *et al.* 2005; Lilley & Bailey 1997). Sundin *et al.* (1994) suggest plasmid mediated resistance to copper and streptomycin persists in *P. syringae* pv. *syringae* when selection pressure is removed. Further experimental testing is required to evaluate plasmid stability in *P. syringae* pv. *tomato* .These copper tolerance stability findings provide an enticing potential strategy to reduce the presence of copper-tolerant *P. syringae* pv. *tomato* in commercial cropping systems. However, given the preliminary nature of results further replication under both greenhouse and field conditions with more isolates is required to clarify this relationship.

Understanding differences in genetic copper tolerance determinants in *P. syringae* pv. *tomato* has the potential to inform more effective resistance management strategies. The *cop* genes described by Bender & Cooksey (1986 and 1987) in American isolates are considered the primary determinants of copper tolerance in *P. syringae* pv. *tomato*. Homologs to these *cop* genes were identified in Australian *P. syringae* pv. *tomato* through PCR assays and genomic analysis (Chapter 6). PCR assays varied in their

ability to detect *cop* genes, however the 3'end region of *copA* was successfully amplified in a majority of Australian copper-tolerant isolates. All of these isolates, except BRIP66810, had significant sequence differences in this region of *copA* compared to American references. Despite these sequence differences multiple copper ion binding regions were still present, indicating protein function is maintained. Isolate BRIP66810 was identical to the American references for this *copA* region. The PCR assays tested have the potential to be further refined for use in the diagnosis of copper tolerance in field isolates.

Genetic analysis of three selected Australian *P. syringae* pv. *tomato* isolates revealed that the genetic basis of copper tolerance in *P. syringae* pv. *tomato* is complex and diverse (Chapter 6). Genome assemblies of these isolates identified putative Cop and CopR/CusS operons homologous to those previously characterised in *P. syringae* pv. *tomato* as mediators of copper-tolerance. Sequence comparisons of these Cop and CusS coding regions identified significant differences at both the nucleotide and amino acid levels between each isolate, with only one isolate (BRIP66810) sharing high homology to plasmid DNA of American reference sequences. Additional CopA and CopB coding regions forming a CopAB complex homologous to chromosomal CopA and CopB in a copper sensitive *P. syringae* pv. *tomato* reference genome was also identified in the three isolates. Further functional analysis is required to clarify the role of these putative Cop and CopR/CusS operons and the CopAB complex in Australian *P. syringae* pv. *tomato*, to determine whether these are expressed and active in the metabolism and detoxification of copper. Genome analysis in this study focused on the Cop proteins, however, a number of other potential copper metabolising coding regions were also identified that could be of interest in future work. Additionally, further DNA preparations are required to fully assemble the genome scaffolds identified in this study into complete genome sequences.

The pathovar identity of herbarium isolates BRIP 38735 and BRIP38826 from the 1970s was ambiguous, with results of PCR based genetic profiling suggesting these isolates were not *P. syringae* pv. *tomato* despite being isolated from tomato (Chapter 5 & Appendix E). Given that *P. syringae* pv. *syringae* has been isolated from tomato in a small number of cases (Garibaldi *et al*. 2007; Gilardi *et al*. 2010; Gullino *et al*. 2009), it was hypothesised that these isolates are *P. syringae* pv. *syringae*. However, genetic analysis using MLST *P. syringae* pathovar profiling (Berge *et al.* 2014; Hwang *et al.* 2005; Morris *et al.* 2010; Sarkar & Guttman 2004), was unable to definitively determine the pathovar identity of these three isolates (Appendix E). Further characterisation of BRIP38735 and BRIP3882 is warranted for future studies, to investigate if they represent a unique *P. syringae* pathovar or are simply genetic variants of an existing phylogroup 2a pathovar. Isolate BRIP66800 also did not conform with expected results as it had the $hrpZ_{Pst}$ profile of *P. syringae* pv. *tomato*, however, the *gltA* sequence analysis suggested otherwise (Appendix E). Both BRIP66801 and BRIP66802, collected from the same property

as BRIP66800, were determined to be *P. syringae* pv. *tomato* (section 5.2.1). Hence further characterisation of BRIP66800 is required to investigate if it represents a genetic variant of *P. syringae* pv. *tomato*.

The genome analysis identified a putative plasmid in isolate BRIP38746 and a plasmid component in BRIP66810, both of these carried the putative Cop and CopR/CusS operons (Chapter 6). *P. syringae* pv. *tomato* isolated in the USA were determined to be carrying two to four plasmids (Bender & Cooksey 1986). These were grouped into five size classes: class A, which range from 95 to 103 kilo base pairs (kb); class B, ranging from 71 to 83 kb; class C, ranging from 59 to 67 kb; class D, ranging from 37 to 39 kb; and class E, which are approximately 29 kb. It is therefore likely that Australian *P. syringae* pv. *tomato* also carry multiple plasmids with a range of cellular functions including copper tolerance. Further investigation into the plasmid profile of a Australian *P. syringae* pv. *tomato* is therefore warranted to clarify whether plasmid DNA is carrying functional Cop proteins or if copper tolerance is being controlled chromosomally as suggested for BRIP66796.

7.2 Conclusion

This study of copper tolerance in *P. syringae* pv. *tomato* contributes to scientific understanding on the prevalence and distribution of copper tolerance domestically and internationally. This is the first systematic study of *P. syringae* pv. *tomato* in Eastern Australia and confirmation of copper tolerance in the regions sampled is of considerable importance to the local horticultural industry. Methodology testing on factors contributing to *in vitro* copper tolerance results has demonstrated how pH can significantly affect the reliability of previously published results and the need for the global standardisation of procedures. Genetic analysis of copper tolerance determinants in Australian *P. syringae* pv. *tomato* revealed tolerance is complex and diverse with analysis suggesting Cop and CopR/CusS operons may be located on either plasmid or chromosomal DNA, depending on the isolate studied. This study provides the first detailed investigation of the genetic basis of tolerance in this species outside of America. Engagement with commercial tomato growers and other industry representatives throughout this research project has contributed to an increased awareness of the need to manage copper tolerance in this important pathogen. Whilst copper tolerance was present in all Australian isolates, the relative level of this tolerance compared to the highly tolerant reference isolate from the USA, suggests there is still potential for producers to prevent the build-up of high tolerance *P. syringae* pv. *tomato* populations. Industry engagement has therefore been aimed at encouraged producers to revise their management programs so that copper is applied more effectively and in conjunction with robust cultural disease management practice.

7.3 Future work

Potentials areas for further investigation into a number of aspects of copper tolerance in *P. syringae* pv. *tomato* are discussed in the main body of this Chapter in the context of study results. Here is a brief summary of these:

- Investigate the relative prevalence of *P. syringae* pv. *tomato* and *Xanthomonas* spp. in Australian tomato growing regions through systematic surveys.
- More wide scale and replicated field studies on the efficacy of alternatives to copper for the control of disease caused by *P. syringae* pv. *tomato*.
- *In vivo* copper tolerance testing on a wider range of copper-sensitive *P. syringae* pv. *tomato* and tolerant isolates with different MIC levels, to clarify MIC thresholds for *in vitro* assays.
- Further investigation into the stability or biological fitness of copper tolerance in *P. syringae* pv. *tomato* under both greenhouse and field conditions.
- Refinement of PCR assays for the detection of *cop* genes for diagnostic purposes.
- Functional analysis on the role the putative Cop and CopR/CusS operons and the CopAB complex in Australian *P. syringae* pv. *tomato*.
- Clarification of cellular location of Cop and CopR/CusS operons and CopAB complex in Australian *P. syringae* pv. *tomato*, including plasmid profiling.
- Investigation into the evolutionary development of copper tolerance in a wider range of herbarium *P. syringae* pv. *tomato* from Australian tomato crops.
- Investigation into copper metabolising proteins other than Cop proteins, and their role in copper tolerance in *P. syringae* pv. *tomato*.
- Further DNA preparations to fully assemble the genome scaffolds identified in this study into complete genome sequences.
- Detailed genetic comparisons of isolates BRIP66800, BRIP66801 and BRIP66802 to investigate differences observed in gene profiling.
- Genetic analysis to determine the pathovar identity of BRIP38826 and BRIP38735.

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Chapter 9 Appendix

Table of Contents

Appendix A: Chapter 2 supplementary material

Supplementary Table 1: Complete list of the 133 publications analysed in the systematic review, separated into categories. Abbreviations: CR = copper tolerance and/or resistance; CE = efficacy of copper based compounds; BA = alternative compounds

Category (#)	Publications
BA (n=34)	Abbasi *et al*., 2002; Abbasi *et al*., 2003; Abo-Elyousr and El-Hendawy, 2008; Aires *et al*., 2009; Al-Dahmani *et al*., 2003; Al-Saleh, 2011; Altundag and Aslim, 2011; An *et al*., 2016; Bashan and Bashan, 2002a; Bhuvaneshwari *et al*., 2015; Campos Silva *et al*., 2008; Cavalcanti *et al*., 2006, 2007; Chandrashekar and Umesha, 2014; Chavez-Dozal *et al*., 2014; Cooksey, 1988; Dawoud *et al*., 2012; El-Hendawy *et al*., 2005; Elkhalfi *et al*., 2013; Fontenelle *et al*., 2011; Goel and Kumar Paul, 2015; Ibrahim, 2012; Indiragandhi *et al*., 2008; Ji and Wilson, 2003; Kavitha and Umesha, 2007; Lanna Filho *et al*., 2010; Mirik and Aysan, 2005; Mirik *et al*., 2008; Nahak and Sahu, 2015; Quattrucci and Balestra, 2006; Reddy *et al*., 2012; Romero *et al*., 2003; Scarponi *et al*., 2001; Vavrina *et al*., 2004
BA and CE (n=55)	Abbasi and Weselowski, 2015; Balestra *et al*., 2009; Balogh *et al*., 2003; Bashan and de-Bashan, 2002b; Briceno-Montero and Miller, 2005; Buonaurio *et al*., 2002; Byrne *et al*., 2005; Conlin and McCarter, 1983; Cuppels *et al*., 2013; da Silva *et al*., 2014; Dougherty, 1978; El-Ghafar and Mosa, 2001; Fayette *et al*., 2012; Flaherty *et al*., 2000; Fousia *et al*., 2015; Garton *et al*., 2007; Hert *et al*., 2009; Huang *et al*., 2012; Itako *et al*., 2014; Itako *et al*., 2012; Itako *et al*., 2015; Ji *et al*., 2006; Jones and Jones, 1985; Jones and Kelly, 1995; Lanna Filho *et al*., 2013; Lewis Ivey *et al*., 2004; Louws *et al*., 2001; Mbega *et al*., 2012; McCarter, 1992; Miller *et al*., 2007; Moss *et al*., 2007; Moura *et al*., 2012; Nascimento *et al*., 2013; Obradovic *et al*., 2004a; Obradovic *et al*., 2005; Ocsoy *et al*., 2013; Paradela *et al*., 2003; Paret *et al*., 2012; Quattrucci and Balestra, 2011; Quattrucci *et al*., 2013; Resende *et al*., 2015; Roberts *et al*., 2008; Romero *et al*., 2001; Saad and Abul Hassan, 2000; Shrestha *et al*., 2014; Stall, 1959; Trueman and May, 2010; Trueman, 2013; Vallad *et al*., 2010; Vallad and Huang, 2012a, b; Wen *et al*., 2009; Wilson *et al*., 2002; Worthington *et al*., 2012
BA, CE and CR (n=2)	Graves and Alexander, 2002; Pontes *et al*., 2012
CE (n=2)	Aguiar *et al*., 2003; Yunis *et al*., 1980
CE and CR (n=4)	Adaskaveg and Hine, 1985; Carmo *et al*., 2001; Jones *et al*., 1991; Jones *et al*., 1993
CR (n=36)	Abbasi *et al*., 2015; Araújo *et al*., 2012; Bender and Cooksey, 1986; Bender *et al*., 1990; Bouzar *et al*., 1996; Bouzar *et al*., 1999; Buonaurio *et al*., 1994; Cooksey *et al*., 1990; Cuppels and Elmhirst, 1999; Gore and O'Garro, 1999; Goto *et al*., 1994; Hall *et al*., 2011; Horvath *et al*., 2012; Ignjatov *et al*., 2010; Kebede *et al*., 2014; Marco and Stall, 1983; Martin *et al*., 2004; Mirik *et al*., 2007; Mitrev *et al*., 2001; Mitrev and Kovačević, 2006; Mixon, 2012; O'Garro, 1998; Obradovic *et al*., 2001; Obradovic *et al*., 2004b; Pernezny *et al*., 1995; Pernezny *et al*., 2008; Pohronezny *et al*., 1992; Quezado-Duval *et al*., 2003; Ritchie and Dittapongpitch, 1991; Sahin and Miller, 1996; Shenge *et al*., 2008; Shenge *et al*., 2014; Silva and Lopes, 1995; Stall *et al*., 1986; Tesoriero *et al*., 1997; Ward and O'Garro, 1992

Appendix B: Optimisation of annealing temperature (T_A) for $hrpZ_{Pst}$ PCR assay

The annealing temperature (T_A) of the PCR reaction was optimised by testing *P. syringae* pv. *tomato* (DAR 75965) and *P. syringae* pv. *syringae* (DAR 77319) reference cultures. Temperatures tested were T_A = 54.3 °C, 57.3 °C, 60.16 °C and 63.2 °C. DNA amplification was carried out in a Kyratec SuperCycler Thermal Cycler (model SC300) using the following programme: denaturation at 95°C for 1 minute (min) followed by 30 cycles of denaturation at 95 °C for 20 s, T_A for 20 s and extension at 72 °C for 30 s, then a final extension at 72 °C for 5 min. Each reaction mixture (50 µL) contained: 0.2 µmol/Lol/L of each primer, 22 µL sterile deionised water and 25 µL MangoMix™ (Bioline, contains 2.5 mmol/L $MgCl_2$, MangoTaq™ DNA Polymerase, dNTPs, 5 x coloured reaction buffer). Bacterial suspensions in water were adjusted to approximately 7.2 x 10^7 CFU/mL, 1 µL of this was added to the reaction mixture.

Agarose gel electrophoresis and EtBr staining were used to visualise the amplification products. A 2.0% w/v agarose gel was made up in 0.5 X Tris/Borate/Ethylenediaminetetraacetic acid buffer (TBE). A 10 µL aliquot of each sample was loaded into the gel and 5 µL of the molecular weight ladder (Bioline HyperLadder™ or EasyLadder™). The gel was run in 0.5X TBE at 80 volts for approximately 80 min or until dye was 80% of the way down the gel. The gel was transferred to a 0.5 µg/mL EtBr solution and soaked for 15-20 min, washed with water for 1-5 min and then visualised under UV light.

The expected amplication product for *P. syringae* pv. *tomato* (532 bp) was observed for the *P. syringae* pv. *tomato* isolate and not in *P. syringae* pv. *syringae*. This suggested a successful reaction supporting the specificity of the test for detecting pv. *tomato* and not pv. *syringae* (Zaccardelli *et al.* 2005). An annealling temperature of 60 °C was selected due to a slight reduction in band intensity at 63 °C (Figure B-1).

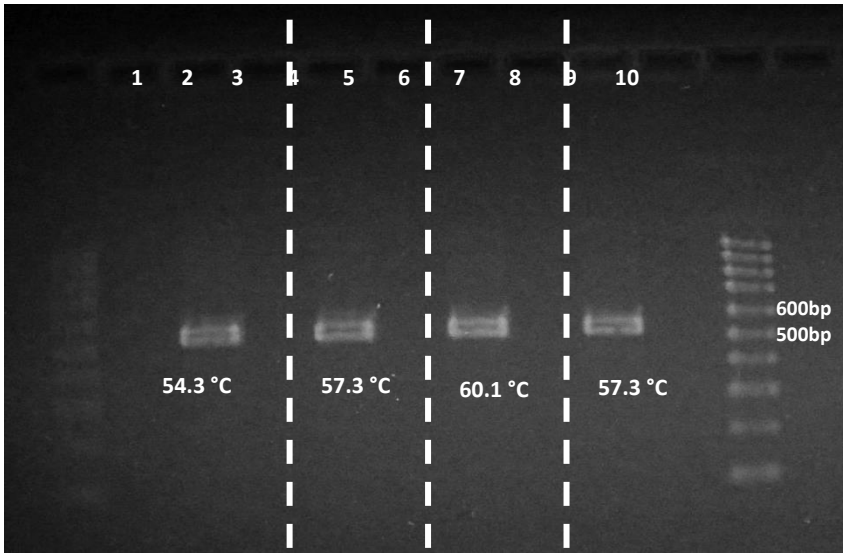

Figure B-1: Optimisation of the T_A of the $hrpZ_{Pst}$ PCR reaction. Temperatures tested: 54.3°C (lanes 2 and 3), 57.3°C (4 and 5), 60.16°C (6 and 7) and 63.2°C (8 and 9). Lane 1 negative control, Lanes 2, 4, 6, 8 and 10 = *P. syringae* pv. *tomato*, Lanes 3, 5, 7 and 9 = *P. syringae* pv. *syringae* Lane 10: Bioline HyperLadder.

Appendix C: Multi-Locus Sequence Typing (MLST) methodology

Primers for the four gene MLST of Hwang *et al* (2005) were used for preliminary testing of 16 *Pseudomonas* isolates (Table C-1). These included 10 herbarium reference samples along with the 3 reference isolates from the ASCT and 3 isolates collected in this study (Table C-2).

Table C-1: Primer details for the MLST analysis.

Primer	Primer type	Sequence	Approximate Fragment Length (bp)
gapA264p	Forward	CCGGCSGARCTGCCSTGG	610
gapA874ps	Reverse	GTGTGRTTGGCRTCGAARATCGA	
gyr271ps	Forward	TCBGCRGCVGARGTSATCATGAC	751
gyrB1022p	Reverse	TTGTCYTTGGTCTGSGAGCTGAA	
gltA174p	Forward	GCCTCBTGCGAGTCGAAGATCACC	1018
gltA1192p	Reverse	CTTGTAVGGRCYGGAGAGCATTTC	
rpoD147p	Forward	CAGGTGGAAGACATCATCCGCATG	1075
rpoD1222ps	Reverse	CCGATGTTGCCTTCCTGGATCAG	
gltA513s	Internal forward	CCTGRTCGCCAAGATGCCGAC	N/A
rpoD364s	Internal forward	GYGAAGGCGARATYGRAATCG	N/A

Universal degeneracy codes: B = C,G,T, V = A,C,G, R = A,G and Y= C,T

Table C-2: *Pseudomonas* isolates used in the testing of MLST. The successful amplification of each gene (*gapA, gyrB, gltA* and *rpoD)* is indicated by '+'. Some isolates failed to produce an amplified product as indicated by '-'.

Sample ID	*Pseudomonas* spp.	*rpoD*	*gltA*	*gyrB*	*gapA*
DAR 75965	*P. syringae* pv. *tomato*	+	+	+	+
DAR 77319	*P. syringae* pv. *syringae*	+	+	+	+
BRIP38735	*P. syringae* pv. *tomato***	+	+	+	+
BRIP38826	*P. syringae* pv. *tomato***	+	+	+	+
BRIP38432	*P. syringae* pv. *striafaciens*	+	+	+	+
BRIP38748	*P. syringae* pv. *syringae*	+	+	+	+
BRIP34814	*P. syringae* pv. *tabaci*	+	+	+	+
BRIP38744	*P. syringae* pv. *tomato*	+	+	+	-
BRIP38746	*P. syringae* pv. *tomato*	-	+	+	-
BRIP66796	Unknown*	+	+	+	+
BRIP66802	Unknown*	+	+	+	+
BRIP66810	Unknown*	-	+	+	+

* $hrpZ_{Pst}$ PCR tests suggested these are *P. syringae* pv. *tomato*

**These samples gave negative results in the $hrpZ_{Pst}$ PCR test, discussed in section 5.9 and Appendix E.

PCR products were submitted to Macrogen Incorporated for purification and sequencing. Two internal forward primers of *rpoD* (*rpoD364s*) and *gltA* (*gltA513s*) (Hwang *et al* 2005), were also used to

overcome potential secondary sequence structures within the amplified DNA. Sequences obtained from the internal primers *rpoD364s* and *gltA513s* were aligned to the consensus sequence as a further check of sequence homology. These tended to provide minimal extra information and no differences compared to the forward and reverse fragment sequences, therefore they were not used in further experiments for this study.

Each primer set was run in separate reactions, each reaction mixture of 50 µL contained 0.2 µmol/L of each primer, 25 µL of MangoMix™ (Bioline) and 1 µL of approximately 7.2 x 10^7 CFU/mL bacterial suspension. The PCR program consisted of a denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s, T_A for 20 s and extension at 72 °C for 45 s, with a final extension at 72°C for 5 min. The T_A was 56 °C for *rpoD* and *gltA*, and 50 °C for *gapA* and *gyrB*.

All four primer sets successfully amplified fragments of the expected size, however, some isolates did not produce a fragment for some genes (Table C-2). In particular the *gapA* reaction only successfully amplified a product in 10 of the 16 isolates tested. A CLUSTALW multiple sequence alignment (Aiyar 1999) was completed with reference pathovar sequences and the sequences collected in this study for each gene. After alignment all sequences were trimmed to the same size (Berge *et al.* 2014; Morris *et al.* 2010), 317 bp for *gltA*, 449 bp for *rpoD*, 506 bp for *gyrB* and 476 bp for *gapA* for further analyses including phylogenetic comparisons.

Comparison of phylogenetic tree building methods and packages

Maximum Likelihood (MaxLH), Bayesian (Mr. Bayes) and Neighbour-Joining methods for the building of phylogenetic trees were compared in this study using the specifications of Berge *et al.* (2014) and Morris *et al.* (2010) as a guide. The 68 *gltA* fragment sequences of *P. syringae* reference pathovars published by Berge *et al.* (2014) were used to compare phylogenetic tree building algorithms and packages. Sequences were aligned using CLUSTALW (Figure D-1). Phylogenetic analysis was completed in UGENE (Okonechnikov *et al.* 2012) with the PhyML package, version 20120412, (Guindon *et al.* 2010) for MaxLH and Mr. Bayes version 3.2.3 (Hulsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) for Bayesian methods. Two MaxLH trees and three Mr. Bayes trees were built using the *gltA* sequence alignment to compare the placement of phylogroups within the trees. MaxLH and Mr. Bayes trees were also compared to a phylogenetic tree constructed in Geneious v 10.2.3 (Kearse *et al.* 2012) using the Neighbour-Joining method (Saitou & Nei 1987).

Specifications for each tree were as follows:

1) MaxLH substitution model Kimura 2-parameter (K80; (Kimura 1981)) with UGENE defaults: empirical equilibrium frequencies, 4 substitution rate categories, aLRT branch support and NNI tree

improvement with optimised topology and branch lengths. The K80 model was used by Berge *et al.* (2014)

2) MaxLH substitution model HKY85 (Hasegawa *et al.* 1985) with UGENE defaults. The HKY85 model was used by Morris *et al.* (2010)

3) Mr. Bayes, substitution model HKY85, gamma 4, chain length 200,000, subsample frequency 20,000 and burn in 50,000. Mr Bayes parameters used by Morris *et al.* (2010).

4) Mr. Bayes, substitution model Jukes-Cantor 69 (JC69; (Jukes & Cantor 1969)) gamma 4, chain length 200,000, subsample frequency 20,000 and burn in 50,000.

5) Mr. Bayes, substitution model HKY85, gamma 4, chain length 500,000, subsample frequency 50,000 and burn in 250,000. Mr Bayes parameters used by Berge *et al.* (2014).

6) Geneious, Neighbour-Joining method, model HKY.

All six trees placed the reference sequences within their expected phylogroups in separate clades, only minor differences were observed between the trees. Phylogroup placement within the MaxLH trees was the same for groups 12, 13, 11, 6, 4, 1 and 3. Two larger groups, (A) 7, 8 and 9 and (B) 5 and 10, had the same configuration within this larger group but were different in their divergence pattern. In the MaxLH substitution model K80 tree (#1), phylogroups 7, 8, 9 were grouped higher than 5 and 10, however, in the MaxLH substitution model HKY85 tree (#2) these groupings were the opposite. Phylogroup placement in the Mr. Bayes trees mirrored many branching features on the MaxLH trees and different phylogroups were still clearly separated. One key difference in the placement of phylogroups with Mr. Bayes was between the HKY85 and JC69 substitution models, in JC69 phylogroup 3 was placed higher than 6 and 4 whilst in HKY85 it was placed lower than 6 and 4. The Neighbour-Joining HKY tree (#6) placed phylogroups in slightly different locations to MaxLH and Mr. Bayes tree, however, general trends in clade organisation were similar.

Given differences were minimal between the trees and the reference sequences were clearly and consistently split into phylogroups it was determined that any of the six tree specifications could be used. The Neighbour-Joining HKY tree constructed in Geneious was used for phylogenetic analysis for the rest of this thesis study (Figure C-2).

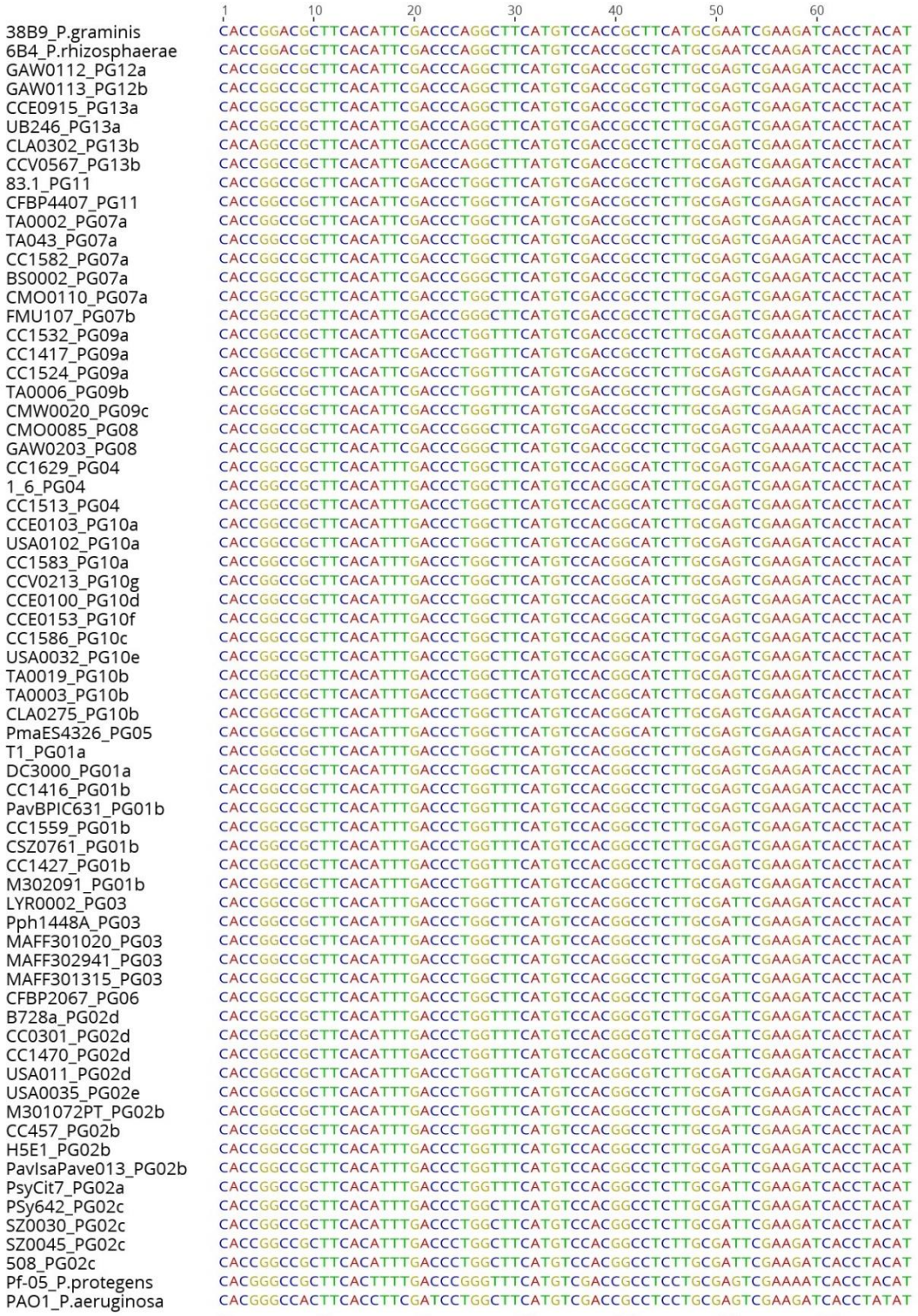

Figure C-1: CLUSTALW multiple sequence alignment of gltA reference sequences. Sequences were sourced from Table S11 Berge *et al.* (2014). Abbreviations: PG## = phylogroup number. a-e = phylogroup clade where applicable.

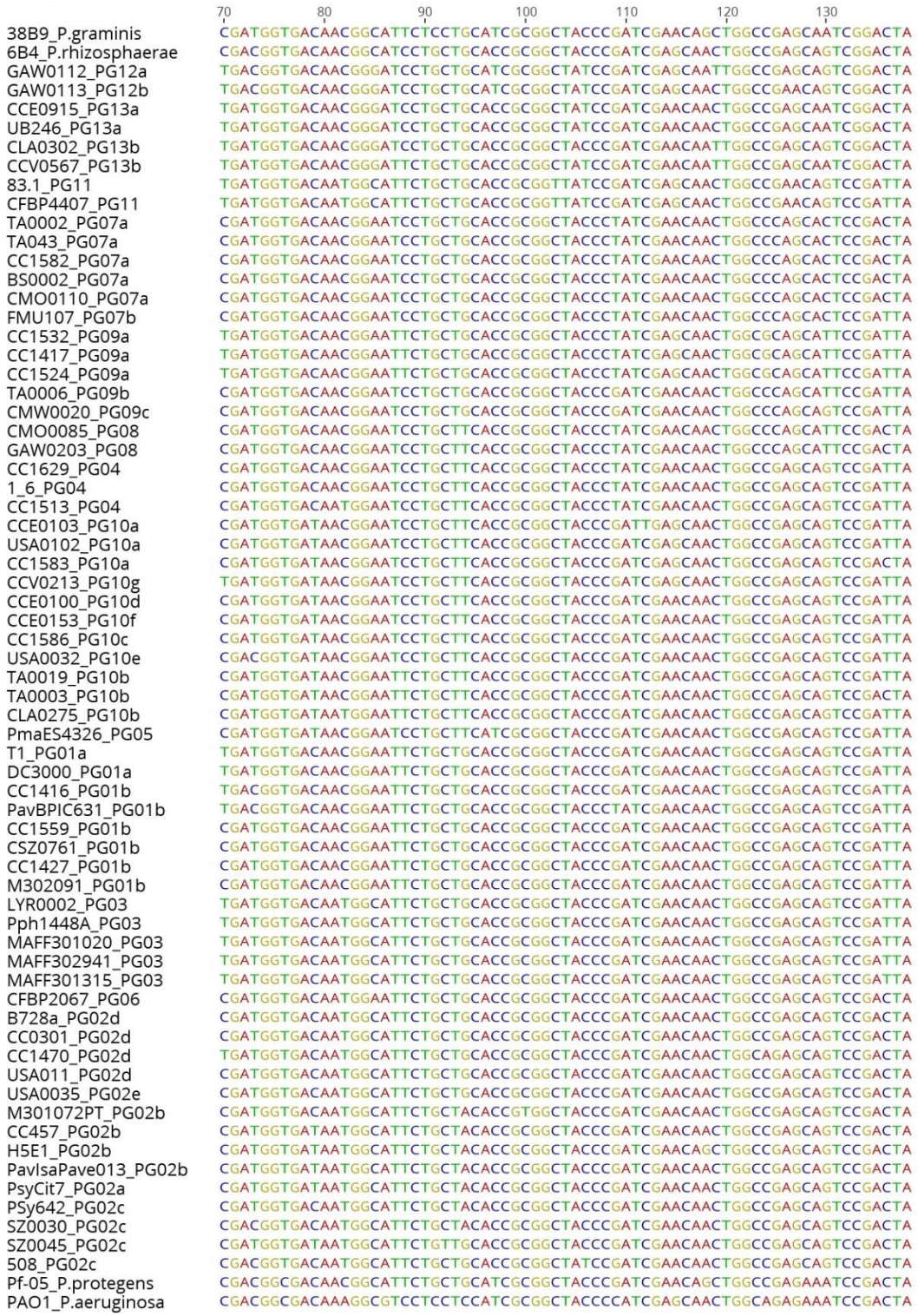

Figure C-1 continued

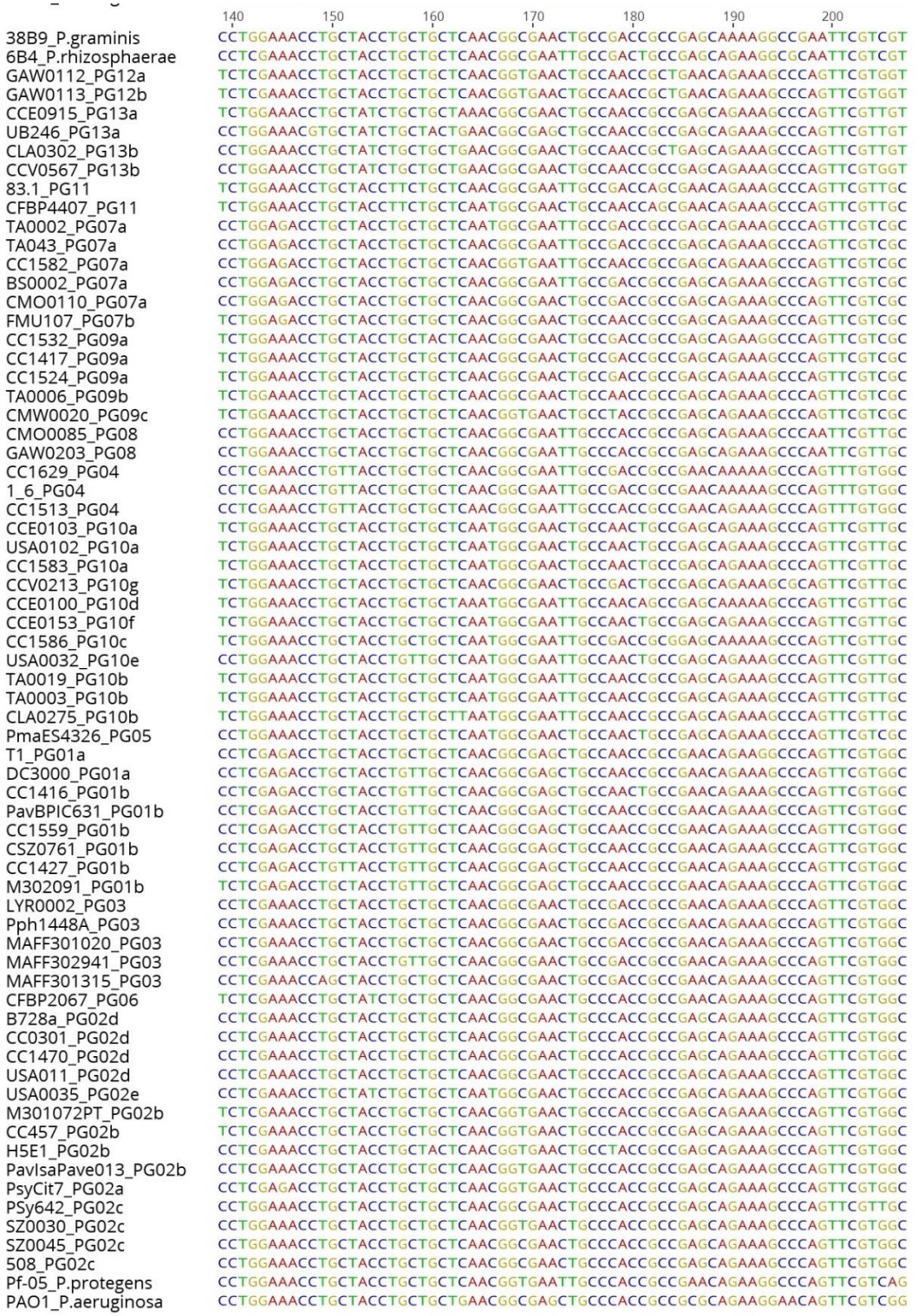

Figure C -1 continued

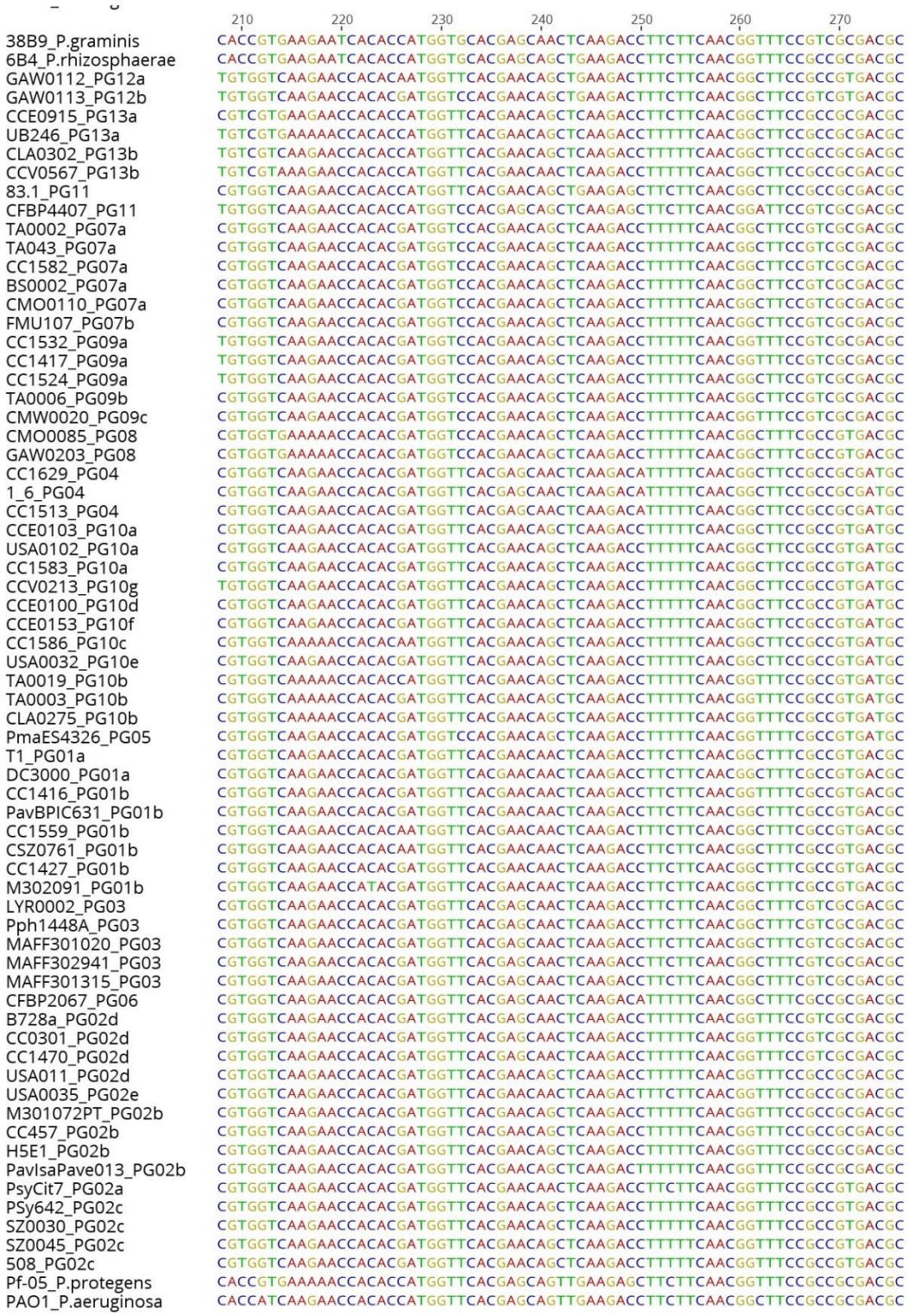

Figure C-1 continued

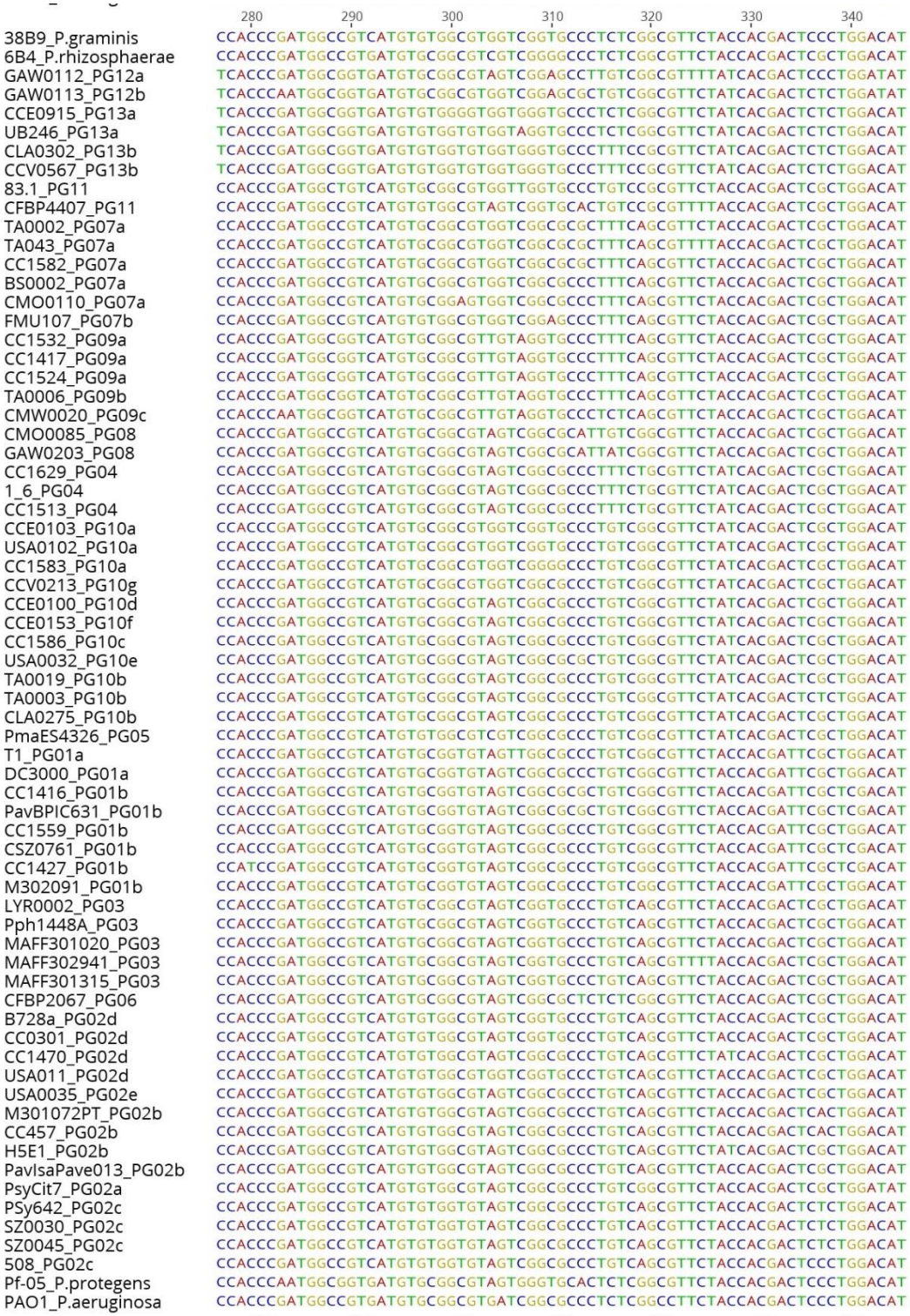

Figure C-1 continued

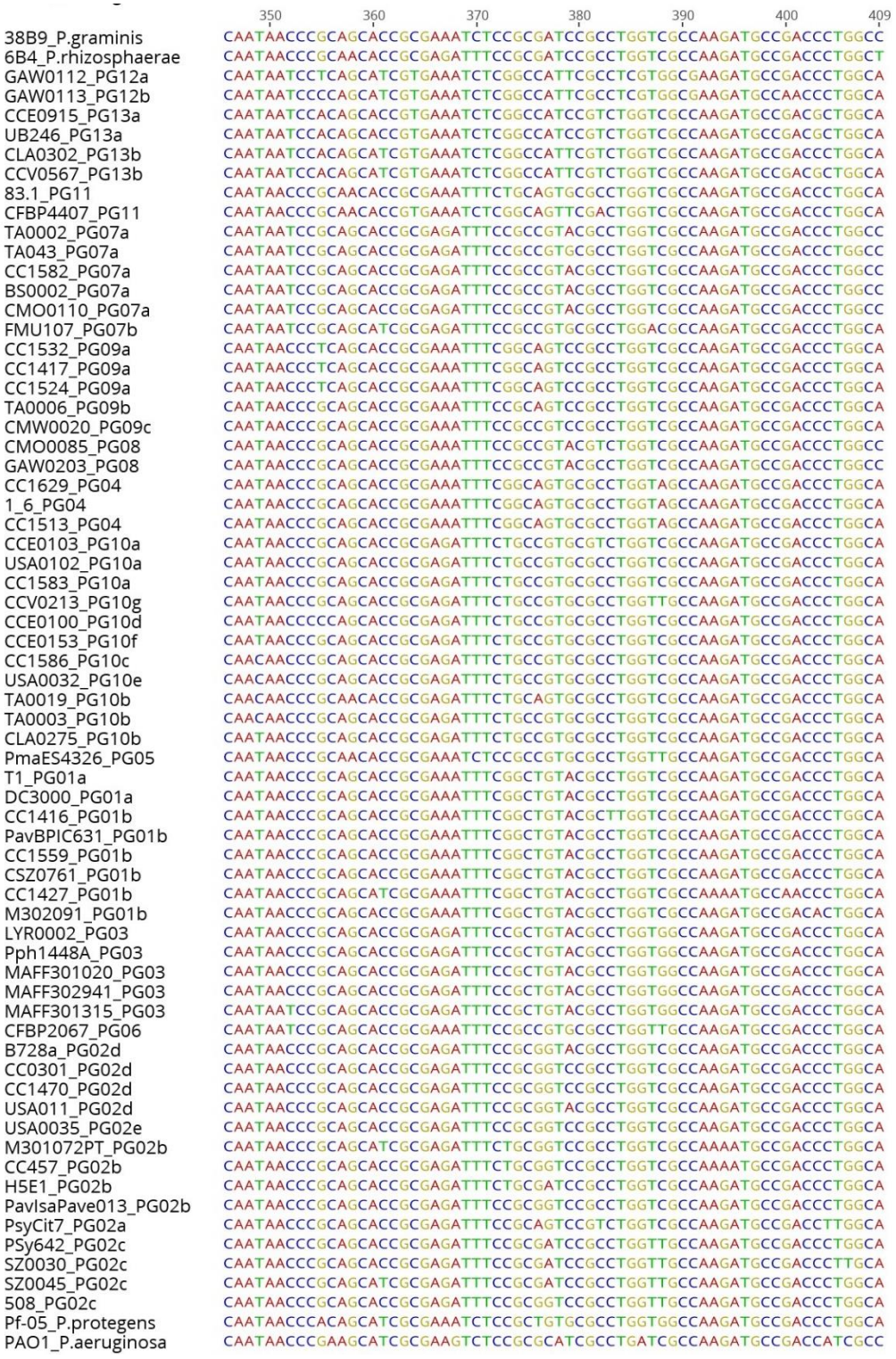

Figure C-1 continued

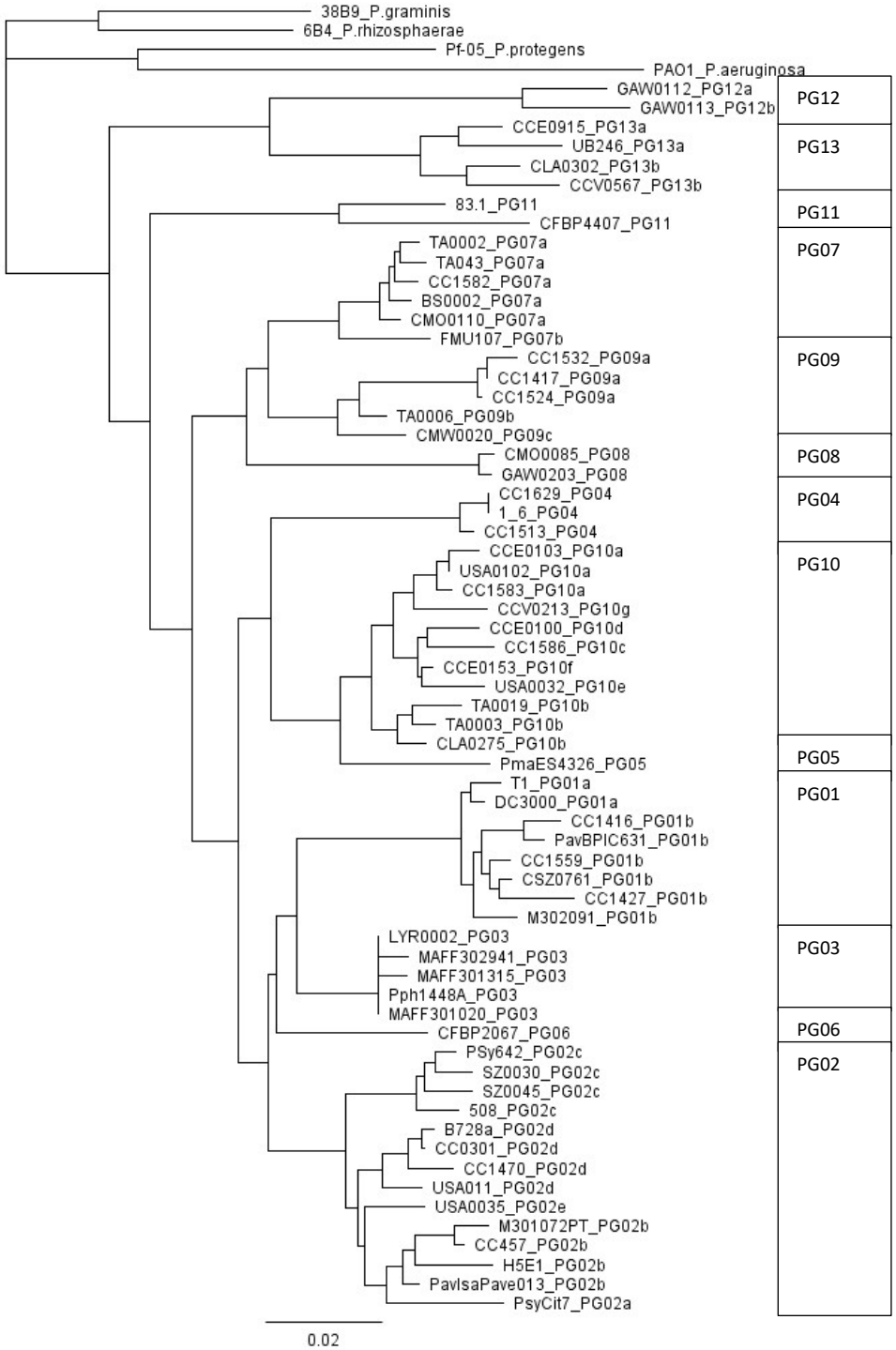

Figure C-2: Phylogenetic tree of *gltA* reference sequences from Berge *et al.* (2014). Constructed in Geneious using the Neighbour-Joining method, model HKY. Abbreviations: PG## = phylogroup number. a-e = phylogroup clade where applicable.

Comparison of phylogenetic trees with four different genes

Using the phenotypic traits of these four genes for grouping pathovars is not always reliable (Berge *et al.* 2014; Sarkar & Guttman 2004). To investigate this further the phylogenetic trees and the positioning of the study isolates (Table C-2) within these trees was compared for each gene i.e. gltA, *rpoD, gapA* and *gyrB*. Each alignment contained the reference sequences of *P. syringae* pathovars suggested by Berge *et al.* (2014), sequences for some pathovars was not unavailable for some genes. Sequences were downloaded from the PAMDB. Analysis was completed in Geneious, multiple sequence alignment with CLUSTALW (default settings IUB cost matrix, gap open cost=15, gap extend cost=6.66 and no 'free end gaps') and tree building with the Neighbour-Joining method and the HKY genetic distance model. The position of study isolates within phylogroups was compared for each gene (Table C-3).

Table C-3: Comparison of the position of study isolates within phylogroups with the MLST genes *rpoD, gltA, gyrB* and *gapA*. Phylogroups were determined through CLUSTALW multiple sequence alignment and Neighbour-Joining HKY phylogenetic analysis in Geneious.

Sample ID	rpoD	gltA	gyrB	gapA
DAR75965	1	1	1	1
DAR77319	2b & 3	2b	2c, b & d	2b
BRIP38735	2a	2a	2a	2a
BRIP38826	2a	2a	2a	2a
BRIP38432	4, 11, 12a	4	4	4
BRIP38748	6, 11 & 12b	3	3	3
BRIP34814	6, 11 & 12b	3	3	3
BRIP38744	1	1	1	NS
BRIP38746	NS	1	1	NS
BRIP66796	1	1	1	1
BRIP66802	1	1	1	1
BRIP66810	NS	1	1	1

NS= no sequence

Comparison of the position of study isolates within phylogroups with different genes demonstrated there was some variability in the phylogenetic placement of isolates (Table C-3). The *rpoD* gene sequences gave the most variable results with DAR77319, BRIP38432, BRIP38748 and BRIP38714 being grouped into a clade with more than one phylogroup. The positioning of reference sequences within the *rpoD* tree was also highly variable with inconsistent phylogroup and clade separation. The *gltA, gyrB* and *gapA* genes gave identical phylogroups for study isolates with the exception of DAR77319. The phylogroup placement of this isolate was only different for the gyrB gene which failed to resolve it into a single subgroup within phylogroup 2. In a detailed analyses by Berge *et al.* (2014) and Sarkar and Guttman (2004), it was demonstrated that *gltA* provided the most consistent

separation of *P. syringae* pathovar phylogroups. Therefore, *gltA* was used for the analysis of all study isolates to determine whether they were *P. syringae* pv. *tomato* or *P. syringae* pv. *syringae*.

The MLST analysis suggests that some of the herbarium isolates may not have been typed accurately at the pathovar level. BRIP38748 was catalogued as *P. syringae* pv. *syringae*, however, this isolate is in phylogroup 3 rather than phylogroup 2 which contains *P. syringae* pv. *syringae* (Berge *et al.* 2014)Table S8). BRIP38735 and BRIP38826 were catalogued as *P. syringae* pv. *tomato*, however, these isolates are in phylogroup 2 rather than phylogroup 1 which contains pv. *tomato*. The positioning of BRIP38735 and BRIP38826 in phylogroup 2 may indicate that these tomato isolates could be *P. syringae* pv. *syringae.* Isolation of *P. syringae* pv. *syringae* from tomato has previously been reported (Garibaldi *et al.* 2007; Gilardi *et al.* 2010; Gullino *et al.* 2009). The pathovar identity of these isolates is further explored in Appendix E.

Appendix D: Distance matrices and phylogenetic analysis of gltA sequence fragments.

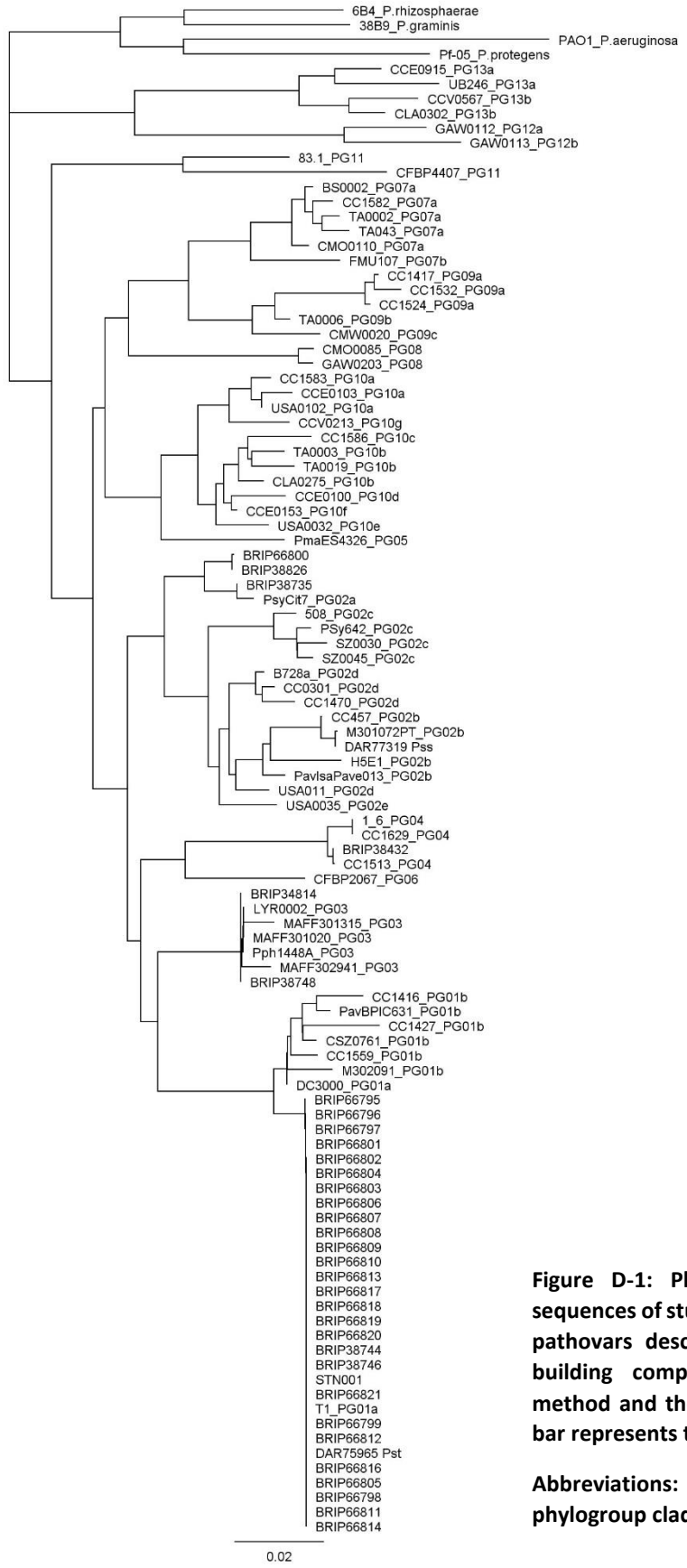

Figure D-1: Phylogenetic analysis of *gltA* gene sequences of study isolates and reference *P. syringae* pathovars described by Berge *et al.* (2014). Tree building completed with the Neighbour-Joining method and the HKY genetic distance model. Scale bar represents the genetic distance.

Abbreviations: PG## = phylogroup number; a-e = phylogroup clade.

Table D-1: Distance matrices of *gltA* gene sequence fragments of study isolates and PAMDB reference isolates from the Hwang MLST schema (529 bp). Data represents the percentage nt sequence identity of sequences based on CLUSTALW multiple sequence alignment.

	GA	B800	GB	STN001	B44	B46	D65	B14	B45	B32	B51	B31	D87	B48	D19	B26	B35	GC	2170	DC-KN
GA		94	100	100	100	100	100	93	93	90	92	93	92	92	92	94	94	100	94	99
B800	94		94	94	94	94	94	95	92	91	94	95	93	96	98	100	99	94	99	94
GB	100	94		100	100	100	100	93	93	90	92	93	92	92	92	94	94	100	94	99
STN001	100	94	100		100	100	100	93	93	90	92	93	92	92	92	94	94	100	94	99
B44	100	94	100	100		100	100	93	93	90	92	93	92	92	92	94	94	100	94	99
B46	100	94	100	100	100		100	93	93	90	92	93	92	92	92	94	94	100	94	99
D65	100	94	100	100	100	100		93	93	90	92	93	92	92	92	94	94	100	94	99
B14	93	95	93	93	93	93	93		92	92	93	95	92	99	95	95	95	93	95	93
B45	93	92	93	93	93	93	93	92		89	93	91	92	91	92	92	92	93	92	93
B32	90	91	90	90	90	90	90	92	89		90	90	89	91	91	91	90	90	90	91
B51	92	94	92	92	92	92	92	93	93	90		94	92	93	93	94	94	92	94	93
B31	93	95	93	93	93	93	93	95	91	90	94		92	95	94	95	95	93	95	93
D87	92	93	92	92	92	92	92	92	92	89	92	92		92	92	93	93	92	93	92
B48	92	96	92	92	92	92	92	99	91	91	93	95	92		96	96	96	92	96	92
D19	92	98	92	92	92	92	92	95	92	91	93	94	92	96		98	97	92	97	93
B26	94	100	94	94	94	94	94	95	92	91	94	95	93	96	98		99	94	99	94
B35	94	99	94	94	94	94	94	95	92	90	94	95	93	96	97	99		94	100	95
GC	100	94	100	100	100	100	100	93	93	90	92	93	92	92	92	94	94		94	99
2170	94	99	94	94	94	94	94	95	92	90	94	95	93	96	97	99	100	94		95
DC-KN	99	94	99	99	99	99	99	93	93	91	93	93	92	92	93	94	95	99	95	

Isolate Abbreviations: GA = group A BRIP66795-BRIP66799; B800 = BRIP66800; GB = group B isolates BRIP66801-BRIP66821; B44 = BRIP38744; B46 = BRIP38746; D65 = DAR75965; B14 = BRIP34814; B45 = BRIP34945; B32 = BRIP38432; B51 = BRIP38751; B31 = BRIP38831; D87 = DAR65987; B48 = BRIP38748; D19 = DAR77319; B26 = BRIP38826; B35 = BRIP38735; GC = group C PAMDB isolates DC84-1, 1318, DC89-4H, DCT6D1, 487, PT23 and TF1; DC-KN = PAMDB isolates DC3000 and KN10

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Table D-2: Distance matrices of *gltA* gene sequence fragments of study isolates and PAMDB reference isolates from the Yan MLST schema (393 bp). Data represents the percentage nt sequence identity of sequences based on CLUSTALW multiple sequence alignment.

	GA	B800	GB	STN001	B44	B46	D65	B14	B45	B32	B51	B31	D87	B48	D19	B26	B35	GC	GD
GA		94	100	100	100	100	100	95	92	92	92	92	86	95	92	94	94	100	99
B800	94		94	94	94	94	94	95	93	92	93	93	87	95	95	100	99	94	95
GB	100	94		100	100	100	100	95	92	92	92	92	86	95	92	94	94	100	99
STN001	100	94	100		100	100	100	95	92	92	92	92	86	95	92	94	94	100	99
B44	100	94	100	100		100	100	95	92	92	92	92	86	95	92	94	94	100	99
B46	100	94	100	100	100		100	95	92	92	92	92	86	95	92	94	94	100	99
D65	100	94	100	100	100	100		95	92	92	92	92	86	95	92	94	94	100	99
B14	95	95	95	95	95	95	95		94	93	94	95	88	100	94	95	95	95	96
B45	92	93	92	92	92	92	92	94		92	94	92	88	94	93	93	93	92	93
B32	92	92	92	92	92	92	92	93	92		91	93	86	93	91	92	92	92	93
B51	92	93	92	92	92	92	92	94	94	91		94	88	94	92	93	93	92	92
B31	92	93	92	92	92	92	92	95	92	93	94		87	95	92	93	93	92	93
D87	86	87	86	86	86	86	86	88	88	86	88	87		88	88	87	87	86	87
B48	95	95	95	95	95	95	95	100	94	93	94	95	88		94	95	95	95	96
D19	92	95	92	92	92	92	92	94	93	91	92	92	88	94		95	96	92	92
B26	94	100	94	94	94	94	94	95	93	92	93	93	87	95	95		99	94	95
B35	94	99	94	94	94	94	94	95	93	92	93	93	87	95	96	99		94	95
GC	100	94	100	100	100	100	100	95	92	92	92	92	86	95	92	94	94		99
GD	99	95	99	99	99	99	99	96	93	93	92	93	87	96	92	95	95	99	

Isolate Abbreviations: GA = group A BRIP66795-BRIP66799; B800 = BRIP66800; GB = group B isolates BRIP66801-BRIP66821; B44 = BRIP38744; B46 = BRIP38746; D65 = DAR75965; B14 = BRIP34814; B45 = BRIP34945; B32 = BRIP38432; B51 = BRIP38751; B31 = BRIP38831; D87 = DAR65987; B48 = BRIP38748; D19 = DAR77319; B26 = BRIP38826; B35 = BRIP38735; GC = group C PAMDB isolates A9, B181, JL1031, JL1065, 16, 19, 20, 21, 22, 23, 24, 315, 316, 407, 410, 836-2, 838-2, 838-4, 838-6, 838-9, 838-16, Max1, Max13, Max14, NCPPB1108, PST6, PST26L, PT2, PT13, PT14, PT18, PT21, PT26, PT28, PT29, PT30, PT32 and T1; GD = group D PAMDB isolates ICMP3435, ICMP3443, ICMP3449, ICMP3455, ICMP9305, 18 and OH314.

Appendix E: Pathovar characterisation of BRIP38735, BRIP38826 and BRIP66800

The pathovar identity of herbarium isolates BRIP 38735 and BRIP38826 from the 1970s and isolate BRIP66800 collected in this study was unclear, with results of PCR based genetic profiling suggesting these isolates are not *P. syringae* pv. *tomato*. Isolates BRIP38735 and BRIP38826 gave negative results for the $hrpZ_{Pst}$ and *cfl* PCR, whilst BRIP66800 gave a positive result for both of these tests. The *gltA* PCR grouped these three isolates within phylogroup 2a, rather than phylogroup 1a were *P. syringae* pv. *tomato* should be located (Figure D-1).

Sequence data from GenBank (NCBI) and the PAMDB for the 12 pathovars of phylogroup 2a as described by Berge *et al.* (2014) were collected for *gltA* (Table E-1). Pathovars included were *aceris, aptata, atrofaciens, avellanae, coryli, dysoxyli, japonica, lapsa, papulans, pisi, solidagae* and *syringae*. Analysis was completed in Geneious, including multiple sequence alignment with CLUSTALW (default settings IUB cost matrix, gap open cost=15, gap extend cost=6.66 and no 'free end gaps') and tree building with the Neighbour-Joining method and the HKY genetic distance model.

Table E-1: Reference *gltA* gene sequences used the analysis to investigate the pathovar identity of BRIP38735, BRIP38826 and BRIP66800. Accessions listed are those corresponding to GenBank unless otherwise indicated.

Accession	*P. syringae pathovar*
AEAO01000561	*aceris*
AY610698.1	*aptata*
KX571937.1	*aptata*
LJPO01000042	*atrofaciens*
NCPPB3487[PAMDB]	*avellanae*
LJQC01000847	*coryli*
LMG5062[PAMDB]	*dysoxyli*
AY610710.1	*japonica*
CP013183.1	*lapsa*
LIHW01000144	*papulans*
AY610735.1	*pisi*
AY610736.1	*pisi*
LJRH0100122	*solidagae*
CP000075.1*	*syringae*
ACXZ01004510	*syringae*
PSy642*	*syringae*
AEAJ00000000*	*syringae*

[PAMDB] Strain number as listed in PAMDB

* *P. syringae* pv. *syringae* isolates used by Berge *et al.* (2014). CP000075.1 = strain B728a and AEAJ00000000 = PsyCit7.

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Table E-2: Distance matrix of *gltA* gene sequences of reference isolates compared to BRIP38735, BRIP38826 and BRIP66800. Data represents the percentage nt sequence identity of sequences based on CLUSTW multiple sequence alignment. Sequences were trimmed to 338 columns prior to analysis

	AE0	P42	CP75	AE61	AY98	KX37	LJ42	NC87	LJ47	LM62	AY10	CP83	LI44	AY35	AY36	LJ22	AC10	B35	B26	B800
AE0		95	96	96	96	97	96	43	97	43	95	96	99	95	95	96	96	100	98	97
P42	95		96	96	97	97	96	44	97	44	96	96	96	96	96	96	97	96	95	94
CP75	96	96		99	97	97	96	42	98	43	96	96	96	96	96	99	97	96	96	95
AE61	96	96	99		97	97	97	43	98	44	96	97	97	97	97	100	97	97	96	95
AY98	96	97	97	97		100	100	44	98	44	99	100	97	98	98	97	100	96	96	94
KX37	97	97	97	97	100		100	44	99	44	100	100	97	98	98	97	100	97	96	95
LJ42	96	96	96	97	100	100		44	98	44	99	99	96	97	97	96	100	96	96	95
NC87	43	44	42	43	44	44	44		43	94	44	44	43	43	43	42	44	43	43	42
LJ47	97	97	98	98	98	99	98	43		44	98	98	98	98	98	98	98	97	97	95
LM62	43	44	43	44	44	44	44	94	44		44	44	44	44	44	43	44	43	43	42
AY10	95	96	96	96	99	100	99	44	98	44		99	96	97	97	96	99	96	95	94
CP83	96	96	96	97	100	100	99	44	98	44	99		96	97	97	96	100	96	95	94
LI44	99	96	96	97	97	97	96	43	98	44	96	96		96	96	96	97	100	99	98
AY35	95	96	96	97	98	98	97	43	98	44	97	97	96		100	97	98	96	95	94
AY36	95	96	96	97	98	98	97	43	98	44	97	97	96	100		97	98	96	95	94
LJ22	96	96	99	100	97	97	96	42	98	43	96	96	96	97	97		97	96	95	94
AC10	96	97	97	97	100	100	100	44	98	44	99	100	97	98	98	97		96	96	94
B35	100	96	96	97	96	97	96	43	97	43	96	96	100	96	96	96	96		99	97
B26	98	95	96	96	96	96	96	43	97	43	95	95	99	95	95	95	96	99		99
B800	97	94	95	95	94	95	95	42	95	42	94	94	98	94	94	94	94	97	99	

Isolate Abbreviations: AE0 = AEAJ00000000; P42 = Psy642; CP75 = CP000075.1; AE61 = AEAO01000561; AY98 = AY610698.1; KX37 = KX571937.1; LJ42 = LJPO01000042; NC87 = NCPPB 3487; LJ47 = LJQC01000847; LM62 = LMG 5062; AY10 = AY610710.1; CP83 = CP013183.1; LI44 = LIHW01000144; AY35 = AY610735.1; AY36 = AY610736.1; LJ22 = LJRH0100122; AC10= ACXZ01004510; B35 = BRIP38735; B26 = BRIP38826 and B800 = BRIP66800

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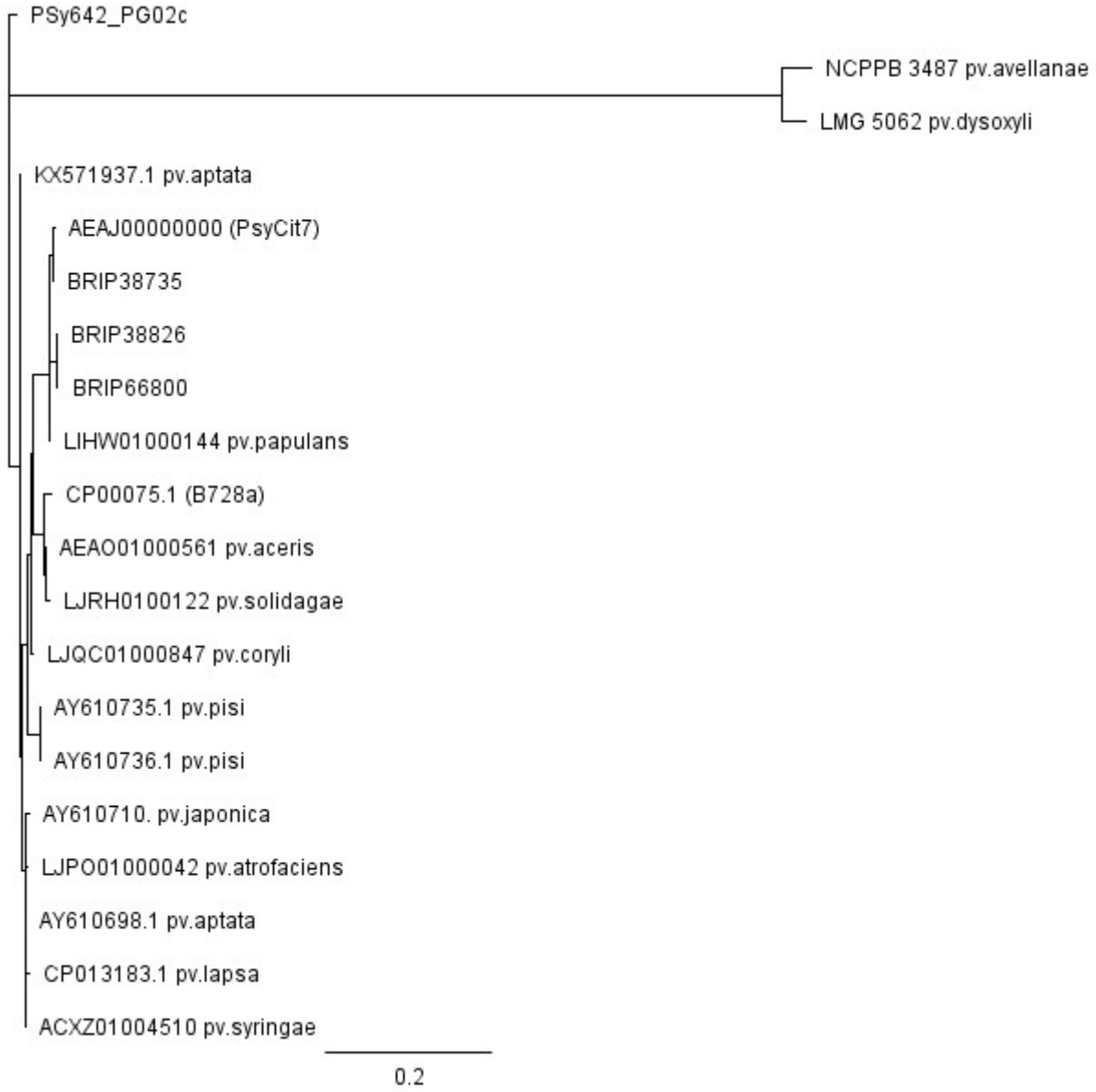

Figure E-1: Phylogenetic tree of *gltA* seqeunce fragments of BRIP38735, BRIP38826 and BRIP66800 compared to *Pseudmonas syringae* pathovars of phylogoup 2 from GenBank and PAMDB. Tree building completed with the Neighbour-Joining method and the HKY genetic distance model. Scale bar represents the genetic distance.

When comparing *gltA* nt sequence, most reference pathovars of phlyogroup 2 had 94-100% seqeunce identity with BRIP38735, BRIP38826 and BRIP66800 (Table E-2). NCPPB3487 pv.*avellanae* and LMG5062 pv.*dysoxyli.* only had 43-44 % identity. AEAJ00000000 and LIHW01000144 shared the highest seqeunce similarity (97-100%) with BRIP38735, BRIP38826 and BRIP66800. However, these two references were from two different pathovars, namely pv. *syringae* and pv. *papulans*. Pathovar *syringae* reference seqeunces were not consitently placed within the same clade (Figure E-1). This is analogous to the phylogentic tree of Berge *et al.* (2014) which places *P. syringae* pv. *syringae* reference isolates into different clades of phylogroup 2; for example PsyCit (AEAJ00000000), Psy642 and B728a

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(CP000075.1) were in clades 2a, 2c and 2d respectively (Figure D-1). Overall, differences in percent seqeunce identity and genetic distances for *P. syringae* pv. *syringae gltA* sequences were small (Table E-2 & Figure E-1). The differences in clade placement and percent seqeunce identity may be attributed to the large diversity of potential hosts that this pathovar can survive on (Scortichini *et al.* 2003).

It is clear that BRIP38735 and BRIP38826 are *P. syringae* and they likely belong to phylogroup 2a as described by Berge *et al.* (2014). However, due to high seqeunce similarity of these isolates with 10 of the 12 pathovars of phylogroup 2, this anlysis was unable to accurately determine the pathovar identity of these isolates. Given these three isolates were obtained from tomato and *P. syringae* pv. *syringae* has previously been reported on tomato (Garibaldi *et al.* 2007; Gilardi *et al.* 2010; Gullino *et al.* 2009) and to the best of my knowledge there are no reports of the other pathovars of phylogroup 2 on tomato. It is hypothesised that these isolates are *P. syringae* pv. *syringae*. Further characterisation of BRIP38735 and BRIP3882 is warranted for future studies, to investigate if they represent a unique *P. syingae* pathovar or are simply genetic variants of an existing phylogroup 2a pathovar.

The PCR analysis of BRIP66800 including the succesfully amplification of $hrpZ_{Pst}$, shown to be a dignostic feature of *P. syringae* pv. *tomato* (Zaccardelli *et al*. 2005), was more consistent with its designation as pv. *tomato*. However, the *gltA* seqeunce analysis suggests otherwise. Additionally, both BRIP66801 and BRIP66802, collected from the same property as BRIP66800, were determined to be *P. syringae* pv. *tomato* (section 5.9). Further characterisation of BRIP66800 is required to investigate if it represents a genetic variant of *P. syringae* pv. *tomato*.

Appendix F: Optimisation of PCR conditions for primers targeting copper processing genes

Evaluation of previously published primers for amplification of copper processing genes

Five primer sets were used in preliminary testing (Table F-1), 4 primer sets designed by Nakajima *et al.* (2002) and a slightly modified version of the degenerate primer set designed by Lejon *et al.* (2007).

Degenerate primers are used when some bases within a target sequence area vary between different species being tested, hence there needs to be a mixture of oligonucleotide sequences so that all possible nt combinations can be targeted in the PCR reaction (Iserte *et al.* 2013). For example this sequence: 5'-ATCGTT[**GC**]AAGTCATC-3', can contain either a G or C at the 7th nt so there needs to be a mixture of 5'-ATCGTT**C**AAGTCATC-3' and 5'-ATCGTT**G**AAGTCATC-3' oligonucleotides in the reaction.

The T_A of the PCR reactions were optimised by testing isolates BRIP66796 and BRIP66810. Sample BRIP66796 was chosen as it was representative of the MIC profile (Table 4-4) of most of the study isolates and *in vivo* testing suggests this isolate is tolerant to copper (Chapter 4). Sample BRIP66810 was chosen as it had a higher MIC compared to all other *P. syringae* pv. *tomato* isolates collected in this study, with a MIC of 1.5 mmol/L on CYEG.

Table F-1: Primer sequence information for the amplification of *P. syringae* pv. *tomato* genes which confer copper tolerance (*cop)*. Including each primers read direction (F=forward, R=reverse), sequence and the expected fragment size.

Primer & Read Direction	Sequence (5'-3')	Expected fragment size (bp)
copA F	ATGGAATCAAGAACTTCTCGACGT	1802
copA R	CTCCTCTACCCGAACTTCGCGGAAC	
copB F	ACTGTTTTGAATAGACTCCAC	979
copB R	AACCACATGCGCACGCCCAGGACTAA	
copC F	CGCATGTTGTTGAACCGCACAAGT	380
copC R	CTTGACCTTAAACGTCACGCT	
copD F	AACATGGAAGATCCGCTCAGCATC	922
copD R	TCCATCTCAGGGGACAGTGT	
Coprun F2.0	GGSASI*TACTGGTRBCAC	1200-1800
Coprun R1.0	TGI*GHCATCATSGTRTCRTT	

Universal degeneracy codes: S = G, C; R = A,G; H = A,C,T and B = G,C,T
I* = 5' Deoxyinosine inserted to replace degeneracy in that position

Each 50 µL reaction mixture contained 0.2 µmol/L of each primer, 22 µL sterile deionised water and 25 µL MangoMix^{TM} (Bioline) and 1 µL of bacterial suspension at approximately 7.2 x 10^7 CFU/mL. DNA amplification was carried out in a Kyratec SuperCycler Thermal Cycler (model SC300), with the following T_A tested; 50, 56 and 60 °C. Reaction conditions for *cop* B, C and D primers were: denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s, T_A for 20 s and extension at 72 °C for 45 s, with a final extension at 72 °C for 5 min. The larger fragment sizes expected

to be produced by *cop* A and Coprun required the extension time within the 30 cycles to be increased. The program was: denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 20 s, T_A for 20 s and extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min.

Agarose gel electrophoresis and EtBr staining were used to visualise the amplification products.

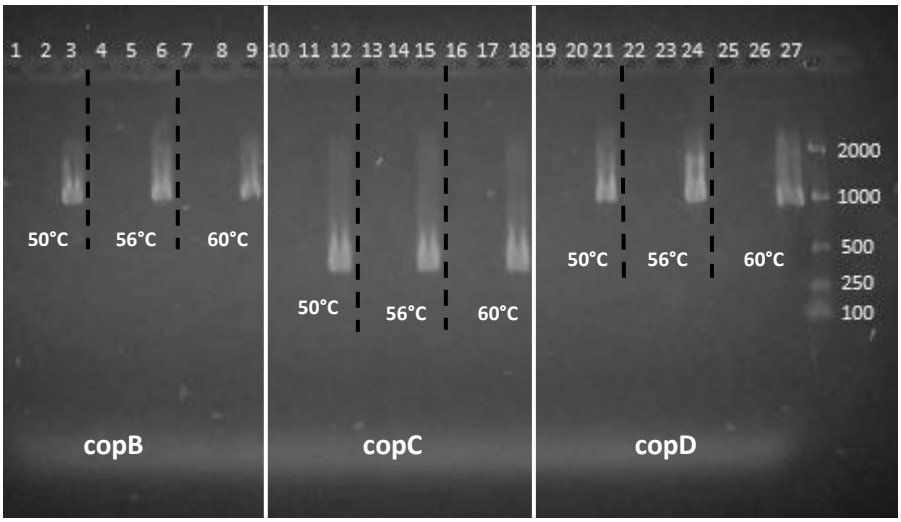

Figure F-1: Gel of *cop B, -C* and *-D* gene fragments. Lane 1- control no template, Lane 2- BRIP66796 and Lane 3- BRIP66810; this sample order is repeated across the gel for each PCR reaction and T_A. 2.0 % w/v agarose gel in 0.5X TBE, run at 80 V for approximately 80 min, with Bioline EasyLadder.

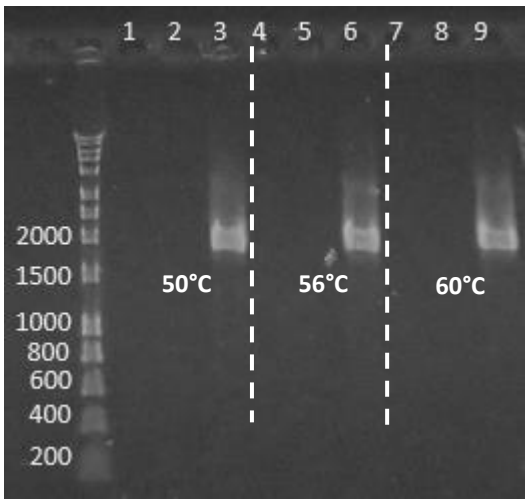

Figure F-2: Gel of *cop A* gene fragments. Lane 1, 4 and 7- control no template, Lane 2, 5 and 8 - BRIP66796 and Lane 3, 6 and 9 - BRIP66810. T_A are shown on the photo. 1.0 % w/v agarose gel in 0.5X TBE, run at 80 V for approximately 80 min, with Bioline Hyperladder.

Sample BRIP66810 produced a fragment of the expected size for all four *cop* primer sets whilst sample BRIP66796 did not (Figure F-1 and F-2). All T_A tested gave strong bands on the gel, therefore a temperature of 60°C was selected for all further testing. An additional 21 study samples were tested with the *copA, -B, -C* and *-D* primer sets, with BRIP66810 and BRIP66796 serving as positive and negative controls respectively. Samples tested were; BRIP66795, BRIP66798, BRIP66802, BRIP66803,

BRIP66807, BRIP66808, BRIP34945, BRIP38735, BRIP38746, BRIP38748, BRIP38751, BRIP38744, DAR 77319, DAR 75965, BRIP66812, 78.1, 79.1, 82.1, 87.1, 89.3 and BRIP66813. None of the isolates produced visible DNA fragments after PCR, except for BRIP66810. Suggesting that they either do not carry the *cop* genes or there is a critical sequence mismatch at the primer binding sites that precludes PCR amplification.

The Coprun primer set was originally designed to amplify the *copA* gene fragment from a wide range of bacteria (Lejon *et al.* 2007). The Coprun PCR failed to produce any detectable DNA fragments for both BRIP66796 and BRIP66810, thus further optimisation of this PCR was completed. This involved increasing the volume of primers added to the mixture, reducing the T_A and increasing the duration of the initial denaturation.

The T_A was optimised with isolate BRIP66810 and two copper-sensitive *P. syringae* pv. *porri* isolates (Psp46 and Psp). The following T_A were tested: 40, 45 and 50 °C. Each 50 µL reaction mixture contained 0.6 µmol/L of each primer, 10 µL 5x Mango*Taq*™ Coloured Reaction Buffer (Bioline), 2.5 mmol/L $MgCl_2$, 0.02 mmol/L dNTPs, 0.3 µL Mango*Taq*™ DNA Polymerase (Bioline) and 1 µL of bacterial suspension at approximately 7.2 x 10^7 CFU/mL. Reaction conditions were denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 20 s, T_A for 20 s and extension at 72°C for 2 min, with a final extension at 72 °C for 5 min. Agarose gel electrophoresis and EtBr staining were used to visualise the amplification products.

Results of the Coprun T_A test (Figure F-3) suggested that a temperature of 40 °C should be used for further testing, as the Psp copper-sensitive isolates should have no amplified products. The double bands in Psp46 at a T_A of 50°C suggested non-specific binding was occurring.

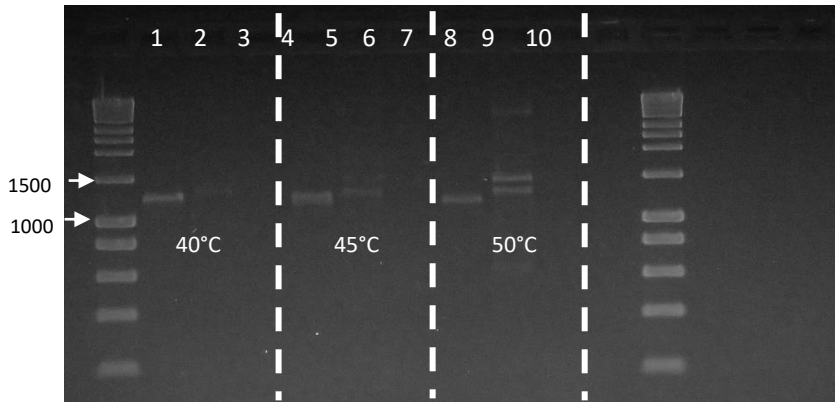

Figure F-3: Gel of T_A optimisation for the Coprun PCR. Lanes 1, 4 & 7 = BRIP66810; lanes 2, 5 & 8 = Psp46; lanes 3, 6 & 9 = Psp; and lane 10 = control no template. T_A are shown on photo. 1.0 % w/v agarose gel in 0.5X TBE, run at 80 V for approximately 80 min with Bioline Hyperladder.

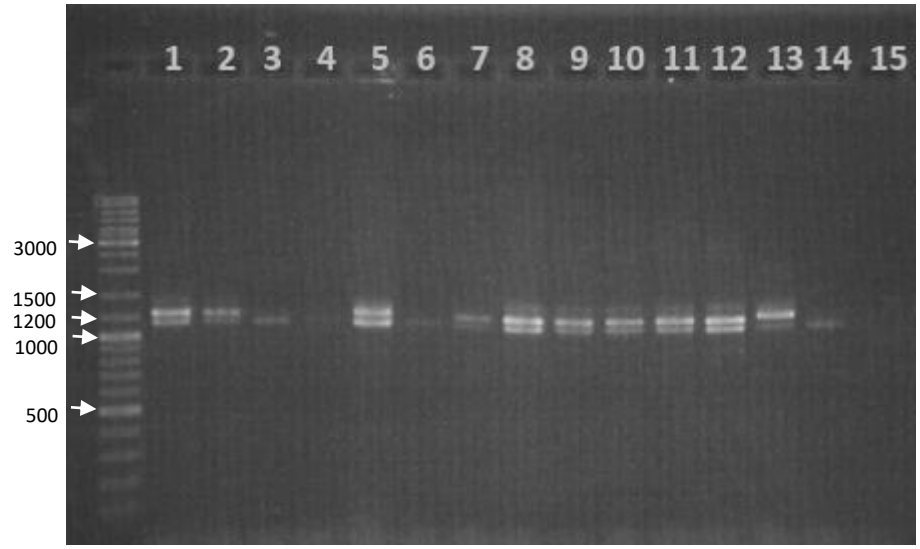

Figure F-4: Gel of Coprun gene fragments. Sample IDs for Lanes 1-14: BRIP66798, BRIP66802, BRIP66810, BRIP38735, BRIP38744, DAR 77319, DAR 75965, BRIP66812, 78.1, 79.1, 82.1, 87.1, A1513R and DC3000. Lane 15- control no template. 1.0 % w/v agarose gel in 0.5X TBE, run at 80 V for approximately 80 min, Thermo Scientific GeneRuler DNA Ladder Mix.

More study isolates were then tested with the Coprun PCR using the same conditions, with a T_A of 40°C. Agarose gel electrophoresis and EtBr staining were used to visualise the amplification products.

DNA fragments were successfully amplified with the Coprun primer set, however, more than one fragment was produced in most samples and the copper sensitive DC3000 had one amplified band on the gel (Figure F-4). These results could be attributed to non-specific binding of DNA in the bacterial isolate due to the relatively low T_A (Vandenbroucke *et al.* 2011) or the degeneracy of the Coprun primer set was allowing for amplification of copA homologs that have been reported in DC3000 (Buell *et al.* 2003). The Coprun primer set was not used in any further PCR testing.

Design of new primers for the amplification of the copper processing gene copA

Given the limited success of the *cop* PCRs using primers published by other researchers, three primer sets were designed in this study. Details of the primer sets PCopAF1/F2, CopAF5'/R5' and CopAF3'/R3' are described in Chapter 6 (Table 6-2). The T_A of the PCR reactions for these new *cop* primer sets were optimised by testing isolates BRIP66796 and BRIP66810. Both freshly prepared and frozen isolates were used as a template for the reaction, to test the potential of using frozen stocks. Each reaction mixture (50 µL) contained: 0.2 µmol/Lol/L of each primer, 0.2 mmol/L dNTPs, 10 µL of 5 x Mango*Taq*™ Coloured Reaction Buffer (Bioline), 2.5 mmol/L $MgCl_2$, 0.3 µL of MangoTaq™ DNA Polymerase (Bioline) and 1 µL of approximately 7.2 x 10^7 CFU/mL bacterial suspension.

The T_A tested were; 45, 50, 55 and 60 °C. DNA amplification was carried out in a Kyratec SuperCycler Thermal Cycler (model SC300) using the following programme: denaturation at 95°C for 1 min followed by 30 cycles of denaturation at 95 °C for 20 seconds (s), T_A for 20 s and extension at 72 °C for

45 s, then a final extension at 72 °C for 5 min. Agarose gel electrophoresis and EtBr staining were used to visualise the amplification products.

At the lower T_A of 45 and 50 °C the PCCopF1/R1 primer set amplified 2 fragments for the fresh preparation of isolate BRIP66810, suggesting there was non-specific primer binding and amplification occurring (Vandenbroucke *et al.* 2011)(Figure F-5). At T_A of 55 °C only one band was visible on the gel with all primer sets and isolates. At 60 °C the band for isolate BRIP66796 was weaker, therefore T_A of 55 °C was chosen. The bands produced for frozen isolates were generally as strong as the freshly prepared isolates, therefore there was potential for bacterial suspensions of 7.2×10^7 CFU/mL to be frozen for later use.

No amplification was observed for isolate BRIP66796 with PCopF1/R1 and CopAF5'/R5', however, there was amplification with CopAF3'/R3'. CopAF5'/R5' and CopAF3'/R3' were designed to target different halves of *copA* (Figure F-5). These results suggested that either one or both primer binding sites of CopAF5'/R5' and PCopF1/R1 are not present in BRIP66796. Given the copAF/R primer set of Nakajima *et al.* (2002) was also unsuccessful for amplification of DNA in BRIP66796 (Figure F-1). The primers designed for this study where specific to *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae* reference sequences from the GenBank database (Chapter 6; Table 6-1). The number of sequences available to design the primers was minimal, therefore, it was not surprising that there were differences in amplification success between isolates. One or more base pair differences in the target binding site means that primers cannot successfully attach. Base pair changes are not uncommon and can be caused by random mutations and a range of other factors than can disrupt primer binding, for example the formation of secondary structures on template DNA (Vandenbroucke *et al.* 2011). A larger selection of *P. syringae* pv. *tomato* isolates from this study were tested with these three primer sets to determine whether the amplification differences in BRIP66796 and BRIP66810 were unique (Chapter 6).

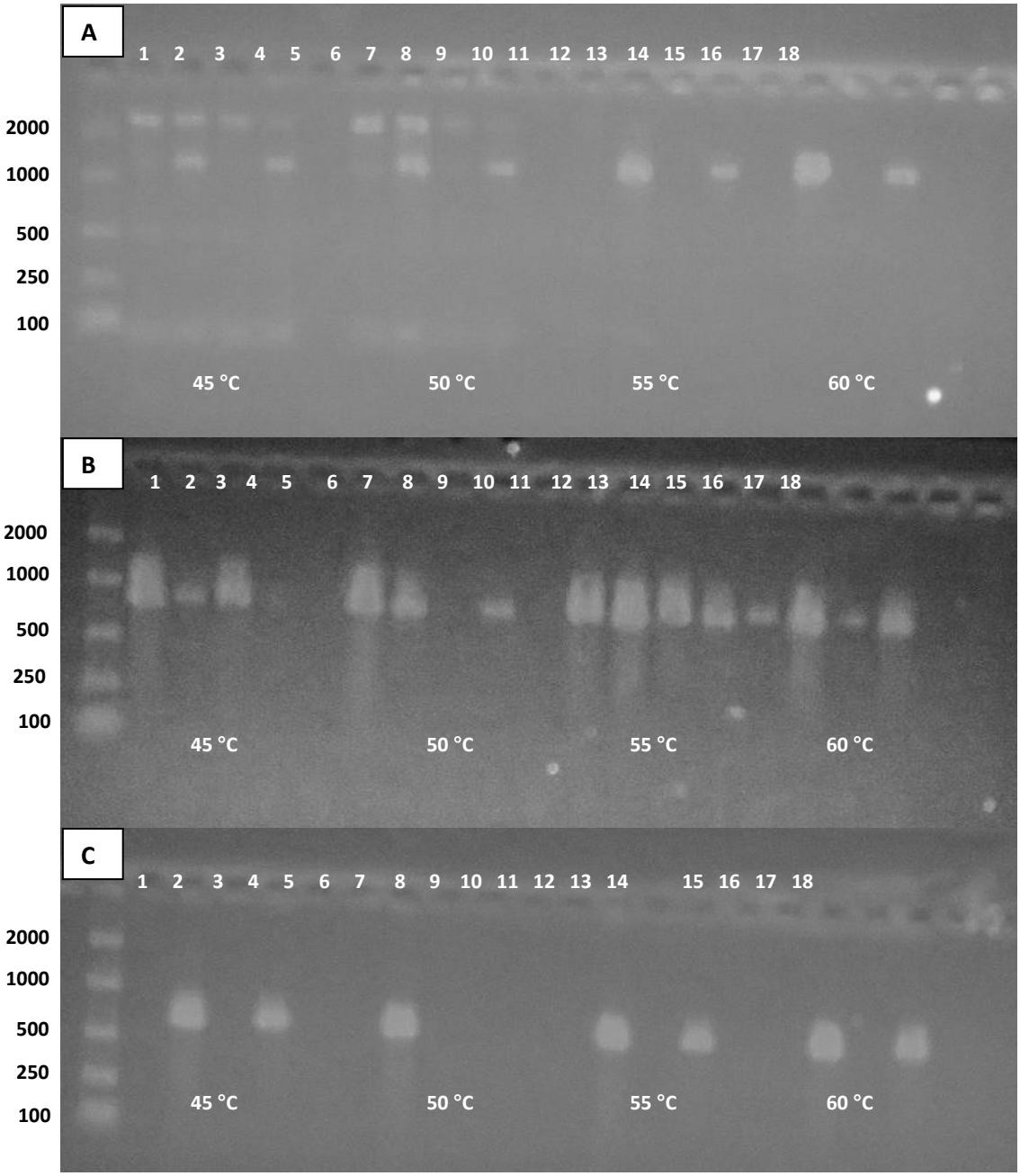

Figure F-5: Optimisation of T_A for *copA* primer sets. (A) PCCopF1/R1, (B) KGCopF1/R1 and (C) KGCOPF2/R2. Temperatures tested: 45 °C (lanes 1-5), 50 °C (6-10), 55 °C (11-14) and 60 °C (15-18). Lanes 1, 6, 11 and 15 - isolate BRIP66796 fresh; lanes 2, 7, 12 and 16 - isolate BRIP66796 fresh; lanes 3, 8, 13 and 17 – isolate BRIP66796 frozen; lanes 4, 9, 14 and 18 – isolate BRIP66810 frozen; lanes 5 and 10 negative control (water). 2 %w/v agarose gel run at 80 V for 80 min with the Bioline EasyLadder.