Hort Innovation

## Final Report

# Management and Detection of Bacterial Leaf Spot in Capsicum and Chili Crops

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**Project code:**

VG14010

**Project:**

Management and Detection of Bacterial Leaf Spot in Capsicum and Chili Crops – VG14010

**Disclaimer:**



**Funding statement:**

This project has been funded by Hort Innovation, using the vegetable research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

**Publishing details:**

ISBN 978 0 7341 4420 1

Published and distributed by: Hort Innovation

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www.horticulture.com.au

## Content

## Summary

This project aimed to increase the capacity of the vegetable industry to develop integrated disease management programs for bacterial leaf spot (BLS) of capsicum and chili field crops. As tomato was considered an alternative host for the BLS pathogens, investigation of the causal agents of the disease in tomato was also completed.

Previously it was assumed *X. campestris* pv. *vesicatoria* (now known as *X. vesicatoria*), was causing BLS in all solanceous crops in Australia. The results of this project clearly indicate this is not the case, instead there are four different *Xanthomonas* species associated with the disease. Furthermore, the species are largely host specific, where *X. euvesicatoria* essentially infects capsicum, chili and tomato, and *X. vesicatoria* and *X. perforans* only tomato. The fourth species, *X. arboricola*, was found in association with BLS symptoms and able to weakly infect tomato only. Given this weak pathogenicity, its ability to cause BLS disease is questionable and as such the bacterium is unlikely to need control. Furthermore, race-typing of *X. euvesicatoria* isolates identified races 1 and 7 in Australia. There are currently several commercial capsicum lines in Australia with resistance to race 1. There are very few, readily available with resistance to race 7. Importantly, the diversity of *X. euvesicatoria* races and other Xanthomonads detected in Australia is very low, however, as there is no regulation for these bacterial pathogens it is likely new races and species could be introduced with seed.

Copper tolerance testing of Australian isolates revealed all were tolerant to highly tolerant. Although the minimum amount of copper used in the tests is well below the amount of copper routinely applied in the field, disease control is ineffective. This highlights that copper tolerance in bacterial populations is not the complete answer as to why copper is ineffective for disease control. Copper may still have some role in management of BLS, however, alternative methods are needed to address the ineffectiveness of using copper alone. To this end, essential oils showed promise as preliminary testing indicated they have a strong antibacterial effect against *X. vesicatoria* and *X. euvesicatoria*, both as a volatile gas and through direct contact.

The literature review on survival of BLS pathogens between cropping cycles highlighted the importance of using disease free planting material. Survival of the bacterial pathogens in the environment is quite low and disease outbreaks are more likely initiated from primary introduction of the pathogen each season rather than transfer of the bacterium from sources within the environment.

Ongoing international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races or species into Australia. This means existing resistance genes managing disease in Australia may become unreliable in the future. Additionally, overuse of a single management strategy such as a resistance gene or a single chemical can provide strong evolutionary pressure on the bacterial pathogen, leading to mutation and the local emergence of resistance-breaking races or chemically tolerant populations.

The major recommendations for the management of BLS are:

- Consult with your seed supplier about assurances on the health status of the seed or alternatively,
- Heat treatment of seed to prevent primary introduction of pathogens into crops and to mitigate the risk of introduction of new bacterial races which may circumvent existing plant host resistance genes. This treatment will also mitigate risk of other potential exotic and endemic pathogen threats
- Use of resistant capsicum lines where possible
- Combining copper with manganese-zinc ethylene bisdithiocarbamate (EBDC) (e.g mancozeb) early in the crop cycle to improve availability of bactericidal forms of copper and thus early infections, later applications are not practical due to with-holding periods of the EBDC

## Keywords

Bacterial leaf spot (BLS), Xanthomonas, disease management, copper tolerance, essential oil

## Introduction

Bacterial diseases of Australian vegetable crops are emerging as an increasing constraint to production. This is partly due to the increasing importance of production in summer rainfall regions and climate variability, and sometimes due to inexperience in identifying the diseases. Limited availability of suitable management strategies for the diseases often result in substantial losses for the grower. The increasing variability of the Australian climate makes prediction of where and when bacterial disease outbreaks are likely to occur more difficult. It is therefore important to have good management practices on stand-by which can be implemented quickly and efficiently. Alternatively, pre-emptive strategies such as heat treatment of seed should be considered.

Previously, the causal agent of Bacterial leaf spot (BLS) in Australia was reported as *Xanthomonas campestris* pv. *vesicatoria* (Martin *et al*. 2004). We now know, however, there are three distinct species which cause BLS in capsicum crops worldwide (Hamza *et al*. 2010). Knowing what causes BLS in Australia is pivotal in developing efficient disease management strategies, particularly as developing robust resistant commercial hybrids is entirely dependent on accurate pathogen identification.

The control of bacterial disease outbreaks is complicated by the presence of copper-tolerant isolates within bacterial populations. Thus, there is a need to reduce the reliance on copper for disease control and for the development of alternative Integrated Pest Management (IPM) compatible control options. To do this, details are required on the epidemiology of BLS including sources of inoculum, variability in bacterial strains, and the survival mechanisms of the bacteria between crops. This information will assist in developing targeted control methods. Results obtained from a previous Hort Innovation project VX99021 “Detection and management of copper tolerance in bacterial diseases of vegetables” (Martin, 2003), showed that copper-tolerant strains of the bacterium *X. campestris* pv. *vesicatoria*, were contributing to poor control of bacterial spot of capsicum, particularly in north Queensland.

This project is aligned to the Plant Biosecurity Cooperative Research Centre (PB CRC) Project PBCRC2002 (New approaches for diagnosing bacterial pathovars). The work in this project supported and extended existing research within the PB CRC program by providing complementary research in areas not explored by the PB CRC project. The PB CRC program provided a PhD student stipend and minimal operating funds to support the PhD student associated with this Hort Innovation project.

The major aim of the project was to increase the capacity of the vegetable industry to implement integrated disease management programs for BLS of capsicum and chili field crops. This was achieved firstly, by comparing past and present isolates of bacteria to identify the causal agent of the disease in Australia and included comparison of biochemical and pathogenicity traits, in addition to whole genome sequences, generated through next generation sequencing techniques. Secondly, previous and current research on the epidemiology of BLS including disease introduction pathways, bacterial survival and spread mechanisms was reviewed to identify areas where disease management can be further improved. This included detecting copper-tolerance in bacterial isolates collected during the study from multiple production areas. Furthermore, a review on potential control methods, alternative to the use of copper, identified essential oils as worthy of further investigation. To this end, selected oils were evaluated in laboratory experiments and then pot trials. The project uses methodologies developed in the previous Hort Innovation project VN13005 “Detection and management of bacterial diseases in Australian allium crops” (Gambley, 2017).

# Methodology

## Investigating species causing bacterial leaf spot of capsicum and chili in Australia:

Details on surveys, isolate collection and characterization are published in Roach *et al.* (2018). In essence, capsicum, chili and tomato crops were surveyed for BLS symptoms during the 2015 growing season in Queensland and New South Wales. Symptomatic tissue was collected and bacteria isolated. A further seven unidentified isolates of *Xanthomonas* spp. from solanaceous hosts were sourced from the DAF herbarium (BRIP) culture collection, giving a total of 64 Australian isolates associated with bacterial leaf spot for diversity studies. The isolates were analysed using multi-locus sequence analysis of atpD, dnaK, efp and gyrB genes, species-specific PCR assays, whole genome sequences, pathogenicity and biochemical tests.

## Review of survival reservoirs of BLS within and between cropping cycles:

In developing a management strategy to control diseases such as BLS, it is important to consider sources for introduction of the bacteria into a field, district or country, how long the bacteria are able to survive without its preferred host plant and where it resides in the environment between cropping periods. A literature review was conducted to identify potential sources of the BLS pathogens within and between cropping cycles. This included published research from the early 1980s to date. This information was captured in a report and a fact sheet describing potential management options for the disease (Appendices 1 and 2).

## Investigating control methods for effectiveness in reducing the impact of BLS:

A literature review (Appendix 1) was conducted to evaluate previously published research on the control of BLS. This included the use of copper as a bactericide, alternatives to copper such as essential oils, defense response initiators, biocontrol agents, and other chemicals to control the disease. Also reviewed was the effectiveness of host plant genetic resistances and cultural practices. From the review, research activities were focused on evaluating the tolerance of Australian BLS pathogens to copper, trialing essential oils for their control and race-typing *X. euvesicatoria* isolates to identify key host resistance genes.

### Copper tolerance testing

To determine the copper tolerance levels of Australian BLS-isolates, the bacterial isolates were exposed to copper sulphate ($CuSO_4$) at varying amounts using *in vitro* culture with amended media. $CuSO_4$ was added to CYEG media containing 20 mM MES buffer at concentrations of 0, 0.1, 0.2, 0.5, 0.8, 1, 1.5, 2 and 5 mM, using the method published by Pernezny *et al*. (2008). The media was adjusted to pH 7 before pouring and used fresh. Duplicate aliquots of 10 µL of $10^8$ CFU/ml bacterial suspensions were spotted onto plates in a grid and growth assessed visually after 2 days incubation at 25 °C. This experiment was repeated twice.

Minimum inhibitory concentration (MIC) of $CuSO_4$ at which no growth of the bacterial isolate was recorded. Ratings are S = susceptible (no growth at any copper concentration), T = tolerant (growth at 0.1-0.5 mM $CuSO_4$) MT = moderately tolerant (growth >0.5 – 1.0 mM $CuSO_4$), HT = highly tolerant (growth >1.0 – 1.5 mM $CuSO_4$), R = resistant (growth >1.5 – 2.0 mM $CuSO_4$).

### Essential oil testing

Essential oils from a range of different plant species were sourced for *in vitro* trials to determine their potential to control bacterial leaf spot. These are listed in Table 1, along with their common name and likely effective chemical component where known.

**Table 1. A list of essential oils selected for *in vitro* testing to determine potential for controlling bacterial leaf spot of solanaceous crops. The plant species and common name is listed for each oil along with the likely effective chemical component where known.**

| Plant species | Common name | Chemical component |
|---|---|---|
| *Thymus vulgaris* | thyme | carvacrol, thymol |
| *Origanum vulgare* | oregano | carvacrol, thymol |
| *Eugenia caryophyllata* (*Syzygium aromaticum*) | clove | eugenol |
| *Foeniculum vulgare* dulce | fennel sweet | |
| *Coriandrum sativum* | coriander | linalool |
| *Lavandula angustifolia* | lavender | linalool |

The oils were assessed *in vitro* using filter paper soaked in the test oil and then applied to the inside of the microtitre culture plate lid to evaluate inhibition of growth from volatiles of the compound. They were also assessed using direct exposure to the oil in broth culture followed by viability assessment in microtitre plate culture. The oils were tested against two causal agents of BLS, *X. euvesicatoria* (BRIP 62425) and *X. vesicatoria* (BRIP 62428). The method for *in vitro* oil evaluation are as published in Appendix 7 of Hort Innovation Final Report for Project VN13005 'Detection and management of bacterial diseases in Australian allium crops' (Gambley, 2017).

From these experiments, selected oils were tested in a pot trial with and without wounding during inoculum application. Twelve replicate capsicum plants were sprayed separately with each treatment solution to run-off and then inoculated 24 h later using a mist application of a suspension of *ca*. 5 x $10^7$ CFU/ml of the *X. euvesicatoria* isolate. One set of six plants were wounded using a sterile syringe needle. The treatment solutions were:

1. Untreated control (UTC)
2. Surfactant control (0.5% DMSO, 0.1% Tween-20)
3. Industry standard (1.5 g/L kocide blue extra copper , 2 g/L mancozeb)
4. Clove oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20
5. Fennel oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20
6. Lavender oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20
7. Oregano oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20
8. Coriander oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20
9. mineral oil 0.2% (v/v), 0.5% DMSO, 0.1% Tween-20

A second pot trial tested clove, fennel, oregano and thyme oil at a concentration of 2.0% and included the above controls.

## Evaluating germplasm for BLS control:

Host resistance or tolerance to bacterial pathogens is long known to be very effective for disease control. To this end, bacterial isolates associated with BLS in Australia were evaluated for pathogenicity. To determine general pathogenicity, all isolates were screened with the susceptible capsicum and tomato lines, Jupiter and Grosse Lisse,

respectively. Differential lines of capsicum ECW (Table 2) were then used to race-type isolates shown to be pathogenic on capsicum (Kousik and Ritchie 1998, 1999; Stall, *et al*. 2009). The method used by Syngenta in their commercial breeding activities was used for this race-typing. The resistance genes used in race-typing are also used in commercial varieties in varying combinations. Essentially, overnight cultures of bacteria were diluted to concentrations of $10^8$ CFU/ml in sterile water and the suspension used to infiltrate two leaves of one plant of each line. Plants were maintained in a glasshouse and the experiment repeated twice. Results were recorded 1-2 days post-inoculation as either resistant or susceptible as described by the method used by Syngenta as mentioned above.

**Table 2 Differential line set of capsicum with genes and race resistance. A positive is noted (+) for a susceptible reaction to that bacterial race and a negative (-) where there was a hypersensitive response to the bacteria. The corresponding bacterial avirulence gene which is recognised by the plant host resistance genes is provided in the final row.**

| Races | Capsicum line/ resistance gene | | | | |
|---|---|---|---|---|---|
| | Jupiter | ECW-10R | ECW-20R | ECW-30R | PI 235047 |
| | none | Bs1 | Bs2 | Bs3 | Bs4 |
| 0 | - | + | + | + | + |
| 1 | - | - | + | + | + |
| 2 | - | + | + | - | - |
| 3 | - | - | + | - | + |
| 4 | - | - | - | + | + |
| 5 | - | + | - | - | - |
| 6 | - | - | - | - | + |
| 7 | - | - | + | + | - |
| 8 | - | - | + | - | - |
| 9 | - | - | - | + | - |
| 10 | - | - | - | - | - |
| Avr gene | na | AvrBs1 | AvrBs2 | AvrBs3 | AvrBs4 (AvrBs3-2) |

## Outputs

The outputs delivered through this project are detailed below.

- A report on potential management options for control of BLS (Appendix 1)
- Two fact sheets prepared, one describing BLS of solanaceous crops and a second detailing available management options for BLS (Appendices 2 and 5)
- Project commencement article for submission to Ausveg magazine (Appendix 3)
- A list of reference bacterial isolates which cause BLS of capsicum and/or chili which are thoroughly characterised and deposited in an Australian national herbarium (Appendix 4, Roach *et al.* 2018)
- Project completion article for submission to Ausveg magazine (provided mid-May 2018, Appendix 6)
- Delivery of project outcomes at two industry field days or meetings (held in the Granite Belt once in 2016 and in 2017).

## Outcomes

This project has delivered four major outcomes. These were:

1. Knowledge of species causing bacterial leaf spot (BLS) of capsicum and chili in Australia
2. Improved understanding of survival reservoirs of BLS within and between cropping cycles
3. Understanding of availability of control methods for effectiveness in reducing the impact of BLS
4. Recommendations on the availability of resistant or tolerant germplasm for BLS control

Prior to this start of this project it was assumed the same pathogen, *X. campestris* pv. *vesicatoria* (now known as *X. vesicatoria*), was causing BLS in all solanceous crops in Australia. The results of this project clearly indicate this is not the case, instead there are four different *Xanthomonas* species associated with the disease. Furthermore, the species are largely host specific, where *X. euvesicatoria* essentially infects capsicum, chili and tomato, and *X. vesicatoria* and *X. perforans* only tomato. Although *X. euvesicatoria* is able to infect tomato in laboratory testing, in the field it was almost always found affecting capsicum or chili, natural infections of tomato were rare. The fourth species, *X. arboricola*, was found in association with BLS symptoms and able to weakly infect tomato only. Given this weak pathogenicity, its ability to cause BLS disease is questionable and as such the bacterium is unlikely to need disease management strategies. Additionally, there was no evidence of the presence of a fifth species associated with BLS overseas, *X. gardneri*, in Australia. Of these species, *X. perforans* represents a new geographical record as it was not previously reported to occur in Australia. It also demonstrates an open entry pathway for xanthomonads affecting Solanaceae to be introduced into Australia. This is concerning as there are multiple races of *X. euvesicatoria* as yet not present here. These races are likely able to overcome the genetic resistance of capsicum varieties currently grown in Australia.

The literature review on survival of BLS pathogens between cropping cycles highlighted the importance of using disease free planting material. Survival of the bacterial pathogens in the environment is quite low and disease outbreaks are more likely initiated from primary introduction of the pathogen each season rather than transfer of the bacterium from sources within the environment. Once established in crops, transfer within and between crops is highly likely, especially during periods of wet weather, particularly windy wet weather. Hail also contributes significantly to disease establishment and spread due to the increased wound sites for bacterial infection.

Copper tolerance testing of Australian isolates revealed all isolates were tolerant and almost all were at least moderately tolerant. Although the minimum inhibitory concentration of copper used in the tests is well below the amount of copper routinely applied in the field, disease control is ineffective. This highlights that copper tolerance in bacterial populations is not the complete answer as to why copper is ineffective for disease control. Review of the mode of copper revealed multiple factors which influence the availability of copper ions, the bactericidal form of copper. It is likely most of the copper applied in field sprays is bound in a form which is not bactericidal. Copper may still have some role in management of BLS, however, alternative methods are needed to address the ineffectiveness of using copper alone. To this end, essential oils show promise as preliminary testing indicated they have a strong antibacterial effect, both as a volatile gas and through direct contact.

Host resistance for BLS is an important tool for disease management. The majority of resistance gene characterization and breeding was, and continues to be done, for *X. euvesicatoria*. There are at least 10 different races of this bacterial species affecting capsicum, and for which resistance genes are identified. The results of this project show that in Australia we have races 1 and 7. Currently available commercial capsicum lines in Australia carry combinations of resistance genes, typically Xcv 0-3, 1-3, 0-5 and 1-5. Although this provides several capsicum lines to choose from with resistance to race 1, there are very few, readily available with resistance to race 7. Although few are currently available in Australia, capsicum lines with resistances that cover both race 1 and 7 are available in the United States.

Importantly, the diversity of *X. euvesicatoria* races and other Xanthomonads detected in Australian Solanaceae crops remains very low, however, as there is no testing of seed for these bacterial pathogens it is possible new races and species could be introduced. This risk of introduction of these exotic seed-borne pathogens can be mitigated through heat treatment of seed. The heat treatment of seed also has a dual purpose in preventing primary introduction of BLS and a range of other diseases into commercial crops.

## Evaluation and discussion

### Investigating species causing bacterial leaf spot of capsicum and chili in Australia:

From the 64 Australian isolates of *Xanthomonas* spp. associated with bacterial leaf spot (BLS) in tomato, capsicum and chili crops in eastern Australia analyzed in this project, at least five species of *Xanthomonas* were identified and their pathogenicity subsequently assessed. Phylogenetic and biochemical analyses identified *Xanthomonas arboricola*, *X. euvesicatoria*, *X. perforans* and *X. vesicatoria* as the most frequently recovered pathogenic species. Non-pathogenic and weakly pathogenic species were also identified.

Of these, only *X. vesicatoria* and *X. euvesicatoria* were previously reported to cause BLS in Australia. This study represents the first report of *X. perforans* causing bacterial leaf spot on tomato in Australia. *Xanthomonas gardneri* is a known pathogen causing BLS in other countries but was not detected amongst the Australian isolates. This represents a biosecurity threat for Australia. The global spread of *X. gardneri* has been rapid and its absence here further emphasizes the importance of seed testing and updated diagnostic protocols and to have treatment procedures in place for decontamination of seed on entry. Similarly, this is needed to mitigate exotic races of the individual bacterial species.

These results were more extensively described in the Roach et al. (2018) publication.

### Review of survival reservoirs of BLS within and between cropping cycles:

In developing a management strategy to control diseases such as BLS, it is important to consider sources for introduction of the bacteria into a field, district or country, how long the bacteria are able to survive without its preferred host plant and where it resides in the environment between cropping periods.

The xanthomonads causing BLS can survive as endophytes within seedlings as asymptomatic infections, causing no signs of disease. They can also survive on seed for at least 10 months and in dried leaf material for at least 14 months. Survival in soil was variable in experiments and ranged from only 16 days to 18 months. The transfer of the bacteria from the infested soil to host plants was reported as questionable and no direct evidence for this was obtained. Infested soil is thus unlikely to be an important source for new disease outbreaks, however, survival in the rhizosphere of a number of different plant species is possible. This includes the non-hosts sorghum, cucumber, bean, pea and wheat and has implications for potential crop-rotation strategies. Crop rotation and/or fallow periods may contribute to better disease management by reducing the numbers of bacteria surviving between cropping seasons. There is limited information, however, on the length of rotation and the useful alternative crops for the rotations.

Xanthomonads are reported to be seed-borne in a number of hosts, including capsicum and a linkage between infested seed and field disease was established (Higgins 1922; Shekhawat, P.S. and Chakravarti, B.P. 1979). To mitigate this risk, seed can be heat treated to eliminate the bacteria; however, germination rates may be compromised. Recently a number of studies have looked at treating seed with essential oil to combat BLS pathogens (Kotan *et al*. 2013, 2014; Lo Cantore *et al*. 2009), however, their efficacy is questionable.

Ongoing high-volume international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races or species into Australia. This represents the highest risk entry pathway for exotic BLS species and/or races to enter the country. No formal mitigation for this risk exists. Seed disinfestation strategies would help mitigate local disease outbreaks each season and more importantly prevent introduction of new disease agents. It is recommended that seed is heat treated to mitigate against introduction of new disease agents and to eliminate primary disease introduction to crops of BLS and a range of other pathogens.

Heat treatment of seed typically involves hot water incubation of tomato and capsicum seed at 50°C for 25-30 min or

52°C for 20 min. This follows a pre-warming of the seed for 10 min at approximately 38°C. After treatment, the seed is placed in cold water to stop the heating action and then dried. Sowing of seed should shortly follow the treatment, as storage after treatment may affect germination efficiency. For capsicum and chili seed hot water treatment is effective in the control of BLS, anthracnose (*Colletotrichum* spp.), *Cucumber mosaic virus, Pepper mild mosaic virus* and *Tobacco* and *Tomato mosaic virus*. For tomato seed it is effective in the control of BLS, *Alfalfa mosaic virus*, anthracnose (*Colletotrichum* spp.), bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis*), bacterial speck (*Pseudomonas syringae* pv. *tomato*), bacterial wilt (*Ralstonia solanacearum*), *Cucumber mosaic virus*, early blight (*Alternaria solani*), fusarium wilt (*Fusarium oxysporum* fsp. *lycopersici*), leaf mold (*Passalora fulva*), septoria leaf spot (*Septoria lycopersici*), *Tomato mosaic virus* and verticillium wilt (*Verticillium albo-atrum* and *Verticillium dahliae*).

## Investigating control methods for effectiveness in reducing the impact of BLS:

The current industry standard for controlling BLS is application of copper or using plant host resistance. Review of literature revealed copper tolerance is likely in BLS pathogen populations and the effectiveness of copper to manage the disease is questionable (Appendix 1). Combining copper with manganese-zinc ethylene bisdithiocarbamate (EBCD), for example, mancozeb, does increase the availability of $Cu^{2+}$ ions which is the bactericidal form of copper. This could be done early in the crop cycle to improve availability of bactericidal forms of copper and thus early infections. Later in the cycle the with-holding period for the EBCD is not compatible with harvest windows. The review also identified methods for evaluating copper tolerance which was subsequently applied to Australian isolates of BLS.

The review further identified a range of alternative management products to copper, including essential oils. Essential oils from a number of plant species were shown to have antimicrobial activity in previous *in vitro* assays to a range of phytobacteria including xanthomonads (Gomah 2008; Oliva *et al*. 2015; Kizil, *et al*. 2005; Kokoskova, *et al*. 2011; Lo Cantore et al. 2004; Kotan et al. 2013; Lopez-Reyes et al. 2013; Mikicinski, *et al*. 2012; da Silva, *et al*. 2014). The essential oil research was current and promising, thus this was chosen as an alternative method for further research in this project. Furthermore, the review identified the effectiveness of genetic resistance in the crop host. This is largely restricted to capsicums to control *X. euvesicatoria*. To evaluate genetic resistance for Australian capsicum production, the isolates of *X. euvesicatoria* from this study were race-typed to inform which resistance genes are likely to be effective.

### Copper tolerance testing

None of the 44 BLS isolates tested grew at copper concentrations equivalent to the resistant control (2 mM), however, all showed tolerance to 0.1 mM copper, the lowest concentration tested and many of the tomato isolates, were able to tolerate levels up to 1.5 mM (Appendix 4). Most of the isolates from capsicum and chili also had high levels of copper tolerance, up to 1.0 mM. This indicates that copper tolerance is present in the bacterial populations that cause BLS in Australia. Furthermore, although there was variation in the level of tolerance observed, there were no isolates identified as susceptible to copper.

The industry standard application of copper is at a concentration of approximately 15 mM, ten times greater than the highest minimum inhibitory concentration (MIC) observed in laboratory testing. The discrepancy between the concentrations of copper required to control bacterial growth *in vitro* compared to in-field is related to the low availability of the bactericidal form of copper (i.e $Cu^{2+}$) in cropping environments. The factors contributing to this low availability are outlined in Appendix 1.

### Essential oil evaluation

A range of essential oils were evaluated for bactericidal activity through direct contact and exposure to volatile gases using *in vitro* assays. When used as a contact there was significant reduction in growth compared to no oil. This was for all oils tested, except mineral oil (Table 1). The responses to the oil were very similar for the two bacterial species

tested thus values were combined for statistical analyses. Similarly, the volatile gases gave significant reduction in bacterial growth compared to no oil. Again this was for all oils tested (Table 2). There were also some minor differences between efficacies of the oils, although these were not consistent across the two experiments. Fennel oil used as a volatile gas significantly reduced bacterial growth compared to the non-oil control but was much less effective than the other oils.

**Table 1 Growth of bacterial isolates when contact exposed to different oil treatments measured by absorbance at $OD_{600nm}$. The mean rating across all three replicate plates of the three replicate exposure tests is listed in the table.**

| Treatment[1] | Average growth of bacterial isolate ($OD_{600nm}$) | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 1 | | | Experiment 2 | | |
| | Xe[2] | Xv[2] | Combined growth data (LSD[3] at 5%) | Xe | Xv | Combined growth data (LSD[3] at 5%) |
| No oil | 0.457 | 0.387 | 0.422b | 0.250 | 0.146 | 0.198b |
| Mineral oil | 0.413 | 0.429 | 0.421b | 0.390 | 0.149 | 0.270c |
| Clove | 0.046 | 0.044 | 0.045a | 0.043 | 0.048 | 0.045a |
| Coriander | 0.045 | 0.046 | 0.045a | 0.046 | 0.060 | 0.053a |
| Fennel | 0.044 | 0.044 | 0.044a | 0.053 | 0.045 | 0.049a |
| Lavender | 0.044 | 0.045 | 0.045a | 0.055 | 0.061 | 0.058a |
| Oregano | 0.050 | 0.045 | 0.045a | 0.044 | 0.046 | 0.045a |
| Thyme | 0.044 | 0.043 | 0.043a | 0.046 | 0.054 | 0.050a |

[1]All oil treatments were at a concentration of 0.2% (v/v), [2]Bacterial isolate evaluated where Xe = Xanthomonas euvesicatoria and Xv = Xanthomonas vesicatoria, [3]Least significant difference (LSD) where letters in common signify no significant difference between the treatments using Fisher's protected LSD method (P = 0.05).

**Table 2 Growth of bacterial isolates when exposed to volatile gas from different oil treatments measured by absorbance at $OD_{600nm}$. The mean rating across all three replicate plates of the three replicate exposure tests is listed in the table.**

| Treatment[1] | Average growth of bacterial isolate ($OD_{600nm}$) | | | | | |
|---|---|---|---|---|---|---|
| | Experiment 1 | | | Experiment 2 | | |
| | Xe[2] | Xv[2] | Combined growth data (LSD[3] at 5%) | Xe | Xv | Combined growth data (LSD[3] at 5%) |
| No oil | 0.583 | 0.656 | 0.620d | 0.433 | 0.529 | 0.481f |
| Clove | 0.075 | 0.084 | 0.080b | 0.084 | 0.092 | 0.088b |
| Coriander | 0.075 | 0.085 | 0.080b | 0.095 | 0.107 | 0.101c |
| Fennel | 0.156 | 0.194 | 0.175c | 0.116 | 0.174 | 0.145e |
| Lavender | 0.066 | 0.071 | 0.069a | 0.070 | 0.084 | 0.077a |
| Oregano | 0.082 | 0.086 | 0.084b | 0.100 | 0.120 | 0.110d |
| Thyme | 0.064 | 0.067 | 0.065a | 0.081 | 0.107 | 0.094b |

[1]All oil treatments were as concentrated solution to the inside of the microtitre plate lid, [2]Bacterial isolate evaluated where Xe = *Xanthomonas euvesicatoria* and Xv = *Xanthomonas vesicatoria*, [3]Least significant difference (LSD) where letters in common signify no significant difference between the treatments using Fisher's protected LSD method (P = 0.05).

The essential oils were further evaluated for bactericidal efficacy in pot trials to control *X. euvesicatoria* on capsicum plants. Disease developed well on control plants and there was some variation in disease severities between the two different inoculation methods (Figure 1). The best performing treatments based on average severities were clove, fennel, lavender and oregano oils (Figure 1, Table 3). Coriander and mineral oil were the least effective of the oil treatments, although generally had less disease than the control plants. There was very consistent ratings of no disease in the un-inoculated plants across both inoculation methods. The industry standard of copper plus mancozeb had higher disease severities than most of the oil treatments, with the exception of plants treated with mineral oil and inoculated with wounding. Thyme oil did induce a slight phytotoxic reaction in the experiment, with some leaves developing holes along the main midrib. This occurred irrespective of inoculation method. The second pot trial evaluated the oils at an applied concentration of 2%. This concentration of oil induced severe phytotoxic reactions for all oils tested.

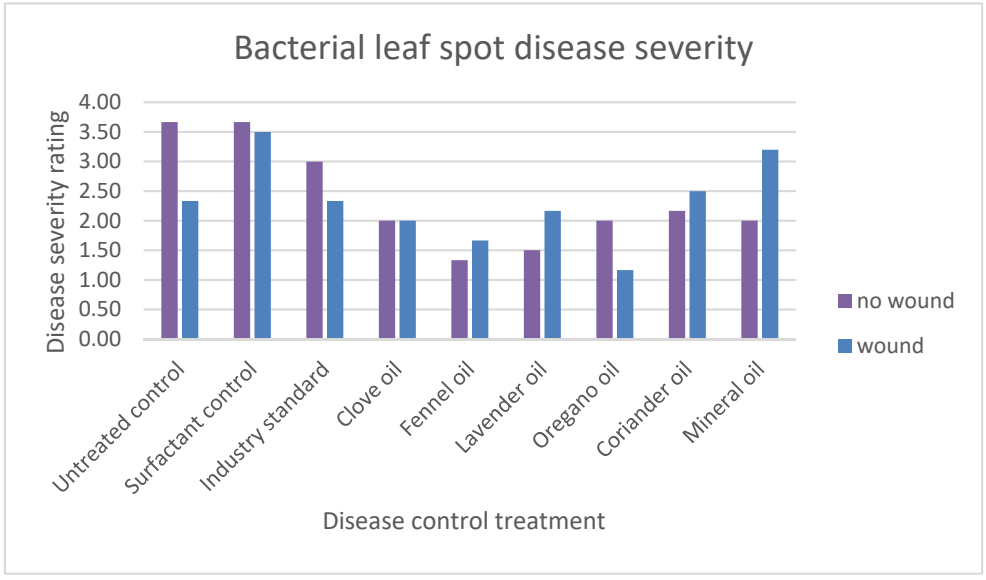


**Figure 1 Graph of bacterial leaf spot disease severity on capsicum plants treated with a range of test control products. Plants were mist inoculated with or without wounding. The disease severity rating is an average from the six replicate plants per treatment. Disease severity was rated on a scale from 0-4, where 0 = no symptoms on inoculated leaves, 1 = 1-10 spots on inoculated leaves, 2 = 11-30 spots on inoculated leaves, 3 = more than 30 spots on inoculated leaves and 4 = confluent necrosis.**

**Table 3 Disease development on capsicum plants when inoculated with *Xanthomonas euvesicatoria* and exposed to different oil treatments. The mean disease rating across all three replicate plants for each test treatment is listed in the table.**

| Treatment[1] | Average disease severity ratings[4] (LSD[5] at 5%) | |
|---|---|---|
| | Inoculated with wounding | Inoculated without wounding |
| Untreated control | 2.33bc | 3.67c |
| Surfactant control[2] | 3.50d | 3.67c |
| Industry standard[3] | 2.33bc | 3.00bc |
| Clove | 1.88ab | 2.00ab |
| Fennel | 1.67ab | 1.33a |
| Lavender | 2.17abc | 1.50a |
| Oregano | 1.17a | 2.00ab |
| Coriander | 2.50bcd | 2.17ab |
| Mineral oil | 3.19cd | 2.00ab |

[1]All oil treatments were 0.2% (v/v) oil plus the surfactant of 0.5% DMSO, 0.1% Tween-20; [2]Surfactant control = water plus 0.5% DMSO, 0.1% Tween-20; [3]Industry standard = 1.5 g/L kocide blue extra + 2 g/L mancozeb. [4]Plants were rated on a disease severity scale from 0-4, where 0 = no symptoms on inoculated leaves, 1 = 1-10 spots on inoculated leaves, 2 = 11-30 spots on inoculated leaves, 3 = more than 30 spots on inoculated leaves and 4 = confluent necrosis and [5]Least significant difference (LSD) where letters in common signify no significant difference between the treatments using Fisher's protected LSD method (P = 0.05).

Although most of the essential oils treatments reduced disease severities, only with oregano was this reduction significant as compared to the untreated control. This was irrespective of the inoculation method used. The wound inoculation technique best represents field infections as wounding commonly occurs during storm activity and from some on-farm activities. This method is preferred for future pot trial evaluations. The best performing oil when plants were wound inoculated was oregano. This oil significantly reduced disease severity compared to both the untreated control, and the industry standard. The pot trial only evaluated one spray application regime. Further evaluation is required to optimize spray timing and the effective concentration of oil to control disease without inducing phytotoxic affects.

## Evaluating germplasm for BLS control:

As highlighted through the literature review on disease control, genetic resistance is a valuable approach. To this end, a pot trial for disease control was completed using differential tomato and capsicum genetic lines. All *Xanthomonas euvesicatoria* isolates tested were pathogenic on the susceptible capsicum variety, Jupiter and mostly non-pathogenic on the tomato variety Grosse Lisse. Conversely, the *X. arboricola, X. perforans* and *X. vesicatoria* isolates were pathogenic to the susceptible tomato variety Grosse Lisse. Some of these isolates were also pathogenic to Jupiter. These results imply there is host-specificity within the BLS pathogen complex and that the disease affecting capsicum and chili is largely caused by a single species, *X. euvesicatoria,* of which multiple races were detected in these experiments. All isolates induced a hypersensitive response on the current industry standard variety which carries all known resistance genes. This indicates there were no new race-types detected from the *X. euvesicatoria* isolates. The races that were detected included race 1 and 7. For further details on pathogenicity testing refer to appendices 4 and 7.

Currently available commercial capsicum lines in Australia carry combinations of resistance genes, typically Xcv 0-3, 1-3, 0-5 and 1-5. Although this provides several capsicum lines to choose from with resistance to race 1, there are very few, readily available with resistance to race 7, thus it is important for growers to know which race they have in their district. The results from this study provides a guide to which races are present in each district (Appendix 4). Specific race testing of the BLS capsicum pathogen is difficult and the only method available is pathogenicity testing on differential host lines as done in this project. This is time consuming and requires ongoing maintenance of the differential lines to ensure seed is available for the testing. Instead, the development of a rapid DNA diagnostic test to replace this system is highly desirable.

Although few are currently available in Australia, capsicum lines with resistances that cover both race 1 and 7 are available in the United States. Future research could involve trialing these varieties in a range of Australian growing districts to evaluate their suitability for our growing conditions. Additionally, many of these lines have multiple pyramided resistance, for example, there are at least seven varieties carrying the resistance genes Xcv 1-5 and 7-9. These pyramided genes are affective to almost all known races of *X. euvesicatoria*.

## Recommendations

The outcomes of this project have identified the major bacterial species causing disease in Australian solanaceous crops. For tomato this is *X. perforans* and *X. vesicatoria*, and for capsicum and chili this is *X. euvesicatoria*. The results show there is copper tolerance within pathogen populations and alternatives to copper for disease control are important. For control of *X. euvesicatoria*, host plant resistance genes are functioning well and control the disease against the specific bacterial race they were developed to. Ongoing international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races or species into Australia. This means existing resistance genes managing disease in Australia may become unreliable in the future. Additionally, overuse of a single management strategy such as a resistance gene or a single chemical can provide strong evolutionary pressure on the bacterial pathogen, leading to mutation and the local emergence of resistance-breaking races or chemically tolerant populations.

Multiple management strategies will help to reduce the evolutionary pressure on the bacterial pathogens, thus prolonging the life of important host-plant resistance genes. It will also provide options to control disease if new races are introduced. A multi-strategy approach to disease management is best. Consideration of other crop management practices is also important for control of any pest or disease. Development of a holistic strategy to control foliar diseases which considers fungal and bacterial pathogens in addition to pests and nutrient requirements would be highly beneficial.

The major recommendations for the management of BLS are:

- Consult with your seed supplier about assurances on the health status of the seed or alternatively,
- Heat treatment of seed to prevent primary introduction of pathogens into crops and to mitigate the risk of introduction of new bacterial races which may circumvent existing plant host resistance genes. This treatment will also mitigate risk of other potential exotic and endemic pathogen threats
- Use of resistant capsicum lines where possible
- Combining copper with manganese-zinc ethylene bisdithiocarbamate (EBDC) (e.g mancozeb) early in the crop cycle to improve availability of bactericidal forms of copper and thus early infections, later applications are not practical due to with-holding periods of the EBDC

To achieve improved disease management the following future research activities are recommended:

- Development of a fast diagnostic test to race-type bacterial pathogens. This will allow informed varietal selection for growers
- Trialing capsicum varieties carrying the resistance genes Xcv 1-5 and 7-9 for suitability to Australian conditions
- Further evaluation of essential oils through investigation of spray application methods and timings
- Continued investigation of other alternatives to copper

The newly contracted Hort Innovation VG16086 will follow up on these future research activities and further investigations of seed treatment strategies thus continuing to leverage the research investment for this project.

## Scientific refereed publications

Roach, R., R. Mann, C. G. Gambley, R. G. Shivas, and B. Rodoni. 2018. Identification of Xanthomonas Species Associated with Bacterial Leaf Spot of Tomato, Capsicum and Chilli Crops in Eastern Australia. *European Journal of Plant Pathology* **150 (3)**: 595–608. https://doi.org/10.1007/s10658-017-1303-9.

Roach, R, R. Mann, C. F. Gambley, Chapman, T., R. G. Shivas, and B. Rodoni. 2018. Genomic Analysis of Australian Xanthomonas Species Reveals Diverse Groups Associated with Bacterial Leaf Spot in Tomato, Capsicum and Chilli Crops. *BMC Genomics* **submitted**.

Roach, R, R. Mann, C.F Gambley, R.J. Shivas, Chapman, T., and B. Rodoni. 2018. Pathogenicity and Copper Tolerance in Australian Xanthomonas Species Associated with Bacterial Leaf Spot. *Crop Protection* **In preparation**.

Roach, R. 2018. "Identification and Classification of Xanthomonas Spp. Causing Bacterial Leaf Spot on Capsicum, Chilli and Tomato in Australia." *PhD thesis*, Bundoora, Victoria, Australia: La Trobe University **Submitted**

## Intellectual property/commercialisation

‘No commercial IP generated’

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## Acknowledgements

We wish to thank our industry partners for allowing access to commercial crops for sample collection and for attending information events. Additionally, we thank Mr Clinton McGrath, DAF for his industry knowledge and networks, without which collection of samples and extension of information would not have been possible. David and Bob Mayer, DAF are also thanked for their biometry advice

The majority of research provided in this report was completed by Ms Rebecca Roach as part of her PhD studies in collaboration with Latrobe University and the PBCRC. Further acknowledgement is to Ms Roach's supervisory team including Professor Brendan Rodoni and Dr Rachel Mann from the Department of Environment and Primary Industries, La Trobe University, Victoria, and Dr Roger Shivas, DAF.

# Appendices

## Appendix 1 : A report on potential management options for control of BLS by project end

### *Introduction*

BLS has been observed in Australia since 1944 (Anon. 1944). General information relating to BLS pathogens in Australia can be found in most guides to vegetable diseases, though generally still under the name Xcv (Jones et al. 2004; Persley, et al., 2010). While this disease is not generally a problem in dry areas, periods of humidity and wet weather can rapidly encourage disease development. The disease is common in Queensland, and isolates have been collected since the 1970s (Martin, et al., 2004). Though outbreaks have been recorded in most growing regions of Australia, very little has been done to investigate the genetic diversity and distribution of the pathogen. A 1982 study of *X. vesicatoria* races in pepper showed that isolates from Queensland were predominantly pepper race 1 (sourced mainly from pepper, one from tomato), with a small number being a tomato race (sourced from tomato) (Cook 1982). Pepper race 1 appears to correspond to the current species designation of *X. euvesicatoria*. There have been no official records of *X. perforans* and *X. gardneri* from Australia, though this does not guarantee they are absent from the country. Within this Hort Innovation project, isolates of *X. perforans* were detected in Australian growing districts and official reporting of this detection is underway. A strain with similarity to *X. gardneri* has been recorded in New Zealand, suggesting this species could possibly be present in Australia (Hamza 2010).

Australia's biggest producer of tomato is Queensland, the main growing regions being Bowen, Bundaberg, the Lockyer Valley and Granite Belt. Capsicum and chili growers are also located in these areas, with the biggest production areas being Bowen and Bundaberg. A smaller amount of chili (hot pepper) types are also grown. BLS has been recorded all over the country, though it is usually not such a problem in cooler climates. BLS is managed in Australia with the use of copper sprays, as antibiotics are not registered for use. Resistance genes are also common in capsicum and chili lines, though not tomato. Strategies to prevent the occurrence of bacterial disease are the most effective control, though less useful once an infestation is present. BLS is a disease of field production and rarely occurs in protected cropping systems.

### *Cultural controls*

In developing a management strategy to control diseases such as BLS, it is important to consider sources for introduction of the bacteria into a field, district or country, how long the bacteria is able to survive without its preferred host plant and where it resides in the environment between cropping periods.

A study in 1982 looked at many of these factors in relation to xanthomonads causing BLS (Bashan, et al, 1982). The important outcomes from this study included survival of the bacteria:

- as an endophyte. The bacteria survived within seedlings as asymptomatic infections, causing no signs of disease. The initial results for this was from laboratory testing with artificially inoculated plants and was then supported by evaluating seeds collected from apparently healthy field plants which gave similar results.
- on seed for at least 10 months and in dried leaf material for at least 14 months.
- in soil was variable in laboratory experiments. In tests using artificial infestation of sandy soil survival was only 16 days, however, viable bacteria were detected in field soils 18 months after infestation. Additionally, the transfer of the bacteria from the infested soil to host plants was questionable and no direct evidence for this was obtained. Thus infested soil may not be an important source for new disease outbreaks.
- in the rhizosphere of a number of different plant species including the non-hosts sorghum, cucumber, bean, pea and wheat. This has implications for potential crop-rotation strategies.

Xanthomonads are reported to be seed-borne in a number of hosts, including capsicum and a linkage between infested

seed and field disease was established (Higgins 1922; Shekhawat, and Chakravarti, 1979). To mitigate this risk, seed can be heat treated to eliminate the bacteria; however, germination rates may be compromised. Recently a number of studies have looked at treating seed with essential oil to combat BLS pathogens (Kotan *et al*. 2013, 2014; Lo Cantore, *et al*. 2009); however, their efficacy is questionable.

Ongoing high-volume international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races into Australia. This represents the highest risk entry pathway for exotic BLS species and/or races to enter the country. No formal mitigation for this risk exists. Seed disinfestation strategies would help mitigate local disease outbreaks each season and more importantly prevent introduction of new disease agents.

Crop rotation and/or fallow periods may contribute to better disease management by reducing the numbers of bacteria surviving between cropping seasons. There is limited information, however, on the length of rotation and the useful alternative crops for the rotations.

### *Genetic host resistance*

Bacterial leaf spot of tomato and pepper has been reported in all major tomato growing regions of the world and as early as 1912. Following the initial reports, a number of studies from around the world detail race shifts and changes in the distribution of BLS species (Sahin 1999, 1995; Ma 2011; Pernezny 1999). For example, pepper race 6 was found in Ohio in a 1994 survey, marking the first time in that region that Xcv overcame the established resistance genes (Sahin 1995). The appearance of resistance breaking races in multiple areas as detailed below suggests the pathogen population changes and moves according to host genetics.

Studies from various countries have attempted to identify the distribution and diversity of races, groups and species of this pathogen causing disease in local crops. The 'race' terminology in not taxonomic but serves to identify strains which are pathogenic on certain host lines. A race distribution study in Taiwan demonstrated the different distributions of three races in both pepper and tomato crops were not due to selection pressure from resistant host lines (Hartman 1990). A resistance breaking strain of Xcv (race 6) was isolated from peppers in Ohio, marking the first time this pathogen overcame the three sources of resistance in pepper (Sahin 1995). An outbreak of race 6 was later recorded in Florida (Pernezny 1999). This new strain was also found to be streptomycin resistant. Race 2 of Xcv was recorded in Puerto Rico (Zapata 1995). These races are described based on responses to differential lines of pepper with 3 resistance genes. *X. vesicatoria* was reported in the eastern region of Turkey in pepper fields and seed lots (Aysan 2003). This report of minor (4-20%) infections in the region was followed some years later by another survey that observed an increase in disease incidence (50-100%) (Mirik 2005). All four species of BLS have been recorded in several countries of Africa (Mbega 2012; Jibrin 2014).

*X. gardneri* was recently reported in Russia as causing the majority of BLS in tomato (Kornev, et al., 2009). A similar situation was reported in Brazil, with low reports of the three other species (Quezado-Duval, et al, 2005). Following the initial report of *X. gardneri* (as *P. gardneri*) in Yugoslavia (Sutic 1957), there have been few other records of this pathogen. There was one record of this species in New Zealand, and several from the island of Reunion (Hamza 2010). Most recently, outbreaks were reported in tomato crops of several states of North America (Ma 2011; Kim 2010). This sudden prevalence of one particular species seemed to indicate a shift from the previously dominant *X. euvesicatoria*.

Although the diversity of the xanthomonads causing BLS is quite high and in many regions largely unknown, studies have identified a number of host resistance genes for incorporation into commercial lines. To this end, breeding activities by commercial seed companies have incorporated many resistance genes for BLS in capsicum. Currently, the varieties of capsicum in Australia have a range of different BLS resistance genes. These are described by the seed companies as resistance to Xcv and include genes Xcv-0, 1, 2, 3, 5, 7, 8 and 9. They are available in varying

combinations, typically, 0-3, 1-3, 0-5, 1-5 and 7-9. There are very few chili and tomato lines listed with BLS or Xcv resistance and often this has no race designation.

### *Copper for BLS control*

#### How does copper protect plants from bacterial infections?

Bacterial populations such as *Xanthomonas* spp. causing bacterial leaf spot in tomato and capsicum and *Pseudomonas syringae* pv. *tomato* causing bacterial speck in tomato, often develop copper tolerance. This means they can tolerate higher concentrations of copper than sensitive populations. It doesn't mean the bacteria are resistant and that copper has no effect.

The availability of free cupric ions ($Cu^{2+}$) is the important component of products to protect against bacterial infections. The concentration of $Cu^{2+}$ on plant surfaces depends on the equilibrium established with the complexed and soluble forms of copper and the chemical reactions releasing the free $Cu^{2+}$ from the soluble forms. There is no strong correlation between the total amount of copper applied and the concentration of $Cu^{2+}$ on leaf or fruit surfaces.

Rainfall or presence of other free water (e.g dew), wind and leaf abrasion, compounds released by the plant and the pH of the leaf surface will affect the amount of soluble copper present. Free water interacts directly with the copper deposits, and indirectly by releasing exudates from the leaf itself, which also interact with the copper. Wind and leaf abrasion can physically remove the copper deposits and/or also release leaf exudates. The spread of the copper on the leaf surface also affects these interactions and this is influenced significantly by the particle size of the product.

As a general rule the half-life of total copper on the leaf surface is about one-month. This is well in excess of the typical application rates of 5 to 10 days. This means more frequent applications will not improve $Cu^{2+}$ availability. Application every 7-10 days is recommended with additional applications if heavy rainfall has occurred or for fast growing plants where additional applications are needed to protect new foliage. Studies on bean indicate that $Cu^{2+}$ concentrations on leaves of about 50 ppb is enough to control copper sensitive populations of *P. syringae* but 10% or more of bacterial cells within copper tolerant populations could survive concentrations of up to 100 ppb $Cu^{2+}$.

Increasing the total amount of copper applied to the leaves will only give a very minor improvement to the amount of soluble copper present, if any at all. Generally, the amount of total copper is often in excess of soluble copper, thus adding more won't provide additional disease control. The exception, is to reapply on fast growing crops to ensure newly developed foliage is covered. Total copper is not a good indicator for the amount of soluble copper present. It is the interaction of the copper product with the plant surfaces that drives solubility of the copper. Furthermore, the amount of free $Cu^{2+}$ present on plant surfaces is only a small fraction of the soluble copper present. In studies on bean, free $Cu^{2+}$ was estimated to be as low as 1% of the soluble copper present. Free $Cu^{2+}$ typically increases with increased amounts of soluble copper.

Application techniques and product type affect the efficacy of copper to protect plants from bacterial infection.

#### *What copper products are commonly used in tomato and capsicum crops?*

Products registered for use against bacterial diseases include Bordeaux mixture, cupric and cuprous hydroxide, cuprous oxide, copper oxychloride, copper salts of fatty acids, copper ammonia acetate complexes, tribasic copper sulphate and mixtures of cupric hydroxide and ethylene bis-dithiocarbamates (EBDC, e.g mancozeb).

The product information usually lists the active ingredient in percent metallic copper which is a measure of the insoluble copper salts and not a measure of free $Cu^{2+}$.

Tank mixes including fungicides such as EBDC (e.g mancozeb) or heavy metals including zinc or iron were shown to improve disease control by increasing the amount of $Cu^{2+}$ in solution. On the other hand, mixing copper products with

organic compounds is highly likely to have the reverse affect and reduce availability of $Cu^{2+}$.

The amount of available $Cu^{2+}$ in a product is a good indicator for efficacy against bacterial pathogens. Commercial products range significantly, from 0.04 to 22.0 µg/ml $Cu^{2+}$. The concentration of $Cu^{2+}$, however, is typically not listed on product labels and requires specific measurement. The metallic copper amount listed on the label is not a good predictor of $Cu^{2+}$ concentration. Products with a $Cu^{2+}$ concentration of 1.5 µg/ml or more were most effective against some bacterial species. In comparing products ask for information on the predicted availability of $Cu^{2+}$.

Copper plus manganese-zinc ethylene bisdithiocarbamate (mancozeb) is consistently better than other copper only products in field studies of a range of bacterial diseases, including bacterial spot and speck of tomato. This is attributed to the ability of the bisdithiocarbamate anion to chelate copper and transport the $Cu^{2+}$ into the bacteria.

Ferric chloride combined with cupric hydroxide improved bacterial disease control in walnut. The ferric chloride increased the sensitivity of the bacterium to the copper. It also increased availability of $Cu^{2+}$ on leaf surfaces by lowering the pH and cation exchange between $Cu^{2+}$ and $Fe^{3+}$. However, lowering the pH with hydrochloric acid or adding a range of other metal ions ($MnSO_4$, $MgCl_2$, $MgSO_4$, $CaCl_2$, NaCl and KCl) did not increase availability of $Cu^{2+}$.

Zinc used instead of or in combination with copper is effective in disease control in walnut. However, further work is needed as again different combinations of product give very different results. Research into alternative chemicals for disease control in capsicum and tomato is underway; however, no products are yet available.

## Suggested spray program

### *What can be mixed with what in the spray tank?*

A tank mix of copper plus an EBDC (ethylene bis-dithiocarbamates, e.g mancozeb) will give the best disease control compared to copper alone. Additionally, avoid mixing the copper with other products that will complex the copper reducing its solubility and ultimately the availability of $Cu^{2+}$

### *What products are likely to perform best and how often/when to use?*

There are various different forms of copper registered for use. These include Bordeaux mixture, cupric and cuprous hydroxide, copper salts of fatty acids, copper ammonia acetate complexes, tribasic copper sulphate and mixtures of cupric hydroxide and ethylene bis-dithiocarbamates (EBDC). There are no strict rules as to which form of copper works best.

Several studies have reported the combination of copper and EBDC (ethylene bis-dithiocarbamates, e.g mancozeb) work best to control bacterial speck of tomato and bacterial spot of capsicum. It is recommended to use this combination early, before harvest as mancozeb has a withholding period of 7 days for tomato and 14 days for capsicum. During harvest, other copper products or alternative control methods should be used.

Products come as wettable powders, wettable granules, liquid flowable suspensions or aqueous liquids. The particular formulation will affect coverage of the product which is an important factor to consider. The formulation may affect solubility of the copper and availability of $Cu^{2+}$. Additivities to products could also potentially affect solubility and/or $Cu^{2+}$ availability. Consult your local supplier for more information about the solubility and $Cu^{2+}$ availability of specific products.

A typical application rate of 7 to 10 days should be adequate as the average half-life of total copper on leaf surfaces is one-month. Applications more frequently are unlikely to improve $Cu^{2+}$ availability and thus disease control. However, in fast growing crops, additional applications might be required to ensure newly developed foliage is protected.

### *Alternative chemical control*

In 2008, a study published findings from evaluations of a range of potential control products for BLS on tomato. These included chemicals such as famoxadone plus cymoxanil (Tanos 50DF®, E.I. du Pont de Nemours and Company), the defence response activator acibenzolar-S-methyl (Actigard 50 WG®, Syngenta Crop Protection) and the biocontrol agent Bacillus subtilis QST 713 (Serenade products, AgraQuest Inc.) with and without copper (Roberts et al. 2008). Although these products looked promising, there were no definitive recommendations from the study. The products have undergone further research and in some instances commercial trials.

Fayette et al. (2012) further investigated the efficacy of Tanos 50DF® (cymoxanil plus famoxadone) in the management of two Xanthomonas spp., which included X. perforans. Neither of these components alone had *in vitro* or in vivo activity against the bacterial species, however, there was a synergistic effect when Tanos was used with copper hydroxide to control a copper-sensitive species of X. perforans. The authors suggest Tanos could be used in replacement of mancozeb to enhance control of bacterial pathogens with copper during the production cycle where mancozeb is excluded due to withholding periods. Further work is required to confirm these results before this recommendation is adopted.

A further study also investigated the efficacy of B. subtilits QST 713 formulations in combination with copper to control BLS of tomato (Abbasi and Weselowski 2015). They concluded there was a moderate positive impact using this product, particularly in combination with copper. This product and/or similar products are being further trialled in Australia on both capsicum and tomato for BLS management.

Worthington et al. (2012) investigated the compound 2-aminoiidazole (2AI) for control of BLS. The compound was shown previously to inhibit and disperse bacterial biofilms. The compound was trialed alone and in combination with a number of other products including copper hydroxide with and without mancozeb, kasugamycin, Regalia™, potassium phosphite and a nonionic surfactant. In leaf disc assays 2AI mixed with copper improved control of a copper resistant isolate. The compound also reduced biofilm formation when tested *in vitro*. Results of the field trials were less conclusive and 2AI requires further evaluation. The first field trial showed significant reduction in disease when 2AI as used in combination with copper hydroxide and potassium phosphite as compared to either of those products alone and to the untreated control. In the second trial, disease reduction of 2AI with copper hydroxide was similar to copper hydroxide mixed with mancozeb and significantly greater than the untreated control and two both components (i.e 2AI and copper hydroxide) used alone. In the same trial, latron a nonionic surfactant was also tested and improved efficacy of 2AI used alone with disease reduction comparable to treatment with a mix of 2AI and copper hydroxide. In the final field trial, the use of 2AI either alone or in combination with copper hydroxide or the biofungicide Regalia™ was significantly greater than the untreated control. The best treatments for the trial were obtained using copper hydroxide (1.4 kg) with 2AI (100 µM), Regalia with 2AI (100 µM) and the industry standard of copper hydroxide (1.4 kg) with mancozeb.

Further work with both 2AI and Regalia would be useful for the Australian industry, particularly to evaluate their effectiveness against a broader range of xanthomonads and other Solanaceae crops such as chili and tomato. Regalia is a 5% extract from the plant species *Reynoutria sachalinensis* sold by Marrone Bio Innovations. The mode of action for Regalia is listed as an induced systemic resistance (i.e a plant defence response activator; http://marronebioinnovations.com/ag-products/brand/regalia/). It has broad spectrum activity for foliar pathogens.

A complication to evaluating disease control products is the unreliability of performance in field trials. This is often due to the strong linkage between bacterial diseases and weather events. If environmental conditions are not favourable for disease development and spread it is often difficult to get robust results from field trials. Pot trials are useful as a preliminary method for evaluating potential products but cannot give good yield impact data.

### *Essential oils*

Essential oils from a range of different plant species were previously shown to have efficacy in control of xanthomonads, including those causing BLS. Sage, clove and BioZell™ (based on thyme oil) performed better at controlling a range of plant pathogenic bacteria than lavender and lemon balm (Mikicinski, *et al*. 2012). Kizil, et al. (2005) reported coriander and hyssop oil were efficacious against *X. campestris* pv. *malvacearum* but not cumin, dill, fennel, mint or anise. The chemical compounds carvacrol and thymol found in a range of essential oils was shown to be antibacterial to a range of plant pathogens including *X. vesicatoria* (Kotan et al. 2014, 2013). Other studies over the past decade have also evaluated essential oils against a range of phytopathogenic bacteria (Oliva et al. 2015; Kokoskova, *et al*. 2011; Lo Cantore, *et al*. 2009; da Silva, *et al*. 2014; Lopez-Reyes *et al*. 2013; Gomah 2008; Lo Cantore *et al*. 2004).

Testing essential oils for efficacy against the BLS causal agents was completed in this current project (VG14010 - Milestone report 104).

## What else needs to be studied to improve BLS disease management?

Improvement in management of bacterial spot of tomato and capsicums will be through improved understanding of disease life-cycles, pathogen diversity and development of novel control products. These products could include formulations which directly interfere with bacterial survivability and/or promote defense responses within the crop plants. Additionally, further plant breeding efforts could identify resistance or tolerance within tomato and capsicum germplasm that could be used instead of or in combination with chemical control methods. Improved understanding of pathogen diversity in Australia will assist with plant breeding efforts and in the development of novel control products. Further research on alternative crops for rotation would also be beneficial. Investigation of different seed disinfestation protocols would be highly desirable.

Ongoing international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races into Australia. This means existing resistance genes managing disease in Australia may become unreliable in the future. Additionally, overuse of a single management strategy such as a resistance gene or a single chemical can provide strong evolutionary pressure on the bacterial pathogen, leading to mutation and the local emergence of resistance-breaking races or chemically tolerant populations. Multiple management strategies will help to reduce evolutionary pressure on the bacterial pathogens to circumvent resistance genes and also provide options to control disease if exotic races are introduced. A multi-strategy approach to disease management is best. Consideration of other crop management practices is also important for control of any pest or disease. Development of a holistic strategy to control foliar diseases which considers fungal and bacterial pathogens in addition to pests and nutrient requirements would be highly beneficial.

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# Management of bacterial leaf spot of tomato and pepper

## What is bacterial leaf spot?

Bacterial leaf spot (BLS) is caused by four species of *Xanthomonas*: *X. vesicatoria*, *X. euvesicatoria*, *X. perforans* and *X. gardneri*. *X. euvesicatoria* infects mainly capsicum and chilli, while the other species primarily infect tomato. Some accounts of species on multiple hosts is recorded. Yield loss is generally a result of decreased photosynthetic area, though in severe infections direct fruit damage is also seen.

Previously it was assumed *X. campestris* pv. *vesicatoria* (now known as *X. vesicatoria*), was causing BLS in all solanceous crops in Australia. The results from the Hort Innovation project (VG14010), clearly indicate this is not the case, instead there are four different *Xanthomonas* species associated with the disease. Furthermore, the species are largely host specific, where *X. euvesicatoria* essentially infects capsicum, chili and tomato, and *X. vesicatoria* and *X. perforans* typically only tomato. The fourth species, *X. arboricola*, was found in association with BLS symptoms and able to weakly infect tomato only. Given this weak pathogenicity, its ability to cause BLS disease is questionable and as such the bacterium is unlikely to need control. Furthermore, race-typing of *X. euvesicatoria* isolates identified races 1 and 7 in Australia. There are currently several commercial capsicum lines in Australia with resistance to race 1. There are very few lines, readily available with resistance to race 7. Importantly, the diversity of X. euvesicatoria races and other Xanthomonads detected in Australia is very low, however, as there is no regulation for these bacterial pathogens it is likely new races and species could be introduced with seed.

## Cultural controls

In developing a management strategy to control BLS, it is important to consider sources for introduction of the bacteria into a field, district or country, how long the bacteria is able to survive without its preferred host plant and where it resides in the environment between cropping periods. Survival of xanthomonads causing BLS include:

- within seedlings but causing no signs of disease
- on seed for at least 10 months and in dried leaf material for at least 14 months
- in the rhizosphere of a number of different plant species including the non-hosts sorghum, cucumber, bean, pea and wheat. This has implications for potential crop-rotation strategies.
- in soil, however, this can vary from only 16 days to 18 months, and the transfer of the bacteria from the infested soil to host plants is questionable, thus infested soil may not be an important source for new disease outbreaks

Xanthomonads are reported to be seed-borne in a number of hosts, including capsicum and a linkage between infested seed and field disease was established many years ago. To mitigate this risk, seed can be heat treated to eliminate the bacteria; however, germination rates may be compromised. Preliminary studies have looked at treating seed with essential oil to combat BLS pathogens.

Ongoing high-volume international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races into Australia. This represents the highest risk entry pathway for exotic BLS species and/or races to enter the country. No formal mitigation for this risk exists. Seed disinfestation strategies would help mitigate local disease outbreaks each season and more importantly prevent introduction of new disease agents.

Crop rotation and/or fallow periods may contribute to better disease management by reducing the numbers of bacteria surviving between cropping seasons. There is limited information, however, on the length of rotation and the useful alternative crops for the rotations.

## Genetic host resistance

BLS of tomato and pepper has been reported in all major tomato growing regions of the world and as early as 1912. Studies from around the world detail race shifts and changes in the distribution of BLS species, e.g. pepper race 6 was found in Ohio in 1994, marking the first time in that region that Xcv overcame the established resistance genes. An outbreak of this race was later recorded in Florida.

Although the diversity of the xanthomonads causing BLS is quite high and in many regions largely unknown, studies have identified a number of host resistance genes for incorporation into commercial lines. To this end, breeding activities by commercial seed companies have incorporated many resistance genes for BLS in capsicum. The results of the first race-typing of *X. euvesicatoria* done with Australian isolates (Hort Innovation VG14010) will assist greatly in variety selection for capsicum growers to combat BLS outbreaks. The results showed that there are at least two races present in Australia, races 1 and 7, and that no new race-types were detected from the *X. euvesicatoria* isolates. Currently, the varieties of capsicum in Australia have a range of different BLS resistance genes. These are described by the seed companies as resistance to Xcv and include genes Xcv-0, 1, 2, 3, 5, 7, 8 and 9. They are available in varying combinations, typically, 0-3, 1-3, 0-5, 1-5 and 7-9. The resistance gene number refers to the race of the bacterial pathogen which it is effective against, for example, Xcv 1 combats race 1. There are very few chilli and tomato lines listed with BLS or Xcv resistance and often this has no race designation.

## Copper for BLS control

### *Are Australian isolates of BLS copper tolerant?*

Bacterial isolates of *X. vesicatoria, X. euvesicatoria* and *X. perforans* were tested in laboratory assays for tolerance to copper (VG14010). All of the 58 isolates tested showed tolerance to the lowest copper concentration tested (0.1 mM). Of the 27 isolated from tomato, 13 were rated as highly tolerant to copper with growth up to 1.5 mM, 11 as moderately tolerant (growth between >0.5-1.0 mM) and 3 as tolerant (growth at 0.1-0.5 mM). All most all of the 31 isolates from capsicum and chilli were rated as moderately tolerant to copper, one isolate was rated as tolerant.

The industry standard application of copper is at a concentration of approximately 15 mM, ten times greater than the highest minimum inhibitory concentration (MIC) observed in laboratory testing. The discrepancy between the concentrations of copper required to control bacterial growth *in vitro* compared to in-field is related to the low availability of the bactericidal form of copper (i.e $Cu^{2+}$) in cropping environments.

### *How does copper protect plants from bacterial infections?*

The evolution of copper tolerance in bacterial populations means they can tolerate higher concentrations of copper than sensitive populations. It doesn't mean the bacteria are resistant and that copper has no effect.

The availability of free cupric ions ($Cu^{2+}$) is the important component of products to protect against bacterial infections. The concentration of $Cu^{2+}$ on plant surfaces depends on the equilibrium established with the complexed and soluble forms of copper and the chemical reactions releasing the free $Cu^{2+}$ from the soluble forms. There is no strong correlation between the total amount of copper applied and the concentration of $Cu^{2+}$ on leaf or fruit surfaces.

Rainfall or presence of other free water (e.g dew), wind and leaf abrasion, compounds released by the plant and the pH of the leaf surface will affect the amount of soluble copper present. Free water interacts directly with the copper deposits, and indirectly by releasing exudates from the leaf itself, which also interact with the copper. Wind and leaf abrasion can physically remove the copper deposits and/or also release leaf exudates. The spread of the copper on the

leaf surface also affects these interactions and this is influenced significantly by the particle size of the product.

As a general rule the half-life of total copper on the leaf surface is about one-month. This is well in excess of the typical application rates of 5 to 10 days. This means more frequent applications will not improve $Cu^{2+}$ availability. Application every 7-10 days is recommended with additional applications if heavy rainfall has occurred or for fast growing plants where additional applications are needed to protect new foliage.

Increasing the total amount of copper applied to the leaves will only give a very minor improvement to the amount of soluble copper present, if any at all. Generally, the amount of total copper is often in excess of soluble copper, thus adding more won't provide additional disease control. Total copper is not a good indicator for the amount of soluble copper present. It is the interaction of the copper product with the plant surfaces that drives solubility of the copper. Furthermore, the amount of free $Cu^{2+}$ present on plant surfaces is only a small fraction of the soluble copper present. In studies on bean, free $Cu^{2+}$ was estimated to be as low as 1% of the soluble copper present. Free $Cu^{2+}$ typically increases with increased amounts of soluble copper.

Application techniques and product type significantly affect the efficacy of copper to protect plants from bacterial infection.

### *What copper products are commonly used in tomato and capsicum crops?*

Products registered for use against bacterial diseases include Bordeaux mixture, cupric and cuprous hydroxide, cuprous oxide, copper oxychloride, copper salts of fatty acids, copper ammonia acetate complexes, tribasic copper sulphate and mixtures of cupric hydroxide and ethylene bis-dithiocarbamates (EBDC, e.g mancozeb).

The product information usually lists the active ingredient in percent metallic copper which is a measure of the insoluble copper salts and not a measure of free $Cu^{2+}$.

Tank mixes including fungicides such as EBDC (e.g mancozeb) or heavy metals including zinc or iron were shown to improve disease control by increasing the amount of $Cu^{2+}$ in solution. On the other hand, mixing copper products with organic compounds is highly likely to have the reverse affect and reduce availability of $Cu^{2+}$.

The amount of available $Cu^{2+}$ in a product is a good indicator for efficacy against bacterial pathogens. Commercial products range significantly, from 0.04 to 22.0 µg/ml $Cu^{2+}$. The concentration of $Cu^{2+}$, however, is typically not listed on product labels and requires specific measurement. The metallic copper amount listed on the label is not a good predictor of $Cu^{2+}$ concentration. Products with a $Cu^{2+}$ concentration of 1.5 µg/ml or more were most effective against some bacterial species. In comparing products ask for information on the predicted availability of $Cu^{2+}$.

Copper plus manganese-zinc ethylene bisdithiocarbamate (mancozeb) is consistently better than other copper only products in field studies of a range of bacterial diseases, including bacterial spot and speck of tomato. This is attributed to the ability of the bisdithiocarbamate anion to chelate copper and transport the $Cu^{2+}$ into the bacteria. As stated above it also improves the concentration of $Cu^{2+}$ in solution.

Ferric chloride combined with cupric hydroxide improved bacterial disease control in walnut. The ferric chloride increased the sensitivity of the bacterium to the copper. It also increased availability of $Cu^{2+}$ on leaf surfaces by lowering the pH and cation exchange between $Cu^{2+}$ and $Fe^{3+}$. However, lowering the pH with hydrochloric acid or adding a range of other metal ions ($MnSO_4$, $MgCl_2$, $MgSO_4$, $CaCl_2$, NaCl and KCl) did not increase availability of $Cu^{2+}$.

Zinc used instead of or in combination with copper is effective in disease control in walnut. However, further work is needed as again different combinations of product give very different results. Research into alternative chemicals for disease control in capsicum and tomato is underway; however, no products are yet available.

*Suggested spray program*

### What can be mixed with what in the spray tank?

A tank mix of copper plus an EBDC (ethylene bis-dithiocarbamates, e.g mancozeb) will give the best disease control compared to copper alone. Additionally, avoid mixing the copper with other products that will complex the copper reducing its solubility and ultimately the availability of $Cu^{2+}$.

### What products are likely to perform best and how often/when to use?

There are various different forms of copper registered for use. These include Bordeaux mixture, cupric and cuprous hydroxide, copper salts of fatty acids, copper ammonia acetate complexes, tribasic copper sulphate and mixtures of cupric hydroxide and ethylene bis-dithiocarbamates (EBDC). There are no strict rules as to which form of copper works best.

Several studies have reported the combination of copper and EBDC (ethylene bis-dithiocarbamates, e.g mancozeb) work best to control bacterial speck of tomato and bacterial spot of capsicum. It is recommended to use this combination early, before harvest as mancozeb has withholding periods which will affect harvest times. During harvest, other copper products or alternative control methods should be used.

Products come as wettable powders, wettable granules, liquid flowable suspensions or aqueous liquids. The particular formulation will affect coverage of the product which is an important factor to consider. The formulation may affect solubility of the copper and availability of $Cu^{2+}$. Additivities to products could also potentially affect solubility and/or $Cu^{2+}$ availability. Consult your local supplier for more information about the solubility and $Cu^{2+}$ availability of specific products

A typical application rate of 7 to 10 days should be adequate as the average half-life of total copper on leaf surfaces is one-month. Applications more frequently are unlikely to improve $Cu^{2+}$ availability and thus disease control. However, in fast growing crops, additional applications might be required to ensure newly developed foliage is protected.

## Alternative chemical control

In 2008, a study published findings from evaluations of a range of potential control products for BLS on tomato. These included chemicals such as famoxadone plus cymoxanil (E.I. du Pont de Nemours and Company), the defence response activator acibenzolar-*S*-methyl (Syngenta Crop Protection) and the biocontrol agent *Bacillus subtilis* QST713 (AgraQuest Inc.) with and without copper. Although these products looked promising, there were no definitive recommendations from the study. The products have undergone further research and in some instances commercial trials, in particular products using *Bacillus amyloliquefaciens* QST713 (previously known as *B. subtilis* QST713). A product containing QST713 was released for use to supress BLS in Australia in 2018.

A subsequent study in 2012 further investigated the efficacy of famoxadone plus cymoxanil in the management of two *Xanthomonas* spp., which included *X. perforans*. Neither of these components alone had *in vitro* or *in vivo* activity against the bacterial species, however, there was a synergistic effect when the product was used with copper hydroxide to control a copper-sensitive species of *X. perforans*. The authors suggest this product could be used in replacement of mancozeb to enhance control of bacterial pathogens with copper during the production cycle where mancozeb is excluded due to withholding periods. Further work is required to confirm these results before this recommendation is adopted.

In 2012, the compound 2-aminoiidazole (2AI) was investigated for control of BLS. The compound was shown previously to inhibit and disperse bacterial biofilms. The compound was trialled alone and in combination with a number of other products including copper hydroxide with and without mancozeb, kasugamycin, Regalia™, potassium phosphite and a

nonionic surfactant. In laboratory assays 2AI mixed with copper improved control of a copper resistant isolate. The compound also reduced biofilm formation when tested *in vitro*. Results of the field trials were less conclusive, in some trials it performed better than copper hydroxide alone and in others it was similar or less, thus 2AI still requires further evaluation. The first field trial showed significant reduction in disease when 2AI as used in combination with copper hydroxide and potassium phosphite as compared to either of those products alone and to the untreated control. In the second trial, disease reduction of 2AI with copper hydroxide was similar to copper hydroxide mixed with mancozeb and significantly greater than the untreated control and the two components (i.e 2AI and copper hydroxide) used alone. In the same trial, latron a nonionic surfactant was also tested and improved efficacy of 2AI used alone with disease reduction comparable to treatment with a mix of 2AI and copper hydroxide. In the final field trial, the use of 2AI either alone or in combination with copper hydroxide or the biofungicide Regalia™ was significantly greater than the untreated control. The best treatments for the trial were obtained using copper hydroxide (1.4 kg) with 2AI (100 µM), Regalia with 2AI (100 µM) and the industry standard of copper hydroxide (1.4 kg) with mancozeb.

Further work with both 2AI and Regalia would be useful for the Australian industry, particularly to evaluate their effectiveness against a broader range of xanthomonads and other solanaceae crops such as chilli and tomato. Regalia is a 5% extract from the plant species *Reynoutria sachalinensis* sold by Marrone Bio Innovations. The mode of action for Regalia is listed as an induced systemic resistance (i.e a plant defence response activator; http://marronebioinnovations.com/ag-products/brand/regalia/). It has broad spectrum activity for foliar pathogens.

A complication to evaluating disease control products is the unreliability of performance in field trials. This is often due to the strong linkage between bacterial diseases and weather events. If environmental conditions are not favourable for disease development and spread it is often difficult to get robust results from field trials. Pot trials are useful as a preliminary method for evaluating potential products but cannot give good yield impact data.

## Essential oils

Essential oils from a range of different plant species were previously shown to have efficacy in control of xanthomonads, including those causing BLS. Sage, clove and BioZell™ (based on thyme oil) performed better at controlling a range of plant pathogenic bacteria than lavender and lemon balm. A 2005 study reported coriander and hyssop oil were efficacious against *X. campestris* pv. *malvacearum* but not cumin, dill, fennel, mint or anise. The chemical compounds carvacrol and thymol found in a range of essential oils was shown to be antibacterial to a range of plant pathogens including *X. vesicatoria*. Other studies over the past decade have also evaluated essential oils against a range of phytopathogenic bacteria.

Testing essential oils for efficacy against the BLS causal agents was completed in Hort Innovation project, VG14010. Clove, coriander, fennel, lavender, oregano and thyme were trialled in laboratory assays for control of *X. vesicatoria* and *X. euvesicatoria*. All the oils showed significant inhibition of bacterial growth for both species as compared to the control when applied as a contact or through exposure to volatile gases from the oils. Fennel was the least effective in the laboratory trials. Further work is needed to investigate the efficacy of these oils as a seed treatment or foliar management option.

## What else needs to be studied to improve BLS disease management?

Improvement in management of bacterial spot of tomato and capsicums will be through improved understanding of disease life-cycles, pathogen diversity and development of novel control products. These products could include formulations which directly interfere with bacterial survivability and/or promote defence responses within the crop plants. Additionally, further plant breeding efforts could identify resistance or tolerance within tomato and capsicum germplasm that could be used instead of or in combination with chemical control methods. The improved understanding of pathogen diversity in Australia will assist with plant breeding efforts and in the development of novel

control products. Further research on alternative crops for rotation would also be beneficial. Investigation of different seed disinfestation protocols would be highly desirable.

Ongoing international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races into Australia. This means existing resistance genes managing disease in Australia may become unreliable in the future. Additionally, overuse of a single management strategy such as a resistance gene or a single chemical can provide strong evolutionary pressure on the bacterial pathogen, leading to mutation and the local emergence of resistance-breaking races or chemically tolerant populations. Multiple management strategies will help to reduce evolutionary pressure on the bacterial pathogens to circumvent resistance genes and also provide options to control disease if exotic races are introduced. A multi-strategy approach to disease management is best. Consideration of other crop management practices is also important for control of any pest or disease. Development of a holistic strategy to control foliar diseases which considers fungal and bacterial pathogens in addition to pests and nutrient requirements would be highly beneficial.

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This project has been funded by Hort Innovation using the vegetable research and development levy and funds from the Australian Government. For more information on the fund and strategic levy investment visit horticulture.com.au

## Appendix 3: Project commencement article - Identification of Xanthomonas species causing bacterial leaf spot disease in Australian tomato and pepper crops – VN14010

The Horticulture Innovation Australia Ltd (Hort Innovation) funded project VN14010 commenced on 29/06/15. This project was initiated as part of a PhD project on detection and identification of Australian Xanthomonas species causing bacterial leaf spot. Bacterial leaf spot (acronym: BLS) affects tomato, chili and capsicum production worldwide, and is caused by four bacterial species of Xanthomonas; *Xanthomonas euvesicatoria* (Xe), *X. vesicatoria* (Xv), *X. gardneri* (Xg) and *X. perforans* (Xp). The aim of this study is to improve the current diagnostic capabilities of rapidly detecting xanthomonads causing BLS, and to investigate the diversity of these pathogens in Australia. This project will result in increased capability for detection and identification of *Xanthomonas* spp. and the capacity of the vegetable industry to implement integrated disease management programs for bacterial leaf spot.

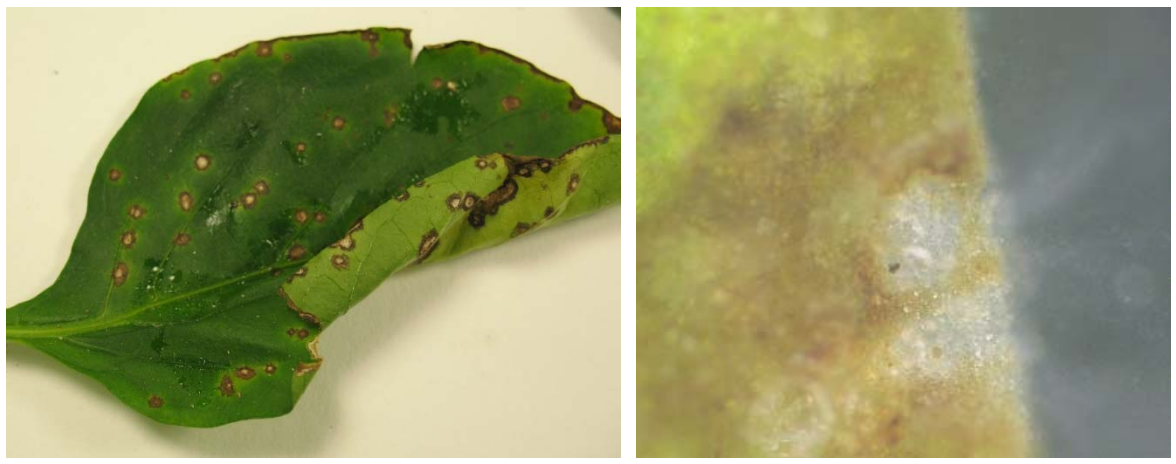

**Figure 1. Bacterial leaf spot lesions on capsicum leaves (left) and bacterial streaming from a lesion (right).**

The main activities within the project involve a range of identification and pathogenicity studies focused on the species causing BLS in Australia. Detection and identification was carried out using a variety of molecular and biochemical methods. Pathogenicity and copper sensitivity studies will provide insight into the ability of Australian BLS strains to overcome certain resistance genes. Another report on Australian BLS populations detailed the copper resistance in Xanthomonas campestris pv. vesicatoria (Xcv), though this does not relate to the new species descriptions.

During the first phase of the project, bacterial isolates were collected from symptomatic plants in Bundaberg, Stanthorpe, Tenterfield, Bowen and the Lockyer valley. This collection was supplemented with isolates from a herbarium collection that represent historical outbreaks. These were identified using specific PCRs, MLSA and biochemical techniques. This study resulted in the identification of Xe and Xv isolates, as well as the first report of Xp and Xg isolates in Australia (Figure 2). Xe isolates were found in south-east QLD (SEQ) and NSW. Xv isolates were found in Bowen, SEQ, and NSW. Xp isolates were found in Bundaberg, Bowen, Brisbane and NSW. Xg isolates were found only in Stanthorpe and Tenterfield. These 'Xg' isolates appear distinctly genetically and physically different from reference strains sourced from other regions. Differences in pathogenicity and climate preferences of these newly reported pathogens will be investigated in further studies.

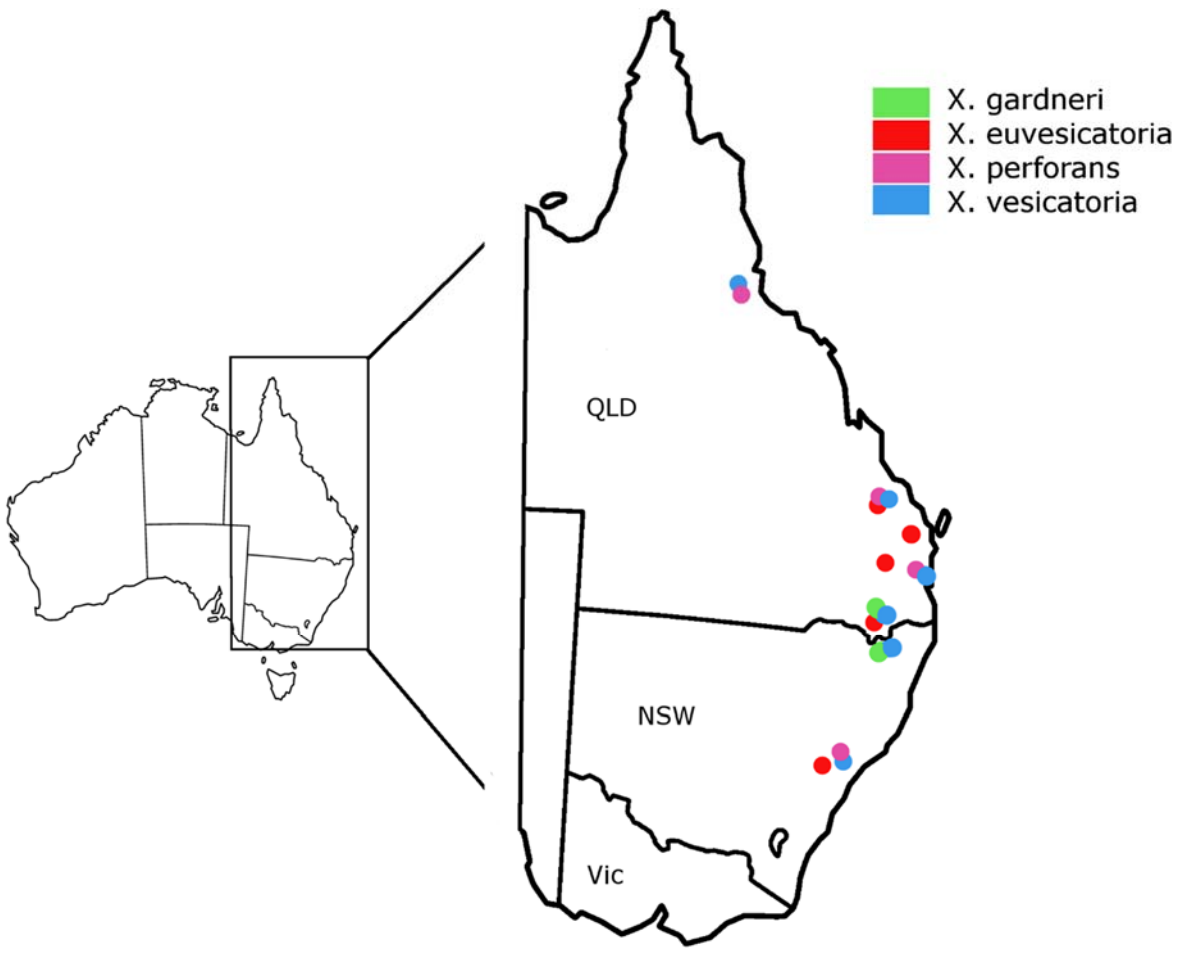


**Figure 2. Map showing the locations of species identified in the study thus far.**

There are several ways a bacterial pathogen may be introduced and persist in a field. Inoculum sources may include seed, crop residue, weeds, volunteers and contaminated equipment. Identifying the vulnerable parts of a pathogen's life cycle has implications for the timing of control measures. BLS pathogens have been shown to persist in crop residue for up to six months, though survivability and transmission in this manner may vary depending on temperatures and water availability. Based on washings from weed species present in tomato crops, weeds are not thought to be a primary source of inoculum as Xcv is rarely found to be associated. Though BLS can be found all over the world, the diversity of the causal pathogen still presents a biosecurity risk, as new strains with different pathogenicity genes could potentially undermine resistance bred into established crops. Aspects of potential management strategies will be investigated in the future.

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## Appendix 4: Results of bacterial isolate characterization for pathogenicity and copper tolerance

The below list of reference bacterial isolates which derived from plants diagnosed with bacterial leaf spot disease. The isolates are deposited in the DAF nationally recognized herbarium. Further details of the isolates are available in Roach et al 2018a, Roach et al 2018b and Roach et al 2018c.

**Table 1. Results of pathogenicity testing of bacterial leaf spot (BLS) isolates. The isolate species identity, reference code and original host it was isolated from are listed with the results of pathogenicity on tomato and capsicum. For isolates identified as X. euvesicatoria, the results of the race-typing are also listed. Positive pathogenicity reactions are shown by a (+) and negative by a (-). Where tests are not applicable this is indicated as (na).**

| Xanthomonas species | Isolation host | Herbarium reference (BRIP) | Location | Copper tolerance[1] | Pathogenicity on capsicum[2] | Pathogenicity on tomato[3] | Race[4] |
|---|---|---|---|---|---|---|---|
| euvesicatoria | capsicum | 62390 | Gatton, QLD | MT | + | | 1 |
| | | 62391 | Gatton, QLD | MT | + | | 1 |
| | | 62392 | Gatton, QLD | MT | + | | 1 |
| | | 62393 | Gatton, QLD | MT | + | - | 7,9 |
| | | 62394 | Gatton, QLD | MT | + | - | ND |
| | | 62395 | Gatton, QLD | MT | + | | 7 |
| | | 62438 | Hawkesbury Heights, NSW | T | + | - | 7 |
| | | 38855 | Bundaberg, QLD | MT | + | - | ND |
| | | 63464 | Bundaberg, QLD | MT | + | - | 7 |
| | chili | 62396 | Gatton, QLD | MT | + | - | 7 |
| | | 62399 | Gatton, QLD | MT | + | - | 7,9 |
| | | 62400 | Gatton, QLD | MT | + | - | 7 |
| | | 62401 | Gatton, QLD | MT | + | - | 7,9 |
| | | 62402 | Gatton, QLD | MT | + | - | 7,9 |
| | | 62403 | Gatton, QLD | MT | + | - | 7 |
| | | 62425 | Stanthorpe, QLD | MT | + | | 1 |
| | | 62434 | Gatton, QLD | MT | + | - | ND |
| | | 62435 | Gatton, QLD | MT | + | - | 7,9 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 62439 | Bundaberg, QLD | MT | + | - | ND |
| | | 62440 | Bundaberg, QLD | MT | + | - | 0,1,4 |
| | | 62441 | Bundaberg, QLD | MT | + | - | 1 |
| | | 62442 | Bundaberg, QLD | MT | + | - | 0,1,4 |
| | | 62443 | Bundaberg, QLD | MT | + | - | 0,1,4 |
| | | 39000 | Glastonbury, QLD | MT | + | - | ND |
| | | 62454 | Bundaberg, QLD | MT | + | - | 0,1,4 |
| | | 62555 | Bundaberg, QLD | MT | + | - | 7 |
| | | 62656 | Bundaberg, QLD | MT | + | - | 7,9 |
| | | 62757 | Bundaberg, QLD | MT | + | - | 7 |
| | | 62858 | Bundaberg, QLD | MT | + | - | 7 |
| | | 62959 | Bundaberg, QLD | MT | + | - | 7 |
| | | 38997 | Bundaberg, QLD | MT | + | | 7 |
| arboricola | tomato | 62410 | Stanthorpe, QLD | T | - | + | na |
| | | 62412 | Stanthorpe, QLD | MT | - | + | na |
| | | 62414 | Stanthorpe, QLD | MT | - | + | na |
| | | 62416 | Stanthorpe, QLD | MT | | + | na |
| | | 62432 | Tenterfield, NSW | T | - | + | na |
| perforans | | 62383 | Bowen, QLD | HT | - | + | na |
| | | 62384 | Bowen, QLD | MT | + | + | na |
| | | 62385 | Bowen, QLD | MT | + | + | na |
| | | 62386 | Bundaberg, QLD | HT | + | + | na |
| | | 62387 | Bowen, QLD | MT | + | + | na |
| | | 62389 | South Turramurra, NSW | HT | - | + | na |
| | | 62397 | Brisbane, QLD | T | - | + | na |
| | | 62398 | Bowen, QLD | MT | - | + | na |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 62404 | Bowen, QLD | MT | + | + | na |
| | | 62405 | Bundaberg, QLD | HT | - | + | na |
| | | 63262 | Bundaberg, QLD | HT | - | + | na |
| | | 63565 | Bundaberg, QLD | MT | | + | na |
| | | 63666 | Bundaberg, QLD | MT | - | + | na |
| vesicatoria | | 62388 | South Turramurra, NSW | HT | + | + | na |
| | | 62413 | Stanthorpe, QLD | MT | - | + | na |
| | | 62423 | Stanthorpe, QLD | HT | + | + | na |
| | | 62428 | Tenterfield, NSW | HT | | + | na |
| | | 62429 | Tenterfield, NSW | HT | - | + | na |
| | | 62430 | Tenterfield, NSW | HT | - | + | na |
| | | 39109 | Cleveland, QLD | HT | - | + | na |
| | | 38861 | Victoria Point, QLD | HT | | + | na |
| | wild tomato | 38864 | Bowen, QLD | HT | - | + | na |
| unknown | tomato | 62409 | Stanthorpe, QLD | T | - | | na |
| | | 62411 | Stanthorpe, QLD | T | - | | na |
| | | 62415 | Stanthorpe, QLD | T | - | | na |
| | | 62417 | Stanthorpe, QLD | HT | - | | na |
| | | 62418 | Stanthorpe, QLD | HT | | | na |
| | apple of peru | 39016 | Christmas Creek, QLD | HT | - | + | na |

[1]Minimum inhibitory concentration (MIC) of $CuSO_4$ at which no growth of the bacterial isolate was observed. Ratings are S = susceptible (no growth at any copper concentration), T = tolerant (growth at 0.1-0.5 mM $CuSO_4$) MT = moderately tolerant (growth >0.5 – 1.0 mM $CuSO_4$), HT = highly tolerant (growth >1.0 – 1.5 mM $CuSO_4$), R = resistant (Growth >1.5 – 2.0 mM $CuSO_4$)

[2]Pathogenicity on capsicum is ascertained from inoculation of the susceptible cultivar Jupitar

[3]Pathogenicity on tomato is ascertained from inoculation of the susceptible cultivar Grosse lisse

[4]Race identity determined through pathogenicity on differential capsicum resistance lines, where multiple races are listed or the race was not determined (ND) this was due to low seed supply of some host lines thus complete testing was not possible

References:

Roach, R., R. Mann, C. G. Gambley, R. G. Shivas, and B. Rodoni. 2018a. “Identification of Xanthomonas species associated with Bacterial leaf spot of tomato, capsicum and chili crops in Eastern Australia.” European Journal of Plant Pathology 150 (3): 595–608. https://doi.org/10.1007/s10658-017-1303-9.

Roach, R, R. Mann, C. F. Gambley, Chapman, T., R. G. Shivas, and B. Rodoni. 2018b. “Genomic analysis of Australian Xanthomonas species reveals diverse groups associated with Bacterial leaf spot in tomato, capsicum and chili crops.” BMC Genomics submitted.

Roach, R, R. Mann, C.F Gambley, R.J. Shivas, Chapman, T., and B. Rodoni. 2018c “Pathogenicity and copper tolerance in Australian Xanthomonas species associated with Bacterial leaf spot.” Crop Protection In preparation.

# Bacterial leaf spot of tomato and pepper

## What is bacterial leaf spot?

Bacterial leaf spot (BLS) is caused by several species of *Xanthomonas*: *X. vesicatoria, X. euvesicatoria, X. perforans* and *X. gardneri.* In Australia, three of these species are known to occur. *X. euvesicatoria* infects mainly capsicum and chilli, while *X. vesicatoria* and *X. perforans* primarily infect tomato. The fourth species, *X. gardneri* was not detected in recent surveys of Australian crops and is thus not known to occur here. Yield loss is generally a result of decreased photosynthetic area, though in severe infections direct fruit damage is also seen.

## What does it look like?

BLS symptoms may occur at any point during plant development. Small (>1mm) tan to black lesions grow to about 2-3 mm in diameter, often with an angular appearance. These spots may coalesce to form large necrotic sections of leaf. Spot may appear on leaves, stems, and calyx. Fruit spots are less common but present in severe infections, and may appear in a variety of sizes. Severe infections can result in entire plant death and can move through a field rapidly. Wilt and rot symptoms are not associated with this disease.

***Angular dark spots on leaves, stem and calyx. Yellow halo around the spot may be suggestive of bacterial disease is not definitive, diagnostic testing is required for confirmation.***

## What can it be confused with?

BLS presents as a leaf spot, which can be easily confused with other bacterial or fungal spots. In particular, bacterial speck lesions (*Pseudomonas syringae* pv. *tomato*) and fungal spots (e.g target spot caused by *Alternaria solani*) on tomato can appear almost identical to BLS. Speck is generally characterised by smaller spots that give the leaf a 'peppered' appearance. Fungal spots are often larger and less angular. Lesions of these diseases may also be present on stems and fruit, though fruit symptoms are distinctly different from the 'bird's eye' spots typical of tomato canker caused by *Clavibacter michagenesis* pv. *michagenesis*. The only way to diagnose BLS with confidence is to get a laboratory identification.

## How does it spread?

BLS development favours warm, humid conditions. The bacteria are spread primarily by water and wind, and persist in the field in volunteer plants and crop residue. BLS species are seed transmissible, but do not survive long in the soil. Physical damage to plants, particularly in association with free water also exacerbates bacterial infection and spread. For example, wind-driven rain or hail.

## Where does this disease occur?

BLS has been reported worldwide since its initial description in 1921. Since the pathogen was reclassified from *X. campestris* pv. *vesicatoria* to four species in 2004, reports of the new species have increased worldwide. Three of these species were recently confirmed in Australia from research co-funded by government and industry (Hort Innovation Project VN14010) and in collaboration with La Trobe University. In addition to these species, two specific races of *X. euvesicatoria*, races 1 and 7, were also confirmed affecting Australian crops. There are up to ten different races of this pathogen reported overseas, some of which can overcome most of the plant resistance genes currently available in Australia.

## How can I protect my farm from bacterial leaf spot?

In Australia, the recommended management strategy for control of BLS in tomato, capsicum and chilli is the use of copper bactericides in combination with a manganese-zinc ethylene bisdithiocarbamate (EBDC) fungicide such as mancozeb. This has provided consistently better control than copper-based products alone in field studies of a range of bacterial diseases, including bacterial spot and speck. This is attributed to the ability of the bisdithiocarbamate anion to chelate copper and transport the $Cu^{2+}$ into the bacteria. Many populations of the BLS-causing bacteria are known, or expected to have, high levels of copper tolerance. The availability of $Cu^{2+}$ on plant surfaces is a major factor affecting efficacy of control of BLS.

Alternative, non-copper products for control of bacterial pathogens such as those causing BLS are under development by major chemical companies. Please check the Australian Pesticides and Veterinary Medicines Authority (APVMA) for a list of currently registered products for use in your crop.

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## Appendix 6: Project completion article: VG14010 'Management and detection of bacterial leaf spot in capsicum and chili crops'

The project VG14010 'Management and detection of bacterial leaf spot in capsicum and chili crops' was completed in May 2018 and has increased the capacity of the vegetable industry to develop integrated disease management programs for bacterial leaf spot (BLS) of capsicum and chili field crops. As tomato was considered an alternative host for the BLS pathogens, investigation of the causal agents of the disease in tomato was also included in the study. This project was also aligned to the Plant Biosecurity Cooperative Research Centre (PB CRC) Project PBCRC2002 (New approaches for diagnosing bacterial pathovars). The work in this Hort Innovation project supported and extended existing research within the PB CRC program by providing complementary research. The PhD student, Ms Rebecca Roach, was co-supervised by Latrobe University, the Department of Environment and Primary Industries, Victoria and the Department of Agriculture and Fisheries.

Previously it was assumed *X. campestris* pv. *vesicatoria* (now known as *X. vesicatoria*), was causing BLS in all solanceous crops in Australia. The results of this project clearly indicate this is not the case, instead there are four different *Xanthomonas* species associated with the disease. Furthermore, the species are largely host specific, where *X. euvesicatoria* essentially infects capsicum, chili and tomato, and *X. vesicatoria* and *X. perforans* only tomato. The fourth species, *X. arboricola*, was found in association with BLS symptoms and able to weakly infect tomato only. Given this weak pathogenicity, its ability to cause BLS disease is questionable and as such the bacterium is unlikely to need control. Furthermore, race-typing of *X. euvesicatoria* isolates identified races 1 and 7 in Australia. There are currently several commercial capsicum lines in Australia with resistance to race 1. There are very few, if any at all, readily available with resistance to race 7. Importantly, the diversity of *X. euvesicatoria* races and other Xanthomonads detected in Australia is very low, however, as there is no regulation for these bacterial pathogens it is likely new races and species could be introduced with seed.

Copper tolerance testing of Australian isolates revealed all were tolerant to highly tolerant. Although the minimum amount of copper used in the tests is well below the amount of copper routinely applied in the field, disease control is ineffective. This highlights that copper tolerance in bacterial populations is not the complete answer as to why copper is ineffective for disease control. Copper may still have some role in management of BLS, however, alternative methods are needed to address the ineffectiveness of using copper alone. To this end, essential oils showed promise as preliminary testing indicated they have a strong antibacterial effect against *X. vesicatoria* and *X. euvesicatoria*, both as a volatile gas and through direct contact.

The literature review on survival of BLS pathogens between cropping cycles highlighted the importance of using disease free planting material. Survival of the bacterial pathogens in the environment is quite low and disease outbreaks are more likely initiated from primary introduction of the pathogen each season rather than transfer of the bacterium from sources within the environment.

Ongoing international trade in seed, together with the high seed-transmissibility of xanthomonads, increases the risk of co-importation of new races or species into Australia. This means existing resistance genes managing disease in Australia may become unreliable in the future. Additionally, overuse of a single management strategy such as a resistance gene or a single chemical can provide strong evolutionary pressure on the bacterial pathogen, leading to mutation and the local emergence of resistance-breaking races or chemically tolerant populations.

The major recommendations for the management of BLS are:

- Consult with your seed supplier about assurances on the health status of the seed or alternatively,
- Heat treatment of seed to prevent primary introduction of pathogens into crops and to mitigate the risk of

introduction of new bacterial races which may circumvent existing plant host resistance genes. This treatment will also mitigate risk of other potential exotic and endemic pathogen threats
- Use of resistant capsicum lines where possible
- Combining copper with manganese-zinc ethylene bisdithiocarbamate (EBDC) (e.g mancozeb) early in the crop cycle to improve availability of bactericidal forms of copper and thus early infections, later applications are not practical due to with-holding periods of the EBDC

Further research activities were also identified by completion of the project and these include:

- Development of a fast diagnostic test to race-type bacterial pathogens. This will allow informed varietal selection for growers
- Trialing capsicum varieties carrying the resistance genes Xcv 1-5 and 7-9 for suitability to Australian conditions
- Further evaluation of essential oils through investigation of spray application methods and timings
- Continued investigation of other alternatives to copper
- Further investigations of seed treatment strategies.

The newly contracted Hort Innovation project VG16086 ‘Area wide management of vegetable diseases: viruses and bacteria’ (nationally focused project, led by DAF) will follow up on these future research activities.