Hort Innovation

Final Report

Detection and Management of Bacterial Diseases in Australian Allium Crops

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Detection and Management of Bacterial Diseases in Australian Allium Crops – VN13005

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Content

Summary

In 2011, shallot and onion growers from the Lockyer production area reported a new disease which was subsequently identified as Bacterial blight of leek, caused by the bacterial pathogen *Pseudomonas syringae* pv. *porri* (Psp). Economic losses were incurred by shallot growers in the Lockyer valley due to development of the yellow leaf symptom, rendering their product downgraded or unmarketable. The impact to red onions during the outbreak was also reported by growers as concerning. Bacterial blight of leek occurred previously in Australia in leek crops and nurseries. It was detected in leek survey samples from SA, Victoria, and WA collected from 2000 to 2003 (Hall et al. 2007; Noble et al. 2006). Results from this project show differences in the bacterial pathogen detected in the early 2000s to that detected from the Lockyer valley in 2011. This strongly indicates there were two separate incursions of Psp into Australia, most likely through contaminated seed. In leek, the bacterium is present externally on the seed and seed was identified as a primary introduction pathway for Psp into California (Koike, et al 1999).

This project has generated new information about the introduction, spread and survival of Psp. Of note was the lack of detection of the bacterium during the project surveys. The lack of detection in the Lockyer valley is either due to lack of survival of this pathogen in the district since the 2011-2012 outbreak or it is below detection limits due to unfavourable environmental conditions for infection and disease development. Experimental evidence shows a strong influence of weather on Psp and disease symptom development and support the observed field data from the 2011-2012 outbreak. Cool, wet conditions promote infection, disease development and production of the coronatine phytotoxin. Evidence also indicates if conditions are dry and warm, plants will recover from infections, the toxin and disease symptoms. This recovery phenomenon has not previously been reported for the coronatine phytotoxin.

The results from this study also indicate multiple introductions of Psp into Australia and diversity in pathogenicity within the bacterial species. Given Psp is spread in seed; there is continued risk of further introductions of the pathogen into onion growing districts. The differences in pathogenicity is also of concern as there could be more aggressive isolates of Psp present elsewhere in the world and thus exotic strains of Psp should be listed in the Onion Industry Biosecurity Plan.

The management of Psp is complicated through lack of availability of suitable bactericides. Results from this project show that copper is likely to be effective. The bacterial isolates in the study were shown to be sensitive to copper in culture, however, more work is needed to determine the appropriate spray regimes and formulations for field control to be successful. Alternative products such as clove oil were also identified has having potential as bactericides, although this research is still in its infancy. Management using commercial varieties is unlikely to be effective as no tolerance/resistance was observed from the eleven varieties tested. Mechanical wounding through equipment or thrips feeding will exacerbate disease under conducive weather conditions. To minimize these impacts appropriate control of thrips and consideration of irrigation regimes to minimize leaf wetness are highly recommended.

A fact sheet for Xanthomonas blight of onion, caused by *Xanthomonas axonopodis* pv. *allii* was developed and subsequently endorsed by Plant Health Australia (http://www.planthealthaustralia.com.au/industries/onions/). The factsheet is also available on the Hort Innovation website (http://horticulture.com.au/wp-content/uploads/2016/11/Bacterial-onion-blight-FS.pdf).

Keywords

Pseudomonas syringae pv. porri, Xanthomonas axonopodis pv. allii, onion, bacterial blight of leek, bacterial blight of onion, coronatine toxin, copper

Introduction

In 2011, shallot and onion growers from the Lockyer production area reported a new disease which was subsequently identified as Bacterial blight of leek, caused by the bacterial pathogen *Pseudomonas syringae* pv. *porri* (Psp), and formally identified a year later (Roach, et al. 2016). The disease was also reported to affect onion in Georgia, USA in 2012 (Gitaitis et al. 2012). Prior to these outbreaks this pathogen was not known to cause disease in onions. In the Netherlands Psp is reported to cause up to 50% yield loss (van Overbeek et al 2010). Economic losses were incurred by shallot growers in the Lockyer valley due to development of the yellow leaf symptom, rendering their product downgraded or unmarketable. The impact to red onions during the outbreak was also reported by growers as concerning.

Bacterial blight of leek occurred previously in Australia in leek crops and nurseries. It was detected in leek survey samples from SA, Victoria, and WA collected from 2000 to 2003 (Hall et al. 2007; Noble et al. 2006). There were no reports of field outbreaks in other allium species. Laboratory tests confirmed the onion cultivars Cream Gold and Hunter River White, spring onion, shallot and garlic were susceptible to the bacterium (Noble et al. 2006). In overseas research, the pathogen is reported to be seed borne in leek; however, seed transmission was not investigated for other important allium species. In leek, the bacterium is present externally on the seed and seed was identified as a primary introduction pathway for Psp into California (Koike, et al 1999). It is highly likely Psp was introduced into QLD as a separate incursion in contaminated onion or shallot seed. Further characterisation of the bacterial isolates from QLD and those from previous outbreaks were needed to clarify if multiple introductions occurred and if there are differences in virulence between isolates from the different outbreaks. A possible link with onion thrips feeding damage and establishment of Psp was also suspected. This is from preliminary field observations in the Lockyer and is also suggested to occur in leek crops overseas (van Overbeek et al. 2010).

The distinctive 'yellow leaf' symptom associated with bacterial blight of leek is thought a response to a phytotoxin produced by the bacterium and is not fully understood. This symptom was present in the Lockyer valley outbreak and also observed to disappear as weather patterns changed. Braun et al. (2008) describe several *P. syringae* pathovars that produce the bacterial phytotoxin coronatine (COR), which is non-host specific and proposed to contribute to disease establishment and symptom development. Although Psp is listed as producing COR there is no direct evidence of this, only similarity of symptoms. The ability of Psp to produce COR and confirmation that this phytotoxin is responsible for the yellowing symptom observed in onion and shallot required confirmation. Once clarified, potential mechanisms for induction of the phytotoxin can be studied which may then influence disease management options. Examples of potential inducers of toxin production include bacterial titre and/or cold conditions.

Methodology

Epidemiology – Understanding *Pseudomonas syringae* pv. *porri* (Psp) distribution within Australia, its introduction and spread into and between production crops, and its survival between cropping periods

Surveys: Surveys were completed in at least five representative production blocks from each growing district during 2014 and 2015. Onion plants were inspected for symptoms of bacterial infection by walking a transect through a large section of the block. Additional plants near irrigation heads or other areas where water may remain or be applied with force were also inspected where possible. Samples of outer leaves with necrotic lesions were collected from at least 30 symptomatic plants per survey site. Alternatively, if the characteristic 'yellowing' leaf symptom was absent, samples were randomly collected from plants with necrotic outer leaves. Samples were evaluated in the laboratory for evidence of bacterial ooze and bacterial isolations attempted from representative samples from each block. Surveys were conducted in each onion growing state. In addition to evaluating isolated bacterial cultures, PCR was used to screen lesion exudates from some survey samples for Psp.

Surveys of onion bulb crops in NSW included the Cream Gold variety in the Murrumbidgee irrigation area and the varieties Lombardi, Jacinta, Rio Red Rock, Red Emperor and Cream Gold in the Griffith area of NSW. Surveys in Victoria were done on bulb onion, spring onion and one leek crop in the Sunraysia district. In SA, bulb onion crops in the Murray Mallee district were surveyed as were production areas in the Lockyer and Fassifern valleys in QLD. Surveys were also conducted in Tasmania.

Environmental conditions for disease development: To investigate possible links between temperature and Psp-induced disease, weather data was obtained from the Bureau of Meteorology weather stations in the Lockyer valley. Studies of the phytotoxin coronatine by *P. syringae* pathovars and strains indicate a temperature of 18 °C for 5-7 days is important for its production (Ullrich et al. 1993; Rangaswamy et al. 1997; Budde et al. 1998; Rohde, et al. 1998). This temperature parameter was used to evaluate the weather data around the time period where the yellow leaf symptom, presumably induced by coronatine production, was observed in field plantings (2011 - 2012).

Additionally, a pot trial was conducted in a temperature controlled growth cabinet at Applethorpe Research Facility, with a 12 h day-night regime to attempt to replicate the yellow-leaf symptom associated with the phytotoxin. Plants were inoculated with either B1039 or B1040 isolate and incubated 18-24 °C for one week followed by incubation at 16-18 °C. Following development of the yellow-leaf symptom incubation conditions were then altered to 12 h each of 18 and 24 °C to attempt to induce recovery from the phytotoxin. A second trial was conducted using a mix of Psp onion isolates (B1038, 39, 40, 41, 42 and 46) and natural incubation temperatures in a glasshouse through winter which were recorded using a Tinytag monitor (Hastings data loggers). The plants were monitored for a four-month period.

Pathogen diversity: To evaluate the potential diversity of strains in Psp, a range of isolates from the Lockyer Valley outbreak were compared to each other and to those obtained from the previous incursion of Psp into southern Australian states. The isolates were compared morphologically, biochemically, molecularly and pathogenically.

Thrips feeding and disease: Thrips feeding was evaluated to determine if it increases incidence of bacterial infection or subsequent disease development in onion. A colony of *Thrips tabaci* (onion thrips) was established and maintained on onion plants. The effect of feeding by *T. tabaci* was evaluated using whole plants grown in thrips proof cages inside the laboratory under grow lights. Onion plants were exposed to the five following treatments:

1. Psp + thrips
2. Psp + wounding (sterile needle or carborundum)
3. Psp only (no wounding)
4. Thrips only

5. Control - no Psp or thrips

Psp inoculum was prepared by resuspending bacteria at a concentration of approximately 10^7 CFU/ml. The inoculum was sprayed onto the onion plants using a small spray bottle as per the above treatments. The plants were kept in thrips proof cages and misted periodically for 2 days to keep humidity high to promote disease development. The final assessment of plants was approximately one-month later. Suspect lesions were tested for bacterial ooze and positive lesions stored in sterile distilled water for PCR testing.

Molecular detection: Specific molecular assays for the detection of Psp were not available. Instead, identification of isolates was confirmed using PCR amplification and subsequent sequencing of bacterial DNA genome fragments. This included fragments of the coronofacate ligase (CFL) and 16SrRNA genes, amplified with assays described by Bereswill et al. (1994) and Gitaitis et al 2012, respectively. Additionally, a selection of other bacterial isolates obtained from survey samples were analysed by 16SrRNA gene sequencing to provide presumptive identification. In particular, cultures with similarity to Xanthomonads were evaluated to check for presence of the exotic species, *X. axonopodis* pv. *allii*.

Management options for control of Psp

Literature review: A literature review was completed on research of Psp. This highlighted an absence of management options for onion crops and the importance of weather conditions in the detection and management of bacterial diseases.

Copper tolerance testing: A new assay was developed to evaluate copper tolerance *in vitro*. The assay uses a microtitre plate system to quantitate bacterial growth after exposure to copper at an absorbance wavelength of OD_{620nm} (where an OD of 0.3 is *ca.* 10^8 CFU/ml). Preliminary experiments evaluated incubation and exposure times to ensure bacteria were given sufficient time to either succumb to the toxicity of the copper or to express genes to protect themselves from it. The final method is described in Appendix 1. Six isolates of Psp from onion and a single isolate from leek were tested. Results from the *in vitro* testing indicated the isolates of Psp were copper sensitive.

To evaluate copper to control Psp *in vivo*, two pot trials were completed using isolates B1046 and DAR75554. The first used prick inoculation of the test plants with the bacterial inoculum and the second used mist inoculation. Inoculum preparation, prick inoculation and symptom ratings were done as per the pathogenicity pot trials (Appendix 2). For mist inoculation, the bacterial suspension was sprayed onto the middle third of the onion plants until obviously wet using a disposable spray bottle with a fine aperture. For the first trial, three replicate pots of *ca*. 10 plants of both Rio Red Rock and Lockyer white onion varieties were inoculated for each test treatment. These were copper sulphate at 190 g/L (equivalent to the copper sulphate concentration in Tri-Base blue; 0.73 M $Cu_2H_4O_6S$), DuPont™ Kocide® Blue and Nufarm Tri-Base Blue Flowable Copper at label rates (1.5 g/L and 2.8 ml/L, respectively) and agral used as a wetting agent at 0.1 ml/L. For the second trial, only Kocide blue and Tri-base blue were evaluated. In this trial three replicate pots were inoculated for the Rio Red Rock and two replicate pots for Lockyer white onion varieties. The severity index was used in statistical analyses (ANOVA and Fisher's protected LSD method, $\alpha = 0.05$) to determine if there were any significant differences in disease control between the treatments.

Varietal testing: There is no specific breeding program in onion for resistance or tolerance to Psp. To determine if there are differences in susceptibility and/or tolerance within current commercial onion lines seedlings of selected white, brown and red commercial varieties, 11 in total, were inoculated with Psp and evaluated for disease development. The varieties included short and intermediate day-length onions for each type. In a preliminary experiment, a range of Psp isolates were evaluated for differences in pathogenicity. From this, the most pathogenic isolates, Psp46-onion (B1046) and Psp-leek (DAR75554), were chosen to screen the different onion varieties. The host range of Psp affecting leek and spring onion is known thus these species were not included in this work. General methodology for these trials was as per the pathogenicity trial detailed in Appendix 2.

Essential oils: To determine their effectiveness to control Psp, essential oils from a number of plant species were selected for testing based on having antimicrobial activity in previous *in vitro* assays to a range of phytobacteria including other pathovars of *P. syringae* or containing compounds previously shown to be efficacious (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, E.O, et al. 2014). The oils were assessed *in vitro* using filter disks impregnated with the compound to evaluate growth inhibition on culture plates, using treated filter paper applied to the inside of the culture plate lid to evaluate inhibition of growth from volatiles of the compound and using direct exposure to the oil followed by viability assessment in microtitre plate culture. From these experiments, clove oil was chosen for a small pot trial evaluation using prick inoculation. Plants were sprayed with treatment solutions to run-off and then prick inoculated 24 h later with bacterial suspensions (B1046 and DAR75554). The treatment solutions all included 0.5% DMSO and 0.1% Tw-20 prepared in water and were:

1. Clove oil at 0.2% (v/v) in
2. Mineral oil at 0.2 % (v/v)
3. No oil

The severity index was used in statistical analyses (ANOVA and Fisher's protected LSD method, $\alpha = 0.05$) to determine if there were any significant differences in disease control between the treatments. General methodology for the pot trial was as per the pathogenicity trial detailed in Appendix 1.

Increased preparedness for phytobacterial biosecurity threats

A review of the biosecurity threat list was completed for exotic bacterial pathogens and a factsheet developed for the key threat. The factsheet was subsequently endorsed and published by Plant Health Australia (PHA).

Communication and extension

The complete project results were extended to industry, Hort Innovation and DAF primarily through project milestone and final reports. Key findings of the research were extended through publication of a factsheet, industry newsletter articles and presentations to grower groups. Additionally, growers were provided with information about Psp and other bacterial diseases through informal conversations during disease surveys.

Outputs

The following outputs were achieved in this project:

1. Documentation of knowledge of survival of *Pseudomonas syringae* pv. *porri* within and between crop cycles in the final report and during industry workshop presentations
2. Evaluation of control methods for reducing the impact of *P. syringae* pv. *porri* on onion crops and risk assessment of cultural practices which may contribute to disease development and/or spread presented in the final report and discussed during industry workshop presentations
3. Grower focussed disease fact sheet and industry website page for the exotic bacteria *Xanthomonas axonopodis* pv. *allii* delivered to and published by PHA, Hort Innovation and Onions Australia (http://www.planthealthaustralia.com.au/pests/bacterial-onion-blight/; http://horticulture.com.au/wp-content/uploads/2016/11/Bacterial-onion-blight-FS.pdf)
4. A minimum of two (2) articles on bacterial diseases of onion for industry newsletter (refer appendices 3-5)

Information sessions were provided in growing districts on disease identification and management options through coordination with Onions Australia and local growers. A presentation was given to local growers in Gatton in 2014, at the Onion Australia Annual General Meeting held in Brisbane on the 15th of October, 2015 and to the Fassifern valley, QLD, growers in September, 2016.

Outcomes

The following outcomes were achieved in this project:

1. An improved understanding of the introduction, spread and survival of bacterial pathogens by project completion

Pseudomonas syringae pv. *porri* (Psp) was not detected during project disease surveys nor were diagnostic samples submitted during this time from any growing regions. Evidence from pathogen diversity studies indicate separate incursions of Psp into Australia and that spread from the leek outbreak in the early 2000s was very unlikely.

The lack of detection of Psp in the Lockyer valley is either due to lack of survival of this pathogen in the district since the 2011-2012 outbreak or it is below detection limits due to unfavourable environmental conditions for infection and disease development. Experiments during this project and field data collected in 2012, indicate a strong influence of temperature on disease symptoms caused by Psp. Warm, dry conditions are not favourable for Psp infection and disease symptom development. Conversely, the cool, wet conditions experienced during the 2011-2012 seasons were very favourable.

Further investigation of Psp disease development found the amount of bacteria used to infect plants affects the level of disease severity but not necessarily disease incidence. In particular, higher bacterial concentrations enhance development of the yellow leaf symptom. Of comfort to growers is the consistent recovery of plants from disease symptoms. This occurs as the temperature warms and/or humidity and free water decreases. If the outer infected leaves senesce under such conditions, pathogen infection of newer leaves is less likely and plants can recover from infection.

Diversity was observed between isolates of Psp collected in the early 2000s from southern Australian disease outbreaks in leek crops and those from the 2011-2012 outbreak in onion and shallot crops from the Lockyer valley, QLD. Differences were observed in morphology, toxin production and aggressiveness on onion. A Psp isolate from leek was more aggressive on onion varieties, inducing more severe leaf lesions, than a Psp isolate derived originally from onion. By contrast, the Psp onion isolate was able to induce a characteristic coronatine phytotoxic affect in onion under cool conditions whereas the leek isolate was not. The reaction of the onion isolate on leek was not tested.

2. Identification of control methods to manage bacterial diseases for the Australian Onion Industry by project completion

Investigation of management strategies for control of Psp has assisted the industry in preparedness if outbreaks occur again. No outbreaks of the disease caused by Psp were reported since the 2012 Lockyer growing season. As weather conditions were not favourable for bacterial infection and disease development since 2012, it is difficult to know if the pathogen is still present. The disease may re-emerge during seasons with prolonged cool wet conditions, however, the risk for most seasons in most growing districts is considered quite low. The experiments described in this report have assisted in identifying products of potential use if disease outbreaks occur in the future. The absence of copper tolerance within Psp populations provides confidence that existing bactericides could manage future outbreaks..

There are no products registered for the control of bacteria in onion crops listed in the Australian Pesticides and Veterinary Medicines Authority (APVMA) database. However, there are 42 products containing copper that are registered for use in onion crops. The registered use of some of these products could be expanded to include control of bacterial diseases. Although laboratory experiments identified Australian isolates of Psp are susceptible to copper, preliminary pot trials did not successfully control the bacterium. This indicates inactivation of the copper ions when applied to onion plants and thus further work is required to look at different application methods, copper formulations

and potential to combine with other compounds such as mancozeb to improve efficacy.

Alternatively, a range of non-copper products could prove efficacious in controlling Psp. Based on other studies of *Pseudomonas syringae* and other phytobacteria, a range of essential oils were tested in vitro for inhibition of Psp. Of these, clove oil was shown to have good contact bactericidal activity in broth cultures. Although the oil did not control Psp infection in pot trials, this was limited to a single experiment. Further optimization of product application would be useful to determine the potential of clove and other essential oils to control bacterial pathogens.

A range of different commercial red, brown, and white onion varieties were trialled for tolerance to Psp. No varieties were resistant to the bacterial species and all varieties evaluated showed similar susceptibility to the known susceptible control.

Results from this project indicate thrips feeding and other mechanical wounding can increase risk of bacterial infections. Increased infection rates occur where wounding sites are co-located on the onion leaves with the bacterium. In the field, cool wet weather or overhead irrigation will disseminate bacteria over the surface of the onion plants and increase the likelihood of bacterial presence at wound sites. Thrips feeding damage in absence of free-water is unlikely to exacerbate the bacterial disease. Appropriate control of thrips within onion crops and consideration of irrigation regimes to minimize leaf wetness is highly recommended.

3. Enhanced preparedness of the industry to incursions of important exotic bacteria by project completion

A fact sheet for Xanthomonas blight of onion, caused by *Xanthomonas axonopodis* pv. *allii* was developed and endorsed by Plant Health Australia. This disease is a key exotic threat for the Australian Onion Industry. It is the only high risk bacterium listed in the industry biosecurity plan to date (http://www.planthealthaustralia.com.au/industries/onions/). Presentations at industry forums on this disease during the project has also increased preparedness through improved awareness of the disease, in particular, how to distinguish it from endemic diseases which will assist with early detection if it is introduced.

The results from this study also indicate multiple introductions of Psp into Australia and diversity in pathogenicity within the bacterial species. Given Psp is spread in seed; there is continued risk of further introductions of the pathogen into onion growing districts. The differences in pathogenicity is also of concern as there could be more aggressive isolates of Psp present elsewhere in the world and thus exotic strains of Psp should be listed in the Onion Industry Biosecurity Plan.

Evaluation and discussion

Epidemiology – Understanding *Pseudomonas syringae* pv. *porri* distribution within Australia, its introduction and spread into and between production crops, and its survival between cropping periods

Surveys: No signs of infection by *Pseudomonas syringae* pv. *porri* were detected from any of the surveys. Other bacterial diseases were observed in some crops and included Pseudomonad-like isolates from Tasmania, *Pseudomonas viridiflava* and *Pseudomonas marginalis* and two *Pantoea* species, *P. ananatis* and *P. agglomerans* from QLD. This indicates the presence of other bacterial diseases affecting onion crops, which may or may not have yield impacts. No evidence of the exotic *Xanthomonas axonopodis* pv. *allii* (Xaa) was detected in any of the surveys.

Environmental conditions for disease development: Coronatine is produced by a range of *Pseudomonas* species and *P. syringae* pathovars (Mitchell 1982). Its biosynthesis pathway has been studied in other *P. syringae* pathovars (Cuppels and Ainsworth 1995; Mitchell, et al. 1994; Ullrich et al. 1993), although very little work on toxin production has been done specifically in Psp. It has been shown that levels of coronatine production significantly impact pathogenicity of some *P. syringae* pathovars, though not all (Bereswill et al. 1994). Multiple studies on the temporal expression of the coronatine gene indicate cool temperatures are important for toxin production. Expression of the gene was high at 18 °C and virtually non-existent at 28 °C (Budde et al. 1998; Ullrich et al. 1993; Rangaswamy et al. 1997; Rohde, et al. 1998). Production of this toxin is confirmed for the Australian isolates of Psp collected from the Lockyer valley outbreak.

Pot trials specifically aimed at inducing the toxin demonstrated development of the 'yellow-leaf' symptom suspected to be associated with the toxin were completed. Bacterial isolates were also confirmed to have the coronatine biosynthesis gene through molecular analyses. Other pot trials, aimed at evaluating management products and conducted under cool conditions, also generated the yellow-leaf symptom from plants infected with a Psp-onion isolate (B1046). Interestingly, the Psp-leek isolate (DAR75554), despite having the gene and being in general more pathogenic than the Psp-onion isolate, did not induce this symptom in the same pot trials. This indicates there is host-specificity in production and/or reaction to the coronatine toxin. Historical weather data from the Lockyer Valley also links the occurrence of the yellow-leaf symptom observed during epidemiology studies with cool conditions. Data from field studies and pot trials also indicates that the toxin effect is reversed with increasing temperature. This toxin reversal phenomenon is not documented in the literature and requires publication. A summary of trials and results is provided in Appendix 6.

Pathogen diversity: The isolates of Psp obtained from the Lockyer onion outbreak were previously identified (Roach et al 2016). These isolates show some morphological and pathogenicity differences from the Australian leek isolates. The onion isolates are slower to grow and induce fluorescence on KB media, are less pathogenic on onion in relation to development of leaf lesions but more pathogenic in relation to toxin production than the leek isolates. All six Psp-onion isolates tested were less pathogenic than Psp-leek isolate DAR75554. Psp-onion isolate B1046 was equally pathogenic as two further Psp-leek isolates DAR61456 and DAR75283. Noble et al. (2006) reported diversity in the 16S rDNA gene of the Psp isolates detected in Australian leek crops based on restriction digestion profiles of PCR amplified gene fragments. In that study, an isolate from onion imported from Japan gave a unique profile compared to the leek isolates. It is likely the Australian Psp-onion isolates will also vary genetically from the leek isolates; however, this requires experimental confirmation.

The differences in pathogenicity and morphology support the notion there were at least two separate incursions of Psp into Australia. It is extremely unlikely Psp was transferred from southern states to QLD, more likely it was introduced into QLD through contaminated onion or shallot seed.

Thrips feeding and disease: Symptoms of bacterial infection were observed on plants from all three treatments that included Psp exposure (Psp + thrips, Psp + wounding and Psp only) whereas the control plants and those exposed only to thrips did not develop symptoms. Psp infection was confirmed from most samples collected from the symptomatic plants. Thrips feeding damage typically occurred on new and expanding leaves. As leaves matured, the wounds became callused and thus prevented further entry by the bacterial cells. The majority of infection was seen on older leaves where the bacterium was present at the time of insect feeding. Bacterial inoculum was not re-applied and plants were watered from the base, thus only the older leaves were exposed to Psp.

This investigation provides preliminary evidence that thrips feeding and other mechanical wounding can increase risk of bacterial infections. In the field, cool wet weather or overhead irrigation will disseminate bacteria over the surface of the onion plants and increase the likelihood of bacterial presence at wound sites. Thrips feeding damage in absence of free-water is unlikely to exacerbate the bacterial disease. In this experiment there was no direct evidence of thrips acquiring bacterial cells from infection foci and then subsequently transferring it to initiate new infections.

Management options for control of Psp

Copper tolerance testing: Tolerance to copper bactericides is well documented for several plant pathogenic bacteria. It is often assumed that other phytobacteria will also have high copper tolerance. Improved understanding of methodologies through this project and others indicate false positives for copper tolerance are highly likely if the wrong media is used or if there is insufficient incubation time to allow bacteria to either combat or succumb to the copper toxicity. Consequently, during this project a new method to evaluate copper tolerance of bacterial isolates was developed. The assay uses a microtitre plate system, to quantitate bacterial growth after exposure to copper. Preliminary experiments evaluated incubation and exposure times. This was to ensure bacteria were given sufficient time to either succumb to the toxicity of the copper or to express genes to protect themselves from it. Six isolates of Psp from onion and a single isolate from leek all showed similar sensitivity to copper as Pss, the sensitive control (Table 1). None of these isolates grew in the presence of 0.25 mM copper sulphate. By contrast, Pst the known tolerant control, showed a high level of copper tolerance as expected and grew in the presence of up to 1 mM copper sulphate.

In addition to *in vitro* screening of isolates for copper tolerance, selected isolates were tested for the presence of copper tolerance genes by molecular assays. The primers, CoprunF2 and CoprunR1, were designed by Altimira et al. (2012) to amplify copper tolerant bacteria from soil. The primers were designed to target the copA gene from a broad range of bacterial species. This gene was not detected in any of the eight Psp isolates tested by PCR using these primers. The isolates included six derived from onion and two from leek. The gene was detected from the positive control Pst isolate used in the in vitro tests.

Table 1 Growth of bacterial isolates when exposed to different amounts of copper sulphate from 0 to 2.0 mM copper. In addition to the seven test isolates of *P. syringae* pv. *porri* (Psp), a known positive (*P. syringae* pv. *tomato*; Pst) and negative (*P. syringae* pv. *syringae*; Pss) control for copper tolerance were also tested. Bacterial growth was considered positive if the absorbance reading exceeded the threshold value of three multiples of the un-inoculated control (i.e OD_{620nm} = 0.15). Positive growth is indicated in bold font.

	Copper concentration (mM)					
	0	0.25	0.5	1.0	1.5	2.0
Isolate	OD_{620nm} absorbance					
Pst	**0.46**	**0.49**	**0.47**	**0.45**	0.05	0.05
Pss	**0.32**	0.05	0.04	0.04	0.04	0.05
Psp38 -onion	**0.33**	0.05	0.05	0.04	0.04	0.05
Psp39 –onion	**0.33**	0.06	0.06	0.07	0.05	0.05
Psp40 –onion	**0.32**	0.05	0.05	0.05	0.05	0.05
Psp41 –onion	**0.33**	0.04	0.04	0.05	0.05	0.05
Psp42 –onion	**0.34**	0.05	0.04	0.04	0.04	0.05
Psp46 -onion	**0.34**	0.05	0.07	0.05	0.05	0.05
Psp-leek	**0.18**	0.04	0.07	0.05	0.05	0.05
control – no bacteria	0.04	0.04	0.04	0.05	0.04	0.05

The results from the copper tolerance testing provided good evidence that tolerance has not yet evolved in Australian Psp populations and thus copper bactericides are likely to be a useful management tool in future outbreaks. Further testing of copper to control Psp was done through pot trials.

To evaluate copper to control Psp *in vivo*, two pot trials were completed. Both tested treatments against Psp-onion (B1046) and Psp-leek (DAR75554) isolates. The first used prick inoculation of the test plants with the bacterial inoculum and the second used mist inoculation.

There was no statistically significant reduction in disease development in either pot trial using copper using Fisher's protected LSD method (P = 0.05). In the first trial, Tri-base blue reduced severity of disease symptoms induced by the Psp-onion isolate on both onion varieties compared to the non-treated control, but this was less evident in the second trial (Table 2). Similarly, kocide was variable in control of Psp-onion between the different varieties and trials. By comparison, the Psp-leek isolate was not controlled at all with most inoculated plants recording a disease severity rating of 3 and all treatments an overall severity of high. These results are in contrast to what was expected from the *in vitro* tolerance testing.

Table 2 Results from in vivo testing of commercial copper products for control of *Pseudomonas syringae* pv. *porri* (Psp). Details include information about varieties used, total number of plants tested for each treatment, bacterial isolate and variety combination and the percent of plants at each disease severity rating. All control plants inoculated with water were rated as 0 and thus not included in the table. Trial 1 was prick inoculated with Psp and trial 2 was mist inoculated.

Trial	Psp-isolate	Variety	Treatment	Plants tested	Disease severity rating (% of plants)[1]				Overall severity rating[2]
					0	1	2	3	
1	onion (B1046)	Rio Red Rocks	Kocide	18	5.56	22.22	22.22	50.00	high
			Tri-base blue	19	0.00	68.42	26.32	5.26	low
			No copper	35	8.57	25.71	45.71	20.00	moderate
		Lockyer white	Kocide	22	0.00	54.55	9.09	36.36	low
			Tri-base blue	27	0.00	48.15	22.22	29.63	low
			No copper	38	0.00	2.63	36.84	60.53	high
	leek (DAR75554)	Rio Red Rocks	Kocide	22	9.09	13.64	0.00	77.27	high
			Tri-base blue	23	0.00	4.35	4.35	69.57	high
			No copper	48	6.25	0.00	27.08	66.67	high
		Lockyer white	Kocide	31	0.00	19.35	3.23	77.42	high
			Tri-base blue	22	0.00	4.55	13.64	81.82	high
			No copper	38	0.00	21.05	26.32	52.63	high
2	onion (B1046)	Rio Red Rocks	Kocide	33	6.06	30.30	45.45	18.18	moderate
			Tri-base blue	32	0.00	21.88	40.63	37.50	high
			No copper	20	0.00	25.00	35.00	40.00	high
		Lockyer white	Kocide	28	7.14	53.57	25.00	14.29	low
			Tri-base blue	23	0.00	30.43	39.13	30.43	moderate
			No copper	27	0.00	25.93	59.26	14.81	moderate
	leek (DAR75554)	Rio Red Rocks	Kocide	37	0.00	10.81	18.92	70.27	high
			Tri-base blue	18	0.00	5.56	0.00	94.44	high

			No copper	21	0.00	0.00	14.29	85.71	high

[1] Disease severity rated as : 0 = wound repair only, 1 = local infection at wound site, 2 = extension of infection 1-2 cm from wound, 3 = extension of infection > 2cm from wound and 4 = complete leaf collapse from infection. This is an average rating across three replicate pots. [2] Overall severity rating was determined by evaluating the spread of disease severities and is coded as: low where >50% of plants had a severity rating of 1 or 2, moderate if >50% of plants had a severity rating of 2 or 3 and high if >50% of plants had a severity rating of 3 or greater.

The *in vivo* trials were limited to a single application of copper, 4 h prior to inoculation. Additional copper applications and/or concentrations may provide benefit; however, the concentration of copper used in the pot trials was greatly in excess of that showing efficacy *in vitro*. This indicates application methodology of copper or copper formulations affect availability of Cu^{2+} ions which are the efficacious form of copper for bacterial control. Further work is needed to understand copper products and application methodologies in relation to bacterial control. There is no evidence of copper tolerance by the Psp isolates from *in vitro* testing and genome analyses thus use of copper for management is not being limited by genetic resistance/tolerance by the bacterium.

Varietal testing: Genetic resistance or tolerance to bacterial pathogens is traditionally a very effective method for mitigating disease impacts. There is no specific breeding program in onion for resistance or tolerance to Psp, presumably, as there are only three formal reports of this pathogen affecting onion crops worldwide. To determine if there are differences in susceptibility and/or tolerance within current commercial lines, seedlings of selected white, brown and red commercial varieties, 11 in total, were inoculated with Psp and evaluated for disease development.

None of the onion varieties tested were resistant to infection by either Psp isolate. Most lines reacted similarly to the known susceptible variety or had more severe reactions. The exception to this was two intermediate day-length brown varieties which displayed lower susceptibility to the Psp-leek isolate than the control. Bacterial infections were confirmed as Psp by molecular testing. With the exception of variety 10, all varieties reacted more severely to the leek isolate of Psp, than the onion isolate, indicating variability in strain severity within this pathovar of bacteria.

Essential oils: Previous work indicates that components of essential oils, in particular terpenes, were identified as having potential antibacterial affects. The two monoterpenes, geraniol and citronellol were inhibitory to *Pseudomonas syringae* pv. *actinidiae* and *Erwinia amylovora*, however, the phytotoxic effects of these compounds is yet to be determined. Geraniol is present in palmarosa, geranium and rose oils whereas citronellol is present in oils from *Boronia citriodora* and *Eucalyptus citridora*. To determine their effectiveness to control Psp, essential oils from a number of plant species were tested by *in vitro* assays and additionally by pot trial for clove oil.

In the first experiment, filter discs infiltrated with an essential oil were placed on lawns of bacteria growing on traditional culture media. The oils were tested against one isolate of Psp from onion and one from leek, on separate culture plates. Of the ten test oils, four showed very good inhibition (thyme, oregano, clove and coriander), a further three showed good inhibition (basil, sage and citronella) and the remaining three showed no inhibition of bacterial growth (eucalyptus, fennel and lavender).

The second experiment included three oils which showed good to very good inhibition (clove, oregano and thyme) and lavender which showed no inhibition of bacterial growth using the above disc assay. The evaluation used a new microtitre plate method where each bacterial isolate was exposed to volatile gas from individual oils eight times per plate in a randomized order. The results of the different oils were compared to each other and to the control plates, which were not exposed to volatile gas, by averaging the replicate wells across three replicate plates. The volatile gas from all four oils tested limited the growth of a Psp isolate obtained from onion. By contrast, growth of the Psp-leek was not always inhibited. The results across the three independent experiments differed slightly which could be a result of variation in the starting concentration of the inoculum. From this experiment, lavender and clove oils were chosen

for contact bactericide evaluation.

To evaluate contact bactericide efficacy, bacterial suspensions were mixed with NA broth and each test treatment which included:

1. broth only
2. broth plus 0.5% DMSO
3. broth plus 0.5% DMSO and 0.2% (v/v) mineral oil
4. broth plus 0.5% DMSO and 0.05% (v/v) clove
5. broth plus 0.5% DMSO and 0.1% (v/v) clove
6. broth plus0.5% DMSO and 0.2% (v/v) clove -
7. broth plus0.5% DMSO and 0.05% (v/v) lavender
8. broth plus 0.5% DMSO and 0.1% (v/v) lavender
9. broth plus 0.5% DMSO and 0.2% (v/v) lavender

In both experiments, clove oil was shown to be efficacious in limiting bacterial growth at the 0.2% concentration. All other treatments were less effective (Results shown in Appendix 6).

The efficacy of essential oils to inhibit bacterial growth varies depending on the type of exposure. Lavender oil was more effective controlling Psp than clove oil when applied as a volatile but not effective when used as a contact bactericide. This was shown through the filter disc contact method and the solution exposure experiments.

Clove oil at a concentration of 0.2% was used in a pot trial to evaluate efficacy *in vivo*. Onion plants were prick inoculated 24 h after the clove oil treatment was applied. There was no significant control of disease in any of the treatments (Table 4), however, clove oil did reduce severity slightly compared to the control. On plants treated with clove oil, disease severity was rated as 1 or 2 compared to the control where it was 2 or more.

Table 3 Results from in vivo testing of clove oil for control of *Pseudomonas syringae* pv. *porri* (Psp). Details include total number of plants tested for each isolate treatment combination and the percent of plants at each disease severity rating. All control plants inoculated with water were rated as 0 and thus not included in the table.

Treatment	Psp isolate	Plants tested[1]	Disease severity rating[2]				
			0	1	2	3	4
Clove oil	onion (B1046)	25	0.0	20.0	72.0	8.0	0.0
	leek (DAR75554)	23	0.0	0.0	13.0	69.6	17.4
Mineral oil	onion (B1046)	24	0.0	29.2	54.2	16.7	0.0
	leek (DAR75554)	19	0.0	10.5	10.5	52.6	26.3
No oil control	onion (B1046)	23	0.0	4.3	56.5	26.1	13.0
	leek (DAR75554)	19	0.0	0.0	15.8	47.4	36.8

[1] The onion variety used was the known susceptible Rio Red Rock. [2] Disease severity rated as : 0 = wound repair only, 1 = local infection at wound site, 2 = extension of infection 1-2 cm from wound, 3 = extension of infection > 2cm from wound and 4 = complete leaf collapse from infection. This is an

average rating across three replicate pots.

Cultural practices: Cultural control of bacterial diseases is generally through management of alternative hosts, reduction in leaf wetness periods through consideration of irrigation practices, and destruction of old crop material. Samson et al. (1998) suggested the existence of different pathovars of *P. syringae* based on host range and biochemical assays. The pathovar designation 'porri' was suggested for strains of *P. syringae* strains from leek as these isolates were only pathogenic to leek. The onion isolates included in the study originated from Japan and were not pathogenic to leek. All other plant species included in the study were non-hosts of the leek isolates. This research is in contrast to results obtained in Australian studies of leek *P. syringae* isolates (Noble et al. 2006). In this study, bacterial isolates obtained from leek were pathogenic to three cultivars of onion, shallot and garlic. Similarly, Koike, et al. (1999) reported pathogenicity of leek strains to onion, chives and garlic. In this project, at least one Australian isolate from leek was pathogenic on eleven different varieties of onion, although differences in pathogenicity were observed between this isolate and one originally derived from onion. No plant species outside the genus *Allium* have as yet been identified as a host of Psp. Koike et al. (1999) tested two non-Allium species of the family *Liliaceae* (lily-of-the-nile and daylily) and six non-*Liliaceae* species (bean, cauliflower, celery, pea, tomato and marigold) for susceptibility to the bacterium and all tested negative.

Spread of foliar bacterial diseases is favoured by wet conditions, through rainfall, irrigation or heavy dew. Damage to plants also increases disease incidence and severity as the wound sites provide entry sites for bacterial infection. Irrigation regimes should consider the duration, timing and type of irrigation used and the movement of people and equipment through the crop, particularly when it is wet. Reduction of leaf wetness periods will assist in limiting bacterial infection and spread.

Crop waste is an important source of Psp for infections of newly planted leek crops in the Netherlands (van Overbeek et al. 2010). In this study, there was an approximate tenfold increase in bacterial infections in leek plants where post-harvest crop waste was returned than in those where no waste was returned. Furthermore, the study identified Psp could survive for at least one month in infected plant material incorporated into the soil. The bacterium was also recovered from soil collected close to infected field plants and found in symptomless plants from 101 of 167 commercial fields surveyed. Incorporation of crop waste with prolonged fallow or rotational non-host crop is beneficial to reduce this risk. Natural microbial degradation of the crop waste was considered to significantly reduce bacterial pathogen populations. Survival of Psp through composting infected crop trash was not evaluated in the study.

Increased preparedness for phytobacterial biosecurity threats

A fact sheet for Xanthomonas blight of onion, caused by *Xanthomonas axonopodis* pv. *allii* was developed and endorsed by Plant Health Australia. This disease is a key exotic threat for the Australian Onion Industry. It is currently the only high risk bacterium listed in the industry biosecurity plan (http://www.planthealthaustralia.com.au/industries/onions/). Presentations at industry forums on this disease during the project have also increased preparedness through improved awareness of the disease, in particular, how to distinguish it from endemic diseases which will assist with early detection if it is introduced.

The results from this study also indicate multiple introductions of Psp into Australia and diversity in pathogenicity within that bacterial species. Given Psp is spread in seed; there is continued risk of further introductions of the pathogen into onion growing districts. The differences in pathogenicity is also of concern as there could be more aggressive isolates of Psp present elsewhere in the world and thus exotic strains of Psp should be listed in the Onion Industry Biosecurity Plan.

Communication and extension

The complete project results were extended to industry, Hort Innovation and DAF primarily through project reports.

Training material on the importance of bacterial diseases of onion was prepared for delivery at industry forums. This included an industry article describing the objectives of the project and the factsheet on the exotic bacterium *Xanthomonas axonopodis* pv. *allii* (Xaa) (refer appendices 3-5). The first of this training was provided as a verbal presentation in 2014 at an industry forum held in Gatton, QLD. The presentation included information on the objectives of the Psp project, a summary of the project results including how to identify the disease caused by Psp and how to differentiate this from that caused by Xaa. It also involved a group discussion on the relative importance of bacterial diseases in onion and what management packages are currently available.

A similar oral presentation was given at the Onion Australia Annual General Meeting held in Brisbane on the 15th of October, 2015 and again at an industry event in the Fassifern Valley of QLD, in September 2016. The training material is available to project collaborators in other major onion growing states for delivery by local staff in those districts.

Recommendations

This project has provided sound research on the detection and management of bacterial crops in Australian allium crops. The main recommendation for industry based on this research is confidence that Bacterial blight of leek is no longer causing impact to Australian onion growers and potential management options are available if it does recur. No outbreaks of this disease caused by Psp were reported since the 2012 Lockyer growing season. As weather conditions were not favourable for bacterial infection and disease development it is difficult to know if the pathogen is still present. The disease may re-emerge during seasons with prolonged cool wet conditions, however, the risk for most seasons in most growing districts is considered quite low. The experiments described in this report have assisted in identifying products of potential use if disease outbreaks occur in the future. The absence of copper tolerance within Psp populations provides confidence that existing bactericides could manage future outbreaks, although there are no registered products for Psp control in onion. If further outbreaks occur, extending the label for copper products currently registered to control fungal diseases of onion should be considered. Further development of essential oils could provide future management options if Psp populations develop copper tolerance.

Results from this project indicate thrips feeding and other mechanical wounding can increase risk of bacterial infections. Increased infection rates occur where wounding sites are co-located on the onion leaves with the bacterium. This risk is further heightened with leaf wetness, particularly during, cool wet weather. Overhead irrigation, rainfall and heavy dew can all disseminate bacteria over the surface of the onion plants and increase the likelihood of bacterial presence at wound sites. Measures to reduce damage to crops by thrips feeding or movement of equipment which contact plants should be implemented where possible. Furthermore, consideration of irrigation regimes to minimize leaf wetness periods is highly recommended.

The fact sheet for Xanthomonas blight of onion, caused by *Xanthomonas axonopodis* pv. *allii* was developed and endorsed by Plant Health Australia. This disease is a key exotic threat for the Australian Onion Industry. It is currently the only high risk bacterium listed in the industry biosecurity plan (http://www.planthealthaustralia.com.au/industries/onions/). It is recommended that this factsheet is distributed annually to industry to ensure awareness continues. The factsheet and the industry biosecurity plan should be reviewed and updated at least every 3-5 years.

Further research in this area is recommended, in particular:

- Investigation of the genetic basis underlying differences in pathogenicity between onion and leek isolates of *Pseudomonas syringae* pv. *porri* (Psp)
- Evaluation of copper for management of bacterial diseases in allium – application methods, formulations and combinations with other compounds
- Improved methodologies to further evaluate bactericides efficacious *in vitro*, in pot and field trials
- Investigation of alternatives to copper such as essential oils
- Detection assay for screening onion seed for the key exotic bacterial threat for onions (*Xanthomonas axonopodis* pv. *allii*) and/or disinfestation strategy developed to treat seed

Scientific refereed publications

None

Intellectual property/commercialisation

'No commercial IP generated' if there is none to report

References

Altimira, Fabiola, Carolina Yáñez, Guillermo Bravo, Myriam González, Luis A. Rojas, and Michael Seeger. 2012. "Characterization of Copper-Resistant Bacteria and Bacterial Communities from Copper-Polluted Agricultural Soils of Central Chile." *BMC Microbiology* 12 (1): 1.

Bender, Carol L., Scott A. Young, and Robin E. Mitchell. 1991. "Conservation of Plasmid DNA Sequences in Coronatine-Producing Pathovars of Pseudomonas Syringae." *Applied and Environmental Microbiology* 57 (4): 993–999.

Bereswill, Stefan, Peter Bugert, B. Völksch, Matthias Ullrich, Carol L. Bender, and Klaus Geider. 1994. "Identification and Relatedness of Coronatine-Producing Pseudomonas Syringae Pathovars by PCR Analysis and Sequence Determination of the Amplification Products." *Applied and Environmental Microbiology* 60 (8): 2924–2930.

Braun, Y., A. V. Smirnova, A. Schenk, H. Weingart, C. Burau, G. Muskhelishvili, and M.S. Ulrich. 2008. "Component and Protein Domain Exchange Analysis of a Thermorepsonsive, Two-Component Regulatory System of Pseudomonas Syringae." *Microbiology* 154: 2700–2708. doi:10.1099/mic.0.2008/018820-0.

Budde, I. P., B. H. Rohde, C. L. Bender, and M. S. Ullrich. 1998. "Growth Phase and Temperature Influence Promoter Activity, Transcript Abundance, and Protein Stability during Biosynthesis of the Pseudomonas Syringae Phytotoxin Coronatine." *Journal of Bacteriology* 180 (6): 1360–67.

Cuppels, Diane A., and Teresa Ainsworth. 1995. "Molecular and Physiological Characterization of Pseudomonas Syringae Pv. Tomato and Pseudomonas Syringae Pv. Maculicola Strains That Produce the Phytotoxin Coronatine." *Applied and Environmental Microbiology* 61 (10): 3530–3536.

da Silva, E.O, Martins, S.J., and E. Alves. 2014. "Essential Oils for the Control of Bacterial Speck in Tomato Crop." *African Journal of Agricultural Research* 9 (34): 2624–29.

Geng, X. Q., L. Jin, M. Shimada, Kim MinGab, and D. Mackey. 2014. "The Phytotoxin Coronatine Is a Multifunctional Component of the Virulence Armament of Pseudomonas Syringae." *Planta* 240 (6): 1149–65. doi:10.1007/s00425-014-2151-x.

Gitaitis, R., S. Mullis, K. Lewis, D. Langston, A. K. Watson, and H. Sanders. 2012. "First Report of a New Disease of Onion in Georgia Caused by a Nonfluorescent Pseudomonas Species." *Plant Disease* 96 (2): 285.

Gomah, A.A. 2008. "Use of Essential Oils for Controlling Bacterial Angular Leaf Spot Disease of Cucumber." *Arab Universities Journal of Agricultural Sciences* 16 (1): 181–90.

Hall, B., C. J. Hitch, E. A. Oxspring, and T. J. Wicks. 2007. "Leek Diseases in Australia." *Australasian Plant Pathology* 36: 383–88.

Kizil, S, F Uyar, and A Sagir. 2005. "Antibacterial Activities of Some Essential Oils against Plant Pathogens." *Asian Journal of Plant Sciences* 4 (3): 225–28.

Koike, S.T., J Barak, and R.L. Gilbertson. 1999. "Bacterial Blight of Leek: A New Disease in California Caused by Pseudomonas Syringae." *Plant Disease* 83: 165–70.

Kokoskova, B., D. Pouvova, and R. Pavela. 2011. "Effectiveness of plant essential oils against *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae* and associated saprophytic bacteria on/in host plants." *Journal of Plant Pathology* 93 (1): 133–39.

Kotan, Recep, Fatih Dadasoğlu, Kenan Karagoz, Ahmet Cakir, Hakan Ozer, Saban Kordali, Ramazan Cakmakci, and Neslihan Dikbas. 2013. "Antibacterial Activity of the Essential Oil and Extracts of Satureja Hortensis against Plant Pathogenic Bacteria and Their Potential Use as Seed Disinfectants." *Scientia Horticulturae* 153 (April): 34–41. doi:10.1016/j.scienta.2013.01.027.

Lo Cantore, Pietro, Nicola S. Iacobellis, Adriana De Marco, Francesco Capasso, and Felice Senatore. 2004. "Antibacterial Activity of Coriandrum Sativum L. and Foeniculum Vulgare Miller Var. Vulgare (Miller) Essential Oils." *Journal of*

Agricultural and Food Chemistry 52 (26): 7862–66. doi:10.1021/jf0493122.
Lopez-Reyes, J.G., D. Spadaro, M. L. Gullino, and A. Garibaldi. 2013. “Synergy between Main Compunds of Essential Oils of Savory and Thyme in the Containment of Some Plant Pathogens.” *Protezione Delle Colture* 2: 78.
Mikicinski, A, P. Sobiczewski, and S Berczynski. 2012. “Efficacy of Fungicides and Essential Oils against Bacterial Diseases of Fruit Trees.” *Journal of Plant Protection Research* 52 (4): 467–71.
Mitchell, Robin E. 1982. “Coronatine Production by Some Phytopathogenic Pseudomonads.” *Physiological Plant Pathology* 20: 83–89.
Mitchell, Robin E., Scott A. Young, and Carol L. Bender. 1994. “CORONAMIC ACID, AN INTERMEDIATE IN CORONATINE BIOSYNTHEsis by Pseudomonas Syringae.” *Phytochemistry* 35 (2): 343–48.
Noble, Dorothy H., Eric J. Cother, Deborah L. Hailstones, Michelle Flack, Liz Oxspring, and Barbara Hall. 2006. “Characterisation of *Pseudomonas Syringae* Strains Associated with a Leaf Disease of Leek in Australia.” *European Journal of Plant Pathology* 115 (4): 419–30. doi:10.1007/s10658-006-9033-4.
Oliva, M., M. E. Carezzano, M. Giuliano, J. Daghero, J. Zygadlo, P. Bogino, W. Giordano, and M. Demo. 2015. “Antimicrobial Activity of Essential Oils of Thymus Vulgaris and Origanum Vulgare on Phytopathogenic Strains Isolated from Soybean.” *Plant Biology* 17 (3): 758–65. doi:10.1111/plb.12282.
Overbeek, Leonard S. van, Els H. M. Nijhuis, Harrie Koenraadt, Johnny Visser, and Gijs van Kruistum. 2010. “The Role of Crop Waste and Soil in Pseudomonas Syringae Pathovar Porri Infection of Leek (Allium Porrum).” *Applied Soil Ecology* 46 (3): 457–63.
Rangaswamy, V., M. Ullrich, W. Jones, R. Mitchell, R. Parry, P. Reynolds, and C. L. Bender. 1997. “Expression and Analysis of Coronafacate Ligase, a Thermoregulated Gene Required for Production of the Phytotoxin Coronatine in Pseudomonas Syringae.” *FEMS Microbiology Letters* 154 (1): 65–72. doi:10.1016/S0378-1097(97)00302-9.
Roach, R, Alistair R. McTaggart, C. F. Gambley, Harper, S., Carey, D, and Duff, J.D. 2016. “Pseudomonas Syringae Pv. Porri: A New Pathogen of Australian Onions | International Society for Horticultural Science.” Accessed May 30. http://www.ishs.org/ishs-article/1105_21.
Rohde, B. H., B. Pohlack, and M. S. Ullrich. 1998. “Occurrence of Thermoregulation of Genes Involved in Coronatine Biosynthesis among Various Pseudomonas Syringae Strains.” *Journal of Basic Microbiology* 38 (1): 41–50. doi:10.1002/(SICI)1521-4028(199803)38:1<41::AID-JOBM41>3.0.CO;2-6.
Samson, R., H. Shafik, A. Benjama, and L. Gardan. 1998. “Description of the Bacterium Causing Blight of Leek as Pseudomonas Syringae Pv. Porri (Pv. Nov.).” *Phytopathology* 88 (8): 844–850.
Ullrich, Matthias, Stefan Bereswill, Beate Volksch, Wolfgang Fritsche, and Klaus Geider. 1993. “Molecular Characterization of Field Isolates of Pseudomonas Syringae Pv. Glycinea Differing in Coronatine Production.” *Journal of General Microbiology* 139 (8): 1927–1937.

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Appendices

Appendix 1 – Copper tolerance testing *in vitro* assay development

All experiments used bacterial suspensions of approximately OD_{620nm} 0.3 prepared in sterile water from 36-48 h cultures grown on KB and copper sulphate solutions of 0.25, 0.5, 1.0, 1.5 and 2.0 mM prepared in water/CYEG containing 20 mM MES, pH adjusted to 7.0 with NaOH and filtered sterilised. After exposure to test treatments, 10 µL aliquots were removed from each 4 ml test media-copper solution and used to inoculate a microtitre plate well containing 180 µL of nutrient broth. Each bacteria copper combination was evaluated in four separate microtitre wells. The plates were incubated for 24 h at 25 °C and then bacterial growth measured using a plate reader at OD_{620nm}. Experiments used bacterial isolates of *Pseudomonas syringae* pv. *porri* (Psp) from onion (B1038 and B1046) and isolate DAR75554 from leek unless otherwise stated.

Experiment 1: Exposing bacterial isolates to different concentrations of copper prepared in water. The treatments were 10 µL of bacterial suspension to 4 ml of test copper solution, incubated at room temperature (ca. 25 °C) for 4 h and overnight. No growth occurred on the microtitre plates for copper exposure treatments whereas the controls which had no copper exposure grew to an OD_{620nm} of 0.15 or greater. The conclusion was possible false positives for copper tolerance if bacteria required active growth to combat the copper toxicity as the exposure incubations were done in water not media.

Experiment 2: This experiment substituted CYEG broth for water in the copper sulphate solutions. Glycerol was used instead of glucose in the media as Pseudomonads are known to grow on glycerol and to prevent any potential interaction between glucose and the copper. Copper solutions were prepared in CYEG containing 20 mM MES, the pH adjusted to 7.0 with NaOH and then filter sterilised. The amount of bacterial suspensions added to the 4 ml test copper treatments was increased to 40 µL of OD_{620nm} 0.3 to give a final dilution of 1:1000 and improve recoverability of viable bacteria. The bacteria were exposed to copper for 1 h at room temperature (ca. 25 °C) and aliquots removed to inoculate microtitre plates containing NA, KB or CYEG broth. This experiment used the isolates B1038, B1046 and DAR75554. Bacterial growth with the control no-copper treatment was good (OD_{620nm} 0.45). There was a general reduction in growth with increased copper; MIC was 1.5 mM for B1038, B1046 and DAR75554 using microtitre plate evaluation. The isolates grow less well on CYEG as compared to NA and complications around fluorescence of pseudomonad growth using KB media, identified NA as the appropriate media for further experiments. The conclusion was the method works quite well, however, there is a possibility of false positives/negatives for copper tolerance if 1 h exposure was insufficient to induce the copper tolerance genes or allow copper toxicity to become lethal.

Experiment 3: This experiment looked at the copper tolerance levels using increased exposure time. The methodology was as per experiment 2 and evaluated 0, 1, 2 and 4 h exposure times to the copper treatments. The isolates used were B1046 and DAR75554 and a known sensitive *P. syringae* pv. *syringae* isolate (BRIP38748). The pH of the treatment solutions was adjusted to 6.5 rather than 7.0 to reduce amounts of NaOH used. In a separate experiment, bacterial growth at pH 6.5 was not significantly different to that at pH 7.0 (results not shown). Additionally, the 24 h recovery incubation period post exposure was compared to 48 h. The media used in the microtitre plates was NA. The two Psp isolates both grew to a MIC of at least 1.5 mM and the sensitive Pss isolate to 1.0 mM at time zero exposure, the MIC threshold decreased with increasing exposure time to 0.5 mM for the two Psp isolates and 0.25 for Pss. Given Pss is a sensitive isolate, the expected MIC was 0 mM, indicating exposure time of 4 h was insufficient and false copper tolerance was recorded. This was using the values from the 48 h recovery incubation which appeared to be more consistent than a 24 h recovery time. The conclusion was to increase exposure time further and include a known copper tolerant isolate of *P. syringae* pv. *tomato* (Pst) which was recently identified in other work. Also to evaluate the recovery incubation time again.

Experiment 4: – This experiment looked at the copper tolerance levels using further increased exposure time. The methodology was as per experiment 3 and evaluated 0, 2, 4, 6 and 24 h exposure times to the copper treatments. The isolates used were B1046, Pss and a Pst isolate. The MIC results from the 48 h recovery incubation were again more consistent than the 24 h recovery period. Similarly, the results from a 24 h exposure time were more consistent with both controls giving expected results. The OD_{620nm} of 0.04 was recorded for both B1046 and Pss when exposed to the minimum copper used in the experiment (0.25 mM) which was identical to that of the un-inoculated control. By contrast, the OD_{620nm} for Pst was 0.5 at a copper exposure of 1.0 mM. The Pst isolate was unable to tolerate a copper at 1.5 mM and had an absorbance reading similar to the un-inoculated controls.

Final methodology: The final method adopted which gave consistent results with no false positives or negatives for copper tolerance is to vary concentrations of copper sulphate from zero to 2.0 mM prepared in Casitone Yeast Extract Glycerol (CYEG) broth containing 20 mM MES (2-(N-morpholino)ethanesulfonic acid). The pH of each solution is adjusted to 6.5 with NaOH prior to filter sterilizing. Bacterial suspensions of approximately 10^9 CFU/ml are mixed with each of the copper treatments at a ratio of 40 µL of suspension to 4 ml of the test media-copper solution. The bacteria are then exposed to the treatments for 24 h at 25 °C with gentle shaking. After exposure, 10 µL aliquots are removed from each 4 ml test media-copper solution and used to inoculate a microtitre plate well containing 180 µL of nutrient broth. Each bacteria copper combination was evaluated in four separate microtitre wells. The plates are then incubated for 48 h at 25 °C and then bacterial growth measured using a plate reader at OD_{620nm}. An isolate known to have high copper tolerance, and one known to have no copper tolerance, are used as controls for the experiment.

Appendix 2 – Methodology and results for pathogenicity pot trials

To determine if Psp isolates varied in pathogenicity, a pot trial was established using the known susceptible onion hybrid Rio Red Rocks. Bacterial inoculum (isolates B1038, 39, 40, 41, 42 and 46, DAR75554, 61456 and 75283) was prepared by suspending bacteria after 24-48 h growth on KB media in 0.85% NaCl and 0.02% Tween 20 to a final concentration of 10^8 CFU/ml (OD_{620nm} 0.3). Three replicates, each of approximately 10 plants were inoculated per isolate. Each plant was inoculated by wounding using a sterile needle pricked through a droplet of inoculum. Plants were maintained under humid conditions in a growth room for 48-72 h then rated approximately two weeks post-inoculation. The average minimum and maximum temperatures for the trial were 15.2 and 18.3 °C, respectively.

Plants were rated using the following disease symptom severity scale: 0 = wound repair only, 1 = local infection at wound site, 2 = extension of infection 1-2 cm from wound, 3 = extension of infection > 2cm from wound and 4 = complete leaf collapse from infection.

The disease symptom severity scale was converted to an overall severity scale. This was determined by assessing the percentage of plants with each symptom rating averaged across the three replicate plants and then evaluating the spread of severities for each treatment. The severities are coded as: low where >50% of plants had a severity rating of 1 or 2, moderate if >50% of plants had a severity rating of 2 or 3 and high if >50% of plants had a severity rating of 3 or greater.

All six Psp-onion isolates tested were less pathogenic than Psp-leek isolate DAR75554. The Psp-onion isolate B1046 was equally pathogenic as two further Psp-leek isolates DAR61456 and DAR75283. From these results isolates B1046 and DAR75554 were used in further pot trial analyses.

Appendix 3 – Industry article for Onions Australia 2015

Detection and management of bacterial diseases in Australian allium crops - VN13005

The Horticulture Innovation Australia Ltd (HIA) funded project VN13005 commenced in July 2014. The main aim of the project is to increase the capacity of the onion industry to manage bacterial diseases, such as Bacterial blight of leek (caused by Pseudomonas syringae pv. porri; acronym: Psp) which affects onions and other alliums such as shallots and leeks. A further aim is to enhance preparedness for potential incursions of key exotic bacterial diseases, such as Xanthomonas leaf blight of onion.

The main activities within the project are to determine the importance of bacterial diseases to the Australian industry, gain a better understanding of how bacteria spread and survive in the environment and review current and potential management strategies for their control. This will involve surveys of Australian onion growing districts for bacterial diseases, investigating links between disease outbreaks and weather and reviewing known research on bacterial survival and spread. Specific experiments will study the influence of temperature on disease for improved early season predictions of potential economic impacts and evaluating bacterial isolates for their tolerance to copper. Potential alternatives to current control methods could include different formulations of copper, new chemical and biological control products and resistant or tolerant plant lines.

This project was initiated following an outbreak of bacterial blight of leek on onion and shallot crops in the Lockyer Valley, Qld a few years ago. The disease is thought to have caused production in these crops problems from 2010 to 2012, with samples sent for diagnosis in late 2011. Initially the disease in the Lockyer was that to be downy mildew caused by the fungus Peronospora destructor as the symptoms can be superficially similar (Figure 1). The preliminary diagnoses in late 2011 was of an exotic bacterial pathogen, Xanthomonas axonopodis pv. alli (Xanthomonas leaf blight of onion) as this was the only disease of onion in the literature at that time that matched the symptoms (Figure 2). Subsequently, there was a report in 2012 from Georgia, USA of a different bacterial species causing these symptoms in onion. Further investigation of the bacteria present in the Lockyer, identified it as Bacterial blight of leek, similar to the pathogen described from Georgia.

Figure 1 Red onion plants affected with downy mildew on the left and Bacterial blight of leek on the right. In both cases there is a yellowing of leaf tissue but with the bacterial infection it is often the whole leaf and always the new leaf, whereas, that caused by the downy mildew fungi is more localised and can occur on any leaf.

Figure 2 - Shallot leaves showing symptoms of bacterial infection. The leaf is typically affected from the tip back with lesions joining together to cause large areas of dying tissue. Within this area, rust-tan coloured individual lesions are often seen. These symptoms were caused by infection with Pseudomonas syringae pv. porri and are very similar to that reported for Xanthomonas axonopodis pv. alli and other bacterial diseases of onion.

Bacterial blight of leek is a well-known disease of leek around the world and was previously reported in Australia affecting leek crops in southern states and WA. The detection from the Lockyer Valley is the first report of this disease affecting onion and shallot for Australia and only the second report affecting onion worldwide. The pathway for introduction into the Lockyer of this disease is uncertain. It is possible it came with trial leek plantings or alternatively the bacteria have contaminated onion or shallot seed production areas elsewhere in the world. The bacterium is known to be seed borne in leek.

During the first phase of the project a literature review was completed. The review highlighted a lack of information on Bacterial blight of leek and Psp. Some information is available on related bacterium and will serve as a good foundation to build specific Psp experiments. The genetic and biological diversity of other related bacteria is quite high and preliminary investigations into Psp also indicate the diversity in Australia is likely to be greater than initially thought. Understanding this diversity is important for development of management strategies.

The review did identify coronatine as a likely toxin produced by Psp which causes the distinctive yellow-leaf symptom observed during the Lockyer disease outbreak (Figure 1). The isolates of Psp detected in the Lockyer in 2012 all tested positive by molecular assays for the presence of the gene that is responsible for producing this toxin. Laboratory experiments by others show that growth at 18 °C for about 5 days is needed to induce coronatine and indicates a strong temperature influence on toxin production. Preliminary experiments support this temperature influence with infected shallot plants developing the yellow-leaf symptom after approximately one-week of growth at a constant temperature of 18 °C. Further experiments are underway.

In addition to Psp, there are many other bacterial diseases which affect onions and often symptoms can be superficially very similar. This includes diseases caused by Pantoea agglomerans, Pseudomonas viridiflava and P. marginalis pv. marginalis. These bacterial species can also cause significant impacts to production if environmental conditions are suitable.

If you see symptoms similar to those described in this article, or other symptoms you are unsure of please contact me.

For further information please contact:

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Appendix 4 – Industry article for Onions Australia April, 2017

Detection and management of bacterial diseases in Australian allium crops

A research project focused on increasing the capacity of the onion industry to manage bacterial diseases has trialed the use of essential oils to three different options to manage disease. This includes the use of copper bactericides, essential oils and host resistance in onion varieties.

Funded by Horticulture Innovation Australia, the Detection and management of bacterial diseases in Australian allium crops project started in 2014 in response to an outbreak of bacterial blight of leek (Pseudomonas syringae pv. porri, or Psp) on onion and shallot crops in Queensland's Lockyer Valley growing region during the 2010 to 2012 seasons.

Lead researcher Dr Cherie Gambley from Queensland's Department of Agriculture and Fisheries said initially the disease was thought to be downy mildew caused by the fungus Peronospora destructor, as the symptoms can be superficially similar to that caused by Psp.

"Bacterial blight of leek is a well-known disease of leek around the world and was previously reported affecting leek crops in southern states and WA," Dr Gambley said.

"But the detection from the Lockyer Valley was the first report of this disease affecting onion and shallot for Australia and only the second report affecting onion worldwide."

Volatile gases from four oils – clove, lavender, oregano and thyme – were tested in laboratory experiments to evaluate their ability to reduce bacterial growth.

Dr Gambley said experiments showed that all four oils limited the growth of a Psp isolate obtained from onion.

"By contrast, the a Psp isolate from a disease outbreak in leek in the early 2000s in southern Australia was only affected by volatiles of clove and lavender," she said.

"Two related bacterial species P. syringae pv. tomato (Pst) and P. syringae pv. syringae (Pss) were included for comparison. Interestingly, Pss was affected by most of the oils except lavender and Pst was not affected by any of the oils."

"Further work is needed to confirm these results and to develop application technology."

Dr Gambley said the research also concluded that further work should be carried out to develop copper spray regimes to control the bacterium and to extend label use of currently registered copper products.

"Experiments show that Australian isolates of Psp had not developed a tolerance to copper as varieties in other countries had, so this and copper based bactericides could be useful to manage the disease, however, there are currently no ensure registered products are available in seasons with disease-favouring weather conditions for this use," she said.

A pot trial experiments were was also conducted around possible resistance to t Psp in a selection of currently available commercial onion varieties to. Psp.

Dr Gambley said the trial included a range of white, brown and red varieties with varying day-length maturity.

"None of the varieties were resistant to Psp and most lines reacted similarly to the known susceptible variety," she said.

"All varieties reacted more severely to the leek isolate of Psp, indicating variability in strain severity within this

bacterium.”

“Overall, the use of resistant or tolerant onion varieties to manage the disease is unlikely to feasible with currently available lines.”

Experiments also indicated cool, wet weather is favourable for disease establishment and spread.

Dr Gambley said while it’s possible the disease will re-emerge if weather conditions are more conducive, the risk for most seasons is considered quite low.

Appendix 5 – Industry article for Onions Australia July, 2017

Detection and management of bacterial diseases in Australian allium crops

Onion Project VN13005 Research Provider: DAF Queensland, Researchers: Cherie Gambley and John Duff

Due for completion later this year, this three-year project has been investigating bacterial diseases of onion crops in order to improve understanding of their introduction, spread and survival. It will build the industry's capacity to manage bacterial diseases – including bacterial blight of leek (caused by Pseudomonas syringae pv. porri), which affects onions and shallots – and to enhance preparedness for potential incursions of exotic diseases, such as Xanthomonas leaf blight of onion.

The project started in 2014 in response to an outbreak of bacterial blight of leek (Pseudomonas syringae pv. porri, or Psp) on onion and shallot crops in Queensland's Lockyer Valley growing region during the 2010 to 2012 seasons.

One of the key areas of this project's work is in relation to control methods. The researchers note that while there are currently no products registered by the Australian Pesticides and Veterinary Medicines Authority specifically for the control of bacteria in onion crops, there are over 40 copper-containing products registered for other uses in onions – with potential to expand the registered use of some of these products for the control of bacterial diseases.

Investigation of these products are continuing along with the testing of non-copper products that may be effective control measures. There was also the ongoing screening of commercial onion varieties for resistance or tolerance to Pseudomonas syringae pv. Porri.

The full report and findings will be published later this year by Hort Innovation.

This project has been funded by Hort Innovation, using the onion research and development levy and contributions from the Australian Government.

Caption for accompanying image for VN13005:

Symptoms induced in onion by Pseudomonas syringae pv. porri. The bright yellow central leaf is a result of a phytotoxin produced by the bacterium under cold conditions. The infection points of the bacterium are seen on the older leaf as tan water-soaked lesions which have joined together.

Appendix 6 – Environmental influences on symptoms and disease development

Coronatine, among other toxins, is produced by a range of *Pseudomonas* species and *P. syringae* pathovars (Mitchell 1982). Its biosynthesis pathway has been studied in other *P. syringae* pathovars (Cuppels and Ainsworth 1995; Mitchell, et al. 1994; Ullrich et al. 1993), though very little work on toxin production has been done specifically in Psp. It has been shown that levels of coronatine production significantly impact pathogenicity of some *P. syringae* pathovars, though not all (Bereswill et al. 1994). It has been established that coronatine's gene cluster may be plasmid borne or chromosomal (Cuppels and Ainsworth 1995), suggesting this may be correlated with host or geographic location. The highly conserved nature of gene products in Bereswill et al. (1994) has proven useful for identifying coronatine production and relatedness. Bender, et al. (1991) has also demonstrated the conservation of coronatine synthesis genes using DNA hybridization on plasmids from a number of different regions. Using the techniques described in these and other papers, coronatine production in Psp can be assessed and used to generate phylogenies. Coronatine toxin is reported to be involved in suppression of systemic acquired resistance defense responses such as the methyl jasmonate pathway and to enhance the duration of stomatal opening (Geng et al. 2014). Both these modes of action enhance likelihood of successful infection and disease development.

Multiple studies on the temporal expression of the coronatine gene indicate cool temperatures are important for toxin production. Expression of the gene was high at 18 °C and virtually non-existent at 28 °C (Ullrich et al. 1993; Rangaswamy et al. 1997; Budde et al. 1998; Rohde, Pohlack, and Ullrich 1998). Published reports are limited to temporal interactions which induce toxin production and its affect, the reversal of toxin and subsequent recovery, however, is not described in the literature. Evaluating historical weather data from the Lockyer Valley was useful to interpret the occurrence of the yellow-leaf symptom observed during epidemiology studies. Additionally, these temporal expression studies provided a useful basis for experiments aimed at determining environmental conditions likely to result in this unique symptom which so far has only been observed in field plants and is most likely caused by coronatine toxin production by the bacterium.

Bulb onion (red and brown) and spring onion were prick inoculated with Psp-onion isolates B1039 and B1040 at 10^8 CFU/ml. Three-four replicate pots, each containing two plants were inoculated for each host variety. Inoculated seedlings were covered with plastic for 48 h at room temperature, uncovered and then kept at 18-24 °C for one week followed by incubation at 16-18 °C. Incubations were done in a temperature controlled growth cabinet with a 12 h day-night regime. To maintain a high level of humidity, plants were misted with water every 2 days and a water-bath placed in the bottom of the cabinet.

The first rating was done approximately 2 weeks after inoculation and bacterial infection was observed on all plants at the inoculation site. There was no evidence of bacterial spread beyond this point. Evidence of the development of the yellow-leaf symptom was observed in spring onion plants only. Four seedlings, two each inoculated per bacterial isolate showed chlorosis. A second rating of plants was done one-week later, with no further symptom development. The growth conditions were then altered to include 12 h each of 18 and 24 °C. Following incubation at this regime for two weeks, no plants showed yellow-leaf symptoms. This indicates there was recovery from chlorosis by the four plants previously affected.

A second experiment was done using a mix of Psp-onion isolates (B1038, B1039, B1040, B1041, B1042 and B1046), at 10^7 to 10^8 CFU/ml. Inoculations were done either by pricking leaves of brown and white onion plants through a *ca*. 5 µL droplet of inoculum using a sterile syringe needle. The water control inoculations were done using a needle prick only. The plants were incubated in a glasshouse and temperature monitored using a Tinytag probe. Plants were overhead watered daily to maintain high levels of humidity and fertilised three weeks post-inoculation (PI). Obvious yellow leaf symptoms were observed three weeks PI. Re-isolations were attempted from selected plants approximately one-month PI. Bacterial infections were successfully induced in both onion hosts. The yellow leaf symptom was first observed in

brown and white onion plants 18 days PI, and most plants had recovered from the symptom by 13.5 weeks PI. No evidence of the yellow leaf symptom was observed at the final rating, done 4.5 months PI (Table 1). The chart of minimum and maximum temperatures recorded during the experiment clearly shows cool temperatures at times when the YLS was present (Figure 1). At the time of inoculation the maximum temperature was above 20 °C . The number of plants with YLS decreased from the 18th to the 30th of June, although temperatures don't appear markedly different to the preceding weeks.

Table 1 The number of plants infected (I) with *Pseudomonas syringae* pv. *porri* (Psp) and those which subsequently developed the severe yellow leaf symptom (YLS) are also shown. Ratings for disease symptoms were done 10, 18 and 26 days post-inoculation (PI) then at 5.5, 10 and 13.5 weeks PI.

Host	Inoculum	10 days PI		18 days PI		5.5 weeks PI	10 weeks PI	13.5 weeks PI
		I	YLS	I	YLS	YLS	YLS	YLS
White onion	Prick	0/15	0/15	10/15	5/15[a]	6/15	8/15	1/15
	water	0/25	0/25	1/25	0/25	0/25	1/25[b]	1/25
Brown onion	Prick	0/21	0/21	7/21	2/21[a]	5/21	3/21	1/21
	water	0/16	0/16	0/16	0/16	0/16	0/16	0/16

[a] yellow colouration on new leaf possibly just starting, [b] infection from neighbouring plants as all grown in the same seedling tray

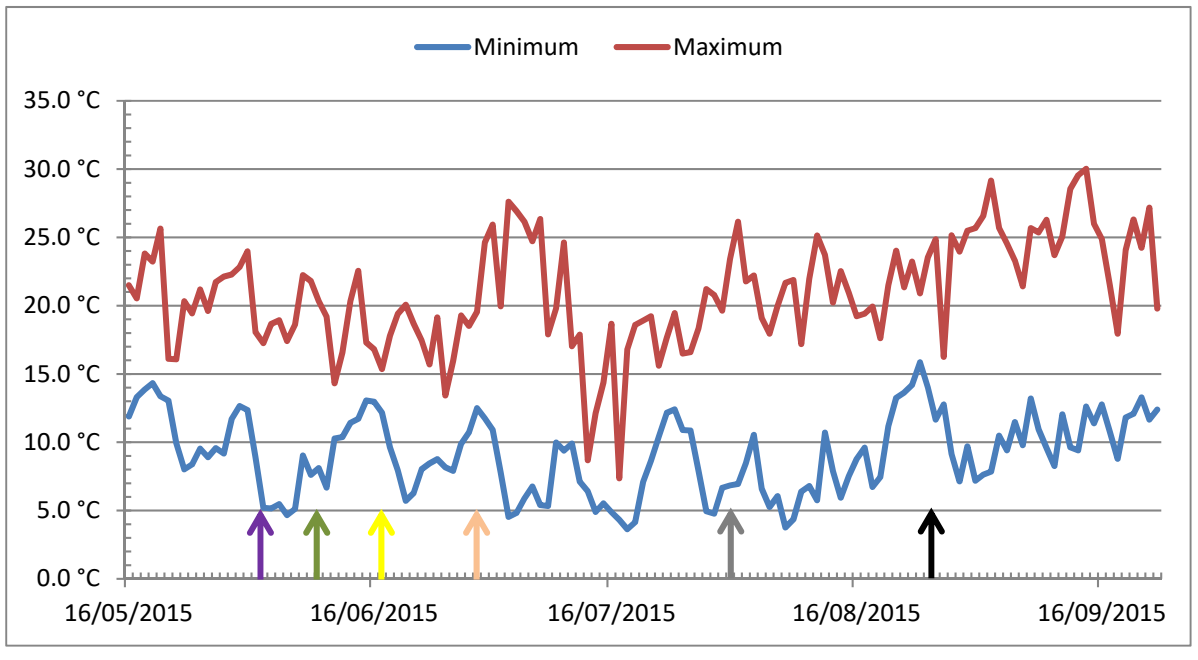

Figure 1 Chart of minimum (blue line) and maximum (red line) temperatures for the duration of the disease development experiment. The purple arrow indicates the inoculation date. The remaining arrows correspond to the rating dates as outlined in Table 1 (green 10 days, yellow 18 days, orange 5.5 weeks, grey 10 weeks and black 13.5 weeks post-inoculation) The yellow leaf symptom was first observed 18 days and had almost completely disappeared 13.5 weeks post-inoculation.

In a third experiment, red onion seedlings were inoculated with a mix of Psp-onion isolates (B1038, B1039, B1040, B1041, B1042 and B1046), using a concentration of *ca*. 10^8 CFU/ml or one-hundredth of this. Three pots each containing approximately 10 seedlings were inoculated for each concentration. Two pots were inoculated with water. Inoculations were done as per experiment 2. Obvious yellow leaf symptoms were observed at the first rating, three weeks post-inoculations. The severity of bacterial infection was related to the bacterial inoculum concentration. More severe symptoms were observed at concentrations of *ca.* 10^8 CFU/ml as compared the inoculum of Psp used at *ca.*10^6 CFU/ml. Lesion development was more severe in these treatments and the incidence of yellow leaf symptom was also higher (Table 2). The infection incidence, however, did not alter greatly. The Psp inoculum at 10^8 CFU/ml produced 86% (25/29) infected plants as compared to 75% (21/28) inoculated with the 10^6 CFU/ml inoculum (Table 2). These results indicate bacterial population levels are important for disease severity and toxin production.

Table 2 The number of plants infected (I) with *Pseudomonas syringae* pv. *porri* (Psp) and the number subsequently showing the severe yellow leaf symptom (YLS) at three, seven and eleven weeks post-inoculation (PI). Plants were inoculated with a mixed isolate suspension of Psp, or a one-hundredth dilution of the this inoculum.

Inoculum	Replicates	3 weeks PI		7 weeks PI	11 weeks PI
		I	YLS	YLS	YLS
Psp	1	7/10	3/10	2/10	0/10
	2	9/9	3/9	0/9	0/9
	3	9/10	2/10	0/10	0/10
1:100 Psp	1	5/8	4/8	0/8	0/8
	2	10/14	0/14	0/14	0/14
	3	6/6	0/6	0/6	0/6
water	1	1/15	0/15	0/15	0/15
	2	0/14	0/14	0/12	0/12

At 11 weeks post-inoculation, all plants were recovered from the yellow leaf symptom and any signs of infection. Outer inoculated leaves had senesced without further infection of newly emerged leaves. This indicates disease induced by Psp in onion is likely to be transitory unless extended periods of cool wet weather persist.

Further yellow leaf symptoms were observed in pot trials established to evaluate efficacy of copper and clove oil as bactericides. On emergence of the symptom, a TinyTag temperature monitor was placed inside the glasshouse. The temperatures recorded and the numbers of plants with the yellow leaf symptom are shown in Figure 2. In this figure experiment 1 (copper) was inoculated on the 25th August and experiment 2 (clove oil) on the 18th September. This data shows a possible link with a drop in maximum temperature which precedes an increase in plants with the symptom. Figure 3 shows the duration of the maximum temperature above 20 °C in relation to when plants with the symptom were observed. Again cooler conditions (i.e. less hours above 20 °C) precede increases in plants with the symptom and conversely, a decrease in numbers coincides with warmer conditions. It is likely temperature influences toxin production in multiple ways, either sustained cool conditions and/or sudden drops in temperature and subsequent

increases in temperature results in amelioration of the yellow leaf symptom.

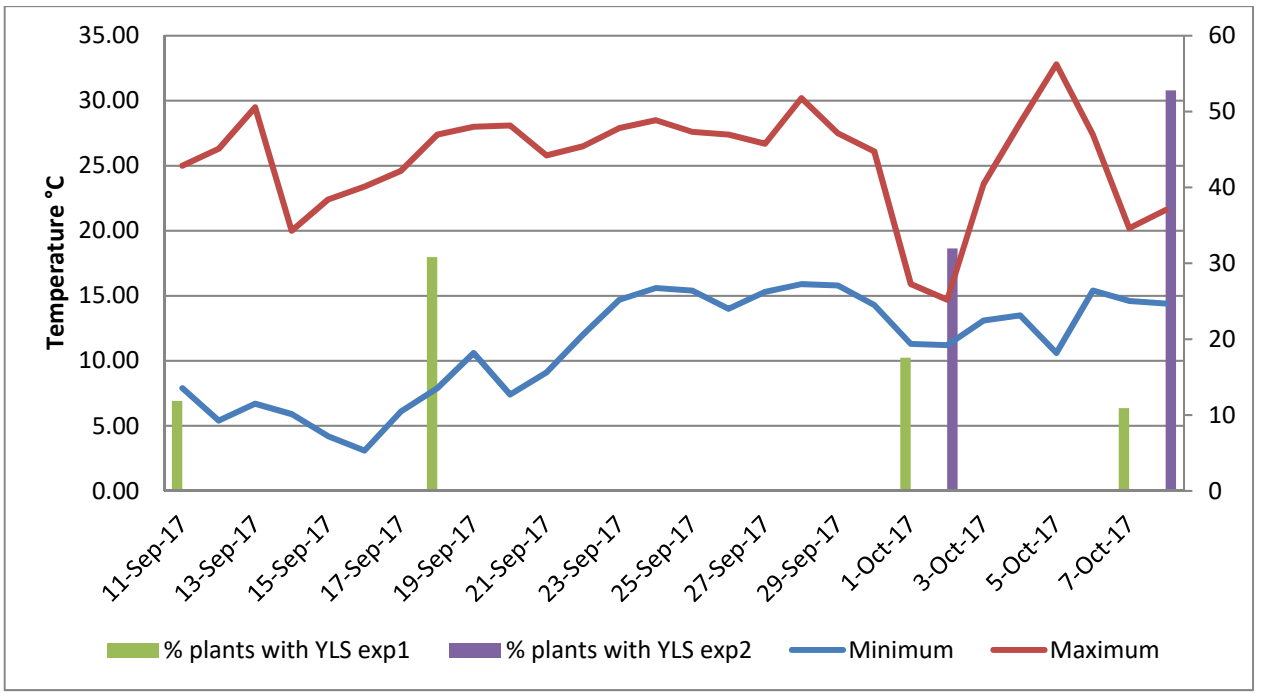

Figure 2 Graph of maximum and minimum temperatures for glasshouse trials. The proportion of plants with the yellow leaf symptom (YLS) at various rating time points is shown. The first experiment (exp 1) was inoculated on the 25th of August and the second experiment (exp 2) on the 18th of September.

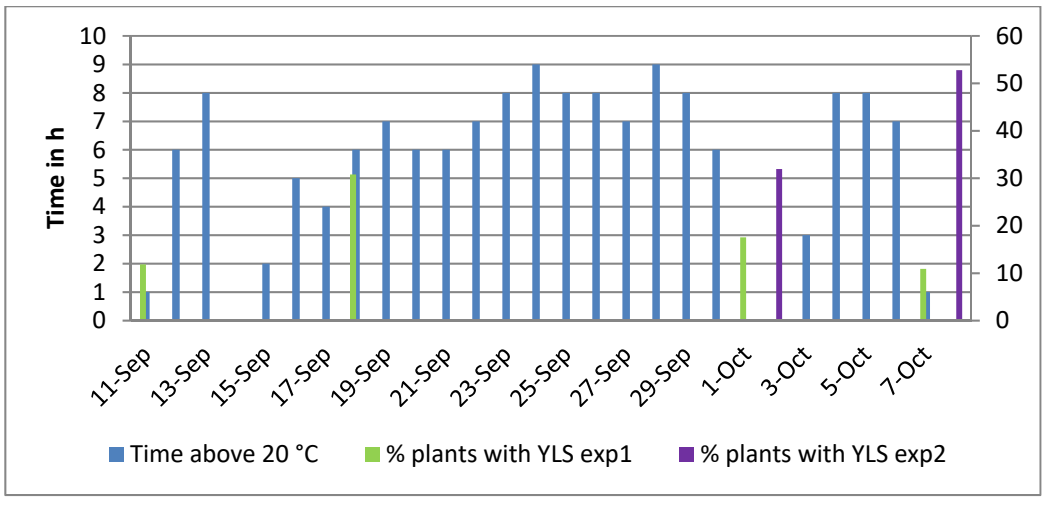

Figure 3 Graph of the duration temperatures in glasshouse trials exceeded 20 °C. The proportion of plants with the yellow leaf symptom (YLS) at various rating time points is shown. The first experiment (exp 1) was inoculated on the 25th of August and the second experiment (exp 2) on the 18th of September.

Appendix 7 – Evaluation of essential oils for efficacy against *Pseudomonas syringae pv. porri* by in vitro assay

Previous work investigating components of essential oils, in particular terpenes were identified as having potential antibacterial affects. The two monoterpenes, geraniol and citronellol were inhibitory to *Pseudomonas syringae* pv. *actinidiae* and *Erwinia amylovora*, however, the phytotoxic effects of these compounds is yet to be determined. Geraniol is present in palmarosa, geranium and rose oils whereas citronellol is present in oils from Boronia citriodora and *Eucalyptus citridora*. To determine their effectiveness to control Psp, essential oils from a number of plant species were selected for testing (Table 1). A range of products were selected based on having antimicrobial activity in previous in vitro assays to a range of phytobacteria including other pathovars of P. syringae or containing compounds previously shown to be efficacious (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).

In the first experiment, filter discs infiltrated with an essential oil was placed on lawns of bacteria growing on traditional culture media. To test each test, 3 of the discs were placed on each culture plate. The oils were tested against one isolate of Psp from onion and one from leek, on separate culture plates. The plates were evaluated for zones of growth inhibition around the disc and the oil rated for inhibition. Of the ten test oils, four showed very good inhibition, a further three showed good inhibition and the remaining three showed no inhibition of bacterial growth (Table 1).

Table 1 List of essential oils sourced for in vitro antibacterial testing. Their antibacterial compounds are listed where known. Oils were tested by placing 3 filter paper discs infiltrated with the test oil onto a lawn of *Pseudomonas syringae* pv. *porri* (Psp) cells. Efficacy is rated as none if there was no zone of inhibition of bacterial growth, poor if the zone was 2 mm or less, good if the zone was 2-5 mm and very good if > 5mm.

Plant species	Common name	Antibacterial compounds[1]	Efficacy to inhibit Psp growth
Thymus vulgaris	thyme (white)	carvacrol, linalool, **thymol**	very good
Origanum vulgare	oregano	**carvacrol, thymol**	very good
Ocimum basilicum	basil	**eugenol**, linalool, citronellol	good
Corymbia citriodora	eucalyptus (lemon)	**citronella[2], citronellol**	none
Syzygium aromaticum[3], syn *Eugenia caryophyllata*	clove (bud)	**eugenol**	very good
Salvia officinalis	sage		good
Foeniculum vulgare Mill	fennel (sweet)		none
Coriandrum sativum	coriander	**linalool**	very good
Cymbopogon winterianus	citronella	**citronella[2], citronellol, geraniol**	good
Lavandula angustifolia	lavender		none

[1] This list of compounds was derived from species descriptions on Wikipedia, the proportions of individual antibacterial compounds may vary with varieties; major compound(s) for the species is shown in bold. In some studies, the essential oil was shown to have bactericidal activity but the individual compounds within the oil were not identified. [2] Known to have antifungal and insect repellent activities but activity against bacteria is not known. [3] Clove essential oil is also known to contain methyl salicylate which can activate host responses in plants.

From this first experiment a selection of four oils were chosen for further work. This included three oils which showed good to very good inhibition and one which showed no inhibition of bacterial growth using the disc assay. The evaluation used a new microtitre plate method where each bacterial isolate was exposed to volatile gas from individual oils eight times per plate in a randomized order. This was repeated using three independent microtitre plates per test oil or control (no oil). To do the assay, nutrient broth was mixed with suspensions of each bacterial isolate at a rate of 10 µL of 10^9 CFU/ml bacterial suspension per 180 µL of broth and then 190 µL of the broth/bacteria mix added per well of the microtitre plate. Filter paper was infiltrated with the test oil and then attached to the inside of the microtitre plate lid. The lids were placed over the inoculated plates and then double bagged and incubated at approximately 25 °C for 24 h. The bacterial concentration in the microtitre plates was then quantified using a standard plate reader, where an OD_{620nm} of 0.3 is about 10^9 CFU/ml. The results of the different oils were compared to each other and to the control plates which were not exposed to volatile gas by averaging the replicate wells across the three replicate plates. The experiment was repeated a further two times.

The volatile gas from all four oils tested limited the growth of a Psp isolate obtained from onion. By contrast, growth of the Psp-leek was not always inhibited (Table 2). The results across the three independent experiments varied slightly which could be a result of variation in the starting concentration of the inoculum. From this experiment, lavender and clove oils were chosen for contact bactericide evaluation.

Table 2 Growth of bacterial isolates when exposed to volatile gases of different essential oils measured by absorbance at OD_{620nm} then converted to a percent growth of the control.

Psp isolate	clove (% growth)[1]			lavender (% growth)			oregano (% growth)			thyme (% growth)		
	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3	Exp1	Exp2	Exp3
onion (B1046)	45	79	57	35	35	52	43	88	58	39	86	60
leek (DAR75554)	63	67	56	56	56	54	99	122	80	96	118	83

[1] the growth of each bacterial isolate – oil combination was calculated as a percent of the growth measured on plates not exposed to oil volatiles.

To evaluate contact bactericide efficacy, bacterial suspensions prepared at *ca*. OD_{620nm} 0.3 were mixed with NA broth at a rate of 100 µL of suspension to 5 ml of broth including test solutions and incubated at room temperature for 3-4 h. Each test exposure suspensions were replicated three times and included:

1. broth only
2. broth plus 0.5% DMSO
3. broth plus 0.5% DMSO and 0.2% (v/v) mineral oil
4. broth plus 0.5% DMSO and 0.05% (v/v) clove
5. broth plus 0.5% DMSO and 0.1% (v/v) clove
6. broth plus0.5% DMSO and 0.2% (v/v) clove -
7. broth plus0.5% DMSO and 0.05% (v/v) lavender
8. broth plus 0.5% DMSO and 0.1% (v/v) lavender
9. broth plus 0.5% DMSO and 0.2% (v/v) lavender

After exposure, aliquots of each treatment combination was removed and evaluated for bacterial viability. This was done in microtitre plates which were inoculated using 10 µL aliquots of the test suspension and 190 µL of NA per well. Three replicate plates were inoculated and incubated at 25°C for 24 h then absorbance measured in a microtitre plate reader. The experiment was completed a second time.

In both experiments, clove oil was shown to be efficacious in limiting bacterial growth at the 0.2% concentration. All other treatments were less effective (Table 3).

Table 3 Growth of bacterial isolates when exposed to different concentrations of clove and lavender oil measured by absorbance at OD_{620nm}. The mean rating across all three replicate plates of the three replicate exposure tests is listed in the table.

Treatment	Average growth of Psp-onion (B1046) (OD_{620nm}) and LSD[1] at 5%		Average growth of Psp-leek (DAR75554) (OD_{620nm}) and LSD at 5%	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
broth only	0.255b	0.257c	0.190bc	0.171c
broth plus 0.5% DMSO	0.278bc	0.258c	0.191bc	0.181cd
broth plus 0.5% DMSO and 0.2% (v/v) mineral oil	0.260bc	0.256c	0.204c	0.180cd
broth plus 0.5% DMSO and 0.05% (v/v) clove	0.251b	0.335d	0.200bc	0.221e
broth plus 0.5% DMSO and 0.1% (v/v) clove	0.258bc	0.189b	0.179b	0.075b
broth plus0.5% DMSO and 0.2% (v/v) clove	**0.060a**	**0.057a**	**0.047a**	**0.043a**
broth plus0.5% DMSO and 0.05% (v/v) lavender	0.287bc	0.258c	0.196bc	0.178cd
broth plus 0.5% DMSO and 0.1% (v/v) lavender	0.261bc	0.243bc	0.244d	0.198d
broth plus 0.5% DMSO and 0.2% (v/v) lavender	0.295c	0.252c	0.271e	0.185cd

[1] 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 efficacy of essential oils to inhibit bacterial growth varies depending on the type of exposure. Lavender oil was more effective controlling Psp than clove oil when applied as a volatile but not effective when used as a contact bactericide. This was shown through the filter disc contact method and the solution exposure experiments.