Identification and monitoring of resistance in vegetable crops in Australia

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FINAL REPORT

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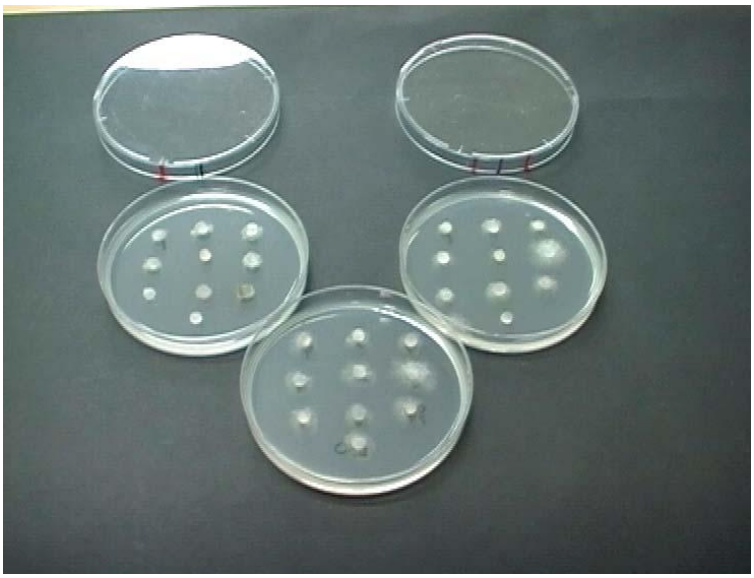

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Purpose of the Report
This report presents a literature review on chemical resistance in several important plant fungal and bacterial pathogens. Results of research to determine the extent of resistance on Australian vegetable crops are presented and discussed.

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Determining copper tolerance in isolates of the bacterium *Clavibacter michiganensis* subsp. *michiganensis* from tomato canker in Australian crops was jointly funded by the Australian Government through the Australian Centre for International Agricultural Research (ACIAR).

1. MEDIA SUMMARY

Resistance to chemical pesticides in microbial plant pathogens could be costing millions of dollars to Australia's $2 billion vegetable industry. In addition there are indirect costs from increased fungicide and copper application. There is limited data from previous Australian studies detailing resistance to chemicals. Those studies assessed chemical classes that have been used for several decades. Many newer specific fungicides have lower environmental impacts and can be safely used in IPM programs. Consequently they are increasingly relied upon by vegetable growers across Australia. This increased use is likely to hasten the likelihood of resistance developing. Furthermore, new and resistant strains of fungal and bacterial pathogens can possibly enter Australia with imported seed. The vegetable industry has no specific information about their occurrence or distribution.

This project set out to establish the resistance status of several important fungal and bacterial pathogens from Australian vegetable crops. Project researchers assessed chemicals from major chemical classes registered or under permit in Australia. In addition base-line sensitivity data was developed for fungicides with potential for future use. This will allow future measurement of shifts in sensitivity.

Project researchers firstly used reference and field isolates to determine the most effective technologies for resistance testing. They then used methods to quantify the extent and diversity of resistant populations. The results were mixed; in several instances populations of fungi and bacteria were susceptible to the chemical tested, however there were a few notable exceptions. Many *Botrytis* and *Sclerotinia* isolates were resistant to cyprodinil and some *Botrytis* isolates were resistant to boscalid. High levels of resistance to copper were found in a wide range of bacterial plant pathogens. Intermediate to high resistance levels were found in nearly 2/3 of isolates of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm)* isolates from tomatoes with bacterial canker disease. To our knowledge this has not been previously reported. Similarly high resistance to copper was detected in bacteria that cause varnish spot of lettuce. *Pseudomonas syringae* isolates from celery, parsley, silver beet and tomato exhibited intermediate levels of copper resistance. These results potentially have important implications for growers. Better control of *Cu*-resistant bacteria can be achieved by tank-mixing *Cu* with a dithiocarbamate fungicide. However, chemicals from that class disrupt certain beneficial insects and mites. This can be problematic for greenhouse vegetable growers and/or anyone using IPM or organic production systems. One alternative might be to tank-mix *Cu* with iron chloride, however this approach would require appropriate experimental testing to validate efficacy and to ensure that there are no significant detrimental consequences.

Findings from this study contributed to the development of guidelines for improved resistance management which were published through VG07110 (Best practice production models (lettuce, brassica)) and VG07109 (Development of effective pesticide strategies compatible with IPM management used on farm). A key recommendation is that growers should continue to follow resistance strategies to minimise further development of resistant populations. It is critical that availability of alternative fungicides from different classes be available for these strategies to be sustained.

2. TECHNICAL SUMMARY

Fifty isolates of *Botrytis cinerea*, 77 *Sclerotinia sclerotiorum*, 24 *Sclerotinia minor*, 19 *Bremia lactucae*, 5 *Albugo candida* and 73 pathogenic bacteria of unknown exposure to chemical controls were collected from Australian vegetable crops and alternative hosts grown adjacent to or in rotation with vegetables.

A discriminatory dose technique was used to test the sensitivity of *Botrytis* and *Sclerotinia* isolates to four fungicide families: benzimidazole (benomyl), dicarboximides (iprodione and procymidone), anilinopyrimidine (cyprodinil) and phenylpyrrole (fludioxonil). In addition, *Botrytis* isolates were tested against the fungicide family hydroxyanilide (fenhexamid) and against a rage of concentrations of two other fungicide families, oxathiin (boscalid) and strobilurin (azoxystrobin) to obtain an EC_{50} value (concentration of the fungicide causing a 50% reduction in the growth rate compared to an unamended control). *Sclerotinia* isolates were also tested against a range of concentrations of the DMI (triazole) tebuconazole. Two *S. sclerotiorum* and four *Botrytis* isolates were also tested against a range of concentrations of fluopyram, a fungicide not yet released in Australia.

Resistance to benzimidazoles was common and detected on 44% of the *Botrytis* isolates, 9% of the *S. sclerotiorum* isolates and 24% of the *S. minor* isolates resistant. Resistance to iprodione was detected in 38% of the *Botrytis*, 7% of *S. sclerotiorum* and 10% *S. minor* isolates. There was variable cross resistance between the two dicarboximide fungicides iprodione and procymidone, with all 12 *Sclerotinia* isolates testing sensitive to procymidone, irrespective of their sensitivity to iprodione. Sixty-six percent of *Botrytis* isolates were sensitive to cyprodinil, but *Sclerotinia* was less sensitive with 88% of *S. sclerotiorum* and 100% of *S. minor* isolates growing on the highest discriminatory dose. All *S. sclerotiorum* and *S. minor* isolates and most of the *Botrytis* isolates were sensitive to fludioxonil. All *Botrytis* isolates were sensitive to fenhexamid. Six percent of the *Botrytis* isolates but none of the *Sclerotinia* isolates were resistant to boscalid. Eleven percent of the *Botrytis* isolates but none of the *Sclerotinia* isolates were resistant to azoxystrobin. All *Sclerotinia* isolates were sensitive to tebuconazole.

The straminopilous fungi, *Bremia lactucae* and *Albugo candida* are both biotrophs and therefore required biological assays to determine their sensitivity to chemicals. Eight of the *Bremia lactuca* isolates established downy mildew infections on lettuce seedlings in the presence of metalaxyl. In 6 cases they were still infective when applied to plants that had been sprayed with double the standard rate of metalaxyl, suggesting they were resistant. When metalaxyl was applied in a formulation with mancozeb, or when mancozeb or copper were applied separately, *Bremia lactucae* failed to establish infections. This confirms the protectant activity of these chemicals and their usefulness when in the presence of metalaxyl resistant populations of downy mildews. All *Albugo candida* isolates were sensitive to strobilurin chemicals except for one isolate that was partially resistant to pyraclostrobin. It should be noted that only five *Albugo candida* isolates were tested as dry weather conditions persisted during the survey period of this project. Any resistance may have been overlooked due to the small population size sampled.

A range of bacterial populations from vegetable crops were demonstrated to be resistant or tolerant to copper when tested *in vitro* on copper (*Cu*)-amended media. One each of *P. syringae* isolates from cucumber and leek were capable of growth on 1mM *Cu*-amended media. This was considered to be a high level of copper resistance and exceeded the tolerance

(0.75mM) of the positive control bacterium (*P. syringae* pv. *tomato*, isolate *PT23*) which is known to contain plasmid-mediated resistance genes. Two isolates of *P. cichorii* from varnish spot affected lettuce were resistant to 0.75mM *Cu*-amended media. Meanwhile, further *P. syringae* isolates from celery, parsley, silver beet and tomato exhibited intermediate levels of copper resistance, tolerating 0.5mM *Cu*-amended media. Several other bacterial isolates were sensitive to low copper concentrations. These included isolates of *Xanthomonas campestris* pv. *campestris* from black rot affected brassicas.

Sixty-two percent (40/65) of tomato bacterial canker isolates of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm)* grew on 0.5 mM *Cu*-amended media. Of these more than half (23) tolerated 0.75mM *Cu*, and a further 3 isolates had an upper tolerance level of 1mM *Cu*-amended media. *Cu* resistance has not been previously reported in *Cmm* isolates. This has some implications for tomato growers, particularly greenhouse growers who are using IPM. Better control of *Cu*-resistant bacteria can be achieved by tank-mixing *Cu* with a dithiocarbamate fungicide. However, chemicals from that class disrupt certain beneficial insects and mites. One alternative might be to tank-mix *Cu* with iron chloride, for which effectiveness to copper resistant bacterial populations was previously demonstrated by Lee et al. (1993) and Scheck & Pscheidt (1998).

Overall this study has shown that fungicide resistant populations exist in the vegetable industry, albeit at low levels for many of the fungicides. Resistance strategies should be followed by all growers to minimise further development of resistant populations. The availability of a wide range of fungicide classes is critical to provide the alternative fungicides necessary for these strategies to be effective.

Table of contents

3. GENERAL INTRODUCTION

Project synopsis

Many growers report disappointing levels of control of fungal and bacterial disease on vegetable crops after spray programs. This can result from incorrect application, incorrect identification of the causal organism(s) and/or the development of genetic resistance in the pathogen. Overuse of fungicides, for example where resistance has developed in *Botrytis*, has been documented to reduce yields by 20% in greenhouse vegetable crops.

The development of widespread resistance to fungicides is a serious problem. Previous research has shown that resistance can occur in some areas even before fungicides have been used, and that the frequency of mutations conferring resistance increases with every application of fungicide. Establishing methods to detect and monitor fungicide resistance in the Australian vegetable industry will identify emerging resistance problems and help formulate recommendations for product use and spray rotation.

This project was undertaken to determine the chemical resistance status in populations of *Botrytis cinerea*, *Sclerotinia* spp., *Bremia lactucae*, *Albugo candida* and a range of bacterial pathogens that occur on Australian vegetable crops. In addition base-line sensitivity data was developed for fungicides that were potential candidates for future use, so any shifts in sensitivity can be monitored. The methods used for testing resistance were chosen where possible from the Fungicide Resistance Action Committee (FRAC) standards (www.frac.info), using discriminatory doses of fungicides in amended agar to determine levels of resistance. Where no discriminatory doses were published, or no FRAC standard was available, organisms were tested against a range of concentrations of each chemical to determine the base-line sensitivity from the dose response curve.

4. LITERATURE REVIEW

4.1 Introduction

Within a population of plant pathogenic fungi (or bacteria), naturally occurring genetic variation results in the presence of both fungicide sensitive and inherently fungicide resistant individuals. Fungicide resistance occurs when the overall fungus population changes genetically following exposure to a fungicide so that it is less susceptible or sensitive to that fungicide (or class of fungicide). Initially a fungicide might kill most individuals in a population except for those bearing natural resistance, and as those resistant individuals multiply, the genetic background of the population changes. Eventually a higher dose of fungicide will be required to kill all of the individuals within that increasingly resistant fungal population, or finally even the highest practical dose may not give control (Martin & Zydenbos 2005).

In the early history of fungicide usage, only copper, sulphur and other protectant fungicides such as thiram, maneb or captan were used, and their physical presence on the surface of a plant typically did not allow fungal spores to germinate, or killed spores upon germination. No resistance was observed with these protectant fungicides (Agrios 1988). In the 1960's, strains resistant to the fungicides diphenyl, hexachlorobenzene and PCNB (all of which contain a benzene ring) were all found to occur naturally. Later, with the introduction and widespread use of systemic fungicides which are translocated throughout the plant providing both protective and curative properties, there was an appearance of numerous strains of fungi resistant to one or more of these fungicides. In many cases this became a widespread issue within only one or two years of use of the chemical. These systemic fungicides tend to be specific in their action, and if they control only one or two steps in the metabolism of the fungus, then a resistant population can rapidly develop with only a single mutation or by selection of resistant individuals (Agrios 1988).

The term 'resistance' should only be used to define a stable and heritable adjustment by a fungus to a fungicide, resulting in a considerably reduced sensitivity to the inhibitor (Köller & Scheinpflug 1987). This resistance should not be confused with a 'momentary' adaptation of a fungal pathogen to a fungicide, as these adaptations are neither heritable nor stable. Thus the term 'field resistance' should only be used when decreased fungicide efficacy is correlated with increased frequency of resistant strains, however this is often difficult to prove or disprove.

Worldwide, studies are carried out and the status of fungicide resistance by different pathogens reported in different crops against the many different classes of fungicides. These studies and reports are numerous and only a limited number will be discussed in this document. However, as an indicator of the magnitude of fungicide resistance, Yourman and Jeffers (1999) found that of *Botrytis* isolates tested and originating from vegetable-growing greenhouses, 32% from Europe and Israel were resistant to benzimidazole and dicarboximide fungicides, 75% from Connecticut USA were also resistant to benzimidazole and dicarboximide, and 63% from England and Wales were resistant to benzimidazole (including 58% from untreated plants).

Within Australia there is limited information about the status of fungicide resistance in vegetable crops. O'Brien (1992) undertook some investigations and developed testing

procedures for 26 fungal pathogens for sensitivity to a few fungicides. Pung et al. (2004) reported pea downy mildew (*Peronospora viciae)* resistance to metalaxyl in northern Tasmania, and failure of azoxystrobin to control white blister in brassicas (*Albugo candida)* suggested resistance had developed (E. Minchinton, pers com). However, the status of this resistance was not confirmed. High levels of *Botrytis* isolates resistant to benomyl (80%), iprodione, and dichlofluanid were reported in Victoria in strawberry crops (Washington et al. 1992), and although it is believed that there is *Botrytis* resistance to other fungicides in many vegetable growing systems, this has not been confirmed.

4.2 Effect on production

Crop losses attributable to pathogenic fungi such as *Botrytis* have been estimated at 30% (De Waard 1993) to 53% (Washington et al. 1992). The effect of fungicide resistance on crop yield has been widely reported as 'significant' and 'severe' (Yourman & Jeffers 1999, Brown et al. 2004, Myresiotis et al. 2008). However, from the figures quoted previously, one can only conclude that if resistant isolates represent up to 80% of a fungal population, then crop losses attributable to fungicide resistance could be in the order of 20-30%. In an Australian vegetable industry worth over $2 billion (Hickey & Hoogers 2006), fungicide resistance could result in losses worth millions of dollars. In addition there are indirect costs from increased fungicide applications.

Despite the often large reported percentages of resistant isolates, poor disease control by a fungicide is not always attributable to the presence of resistant strains in the field, and can often be caused by misidentification of the pathogen, improper application, deteriorated product and extremely high infection pressure.

4.3 Mechanisms of resistance and monitoring

Considerable effort has focussed on determining the mechanisms of resistance, and the mechanisms are well understood for many groups of fungicides, including the benzimidazoles and dicarboximides. Many types of resistance mechanisms are known for fungi, and these include alteration of the biochemical target site so that it is no longer sensitive, increased production of the target protein, developing an alternative metabolic pathway that bypasses the target site, metabolic breakdown of the target fungicide, and exclusion or expulsion of the fungicide through ATP-ase dependent transporter proteins (Brent & Holloman 2007). For bacteria, resistance mechanisms include sequestering of copper in the periplasm and outer membrane (Cooksey 1994) and removal of excess copper from the cells (Teixeira et al. 2008).

Monitoring for fungicide resistance involves testing of field populations of target pathogens for their level of sensitivity to one or more fungicides, and this is a means of gaining an early warning on possible control failure.

In order to detect a change in the sensitivity of a population, it is crucial that monitoring methods are developed early such that base-line data on typical pathogen populations can be obtained before they are exposed to widespread use of a new fungicide. This can be used to determine dose response levels and discriminatory doses for testing large populations of fungi. Practical performance of a fungicide must be systematically observed and documented in order to establish a clear correlation between the incidence

of resistant biotypes and the deterioration of field performance of the fungicide (Brent & Holloman 2007).

4.4 Techniques to detect resistance

Internationally, the Fungicide Resistance Action Committee (FRAC) sets the standards for assessing and monitoring fungicide resistance in Europe (de Waard et al. 1993), and maintains a catalogue of recommended methods on its' website (www.frac.info). Such standardisation of test method enables direct comparisons between results from different research centres.

In most cases, a bioassay is used to determine the sensitivity of a population. These bioassays might use leaf disc, whole plant testing or mycelial growth, spore germination or germ tube elongation on fungicide-amended artificial media. The development of methods which allow larger numbers of samples to be tested can be applied, including spore germination assays in multi-well plates, growth in liquid medium of fungi using a spectrophotometer, and where molecular methods of resistance are known and point mutations causing them are defined, various real-time PCR diagnostic technologies (Brent & Holloman 2007).

Whilst many fungi and bacteria can be easily tested using current technology on artificial media, obligate parasites (such as powdery and downy mildew) are unable to be grown on artificial media, and in these cases molecular methods are the ideal option. Some of the new generation of fungicides, such as the strobilurins, are difficult to test *in vitro* as the fungus has developed alternative metabolic pathways when grown on artificial media and hence will mimic resistance. For example, when *in vitro* testing the fungicide azoxystrobin, the chemical SHAM (salicyl hydroxamic acid) must be added to inhibit the induction of alternative oxidase respiration (Olaya & Koller 1999).

In the current study, the organisms tested against various chemicals were grey mould (*Botrytis cinerea*), white mould (*Sclerotinia* spp.), downy mildew on lettuce (*Bremia lactucae*), white blister on Brassica (*Albugo candida*) and various bacteria from a range of vegetable hosts.

4.4.1. Fungicide sensitivity of Botrytis and Sclerotinia

Grey mould, caused by *Botrytis cinerea*, the anamorph of *Botryotinia fuckeliana*, is one of the most important diseases of crops worldwide. The fungus attacks a wide range of plant species, including many vegetables (Koike et al. 2007, Sun et al. 2010), infecting plants through wound sites and resulting in stem and plant death. In some plants, such as cucumber (Fig. 1), infection occurs through flowers and causes direct fruit loss (Elad & Yunis 1993, Yunis & Elad 1991). The infection begins as a watery rot which in the presence of humidity develops into a characteristic grey fluffy growth. *B. cinerea* requires high humidity and temperatures from 10-20°C for an infection to develop, however sporulation of the fungus and dispersal of spores occurs at lower humidity and across a wide range of temperatures (Jarvis 1980).

Diseases caused by *Sclerotinia sclerotiorum* (Fig 2) and *Sclerotinia minor* result in serious yield losses in a wide range of vegetable crops (Persley et al 2010). They survive as sclerotia and microsclerotia in the soil or on plant debris for up to 10 years

(Adams & Ayres 1979, Punja 1985) and can be spread by movement of the infected soil. The sclerotia germinate, producing mycelia which colonise roots and stems of the host. Sclerotia of *S. sclerotiorum* also produce apothecia, from which airborne spores are splash and wind dispersed causing infection of foliage (Adams & Ayres 1979).

Figure 1. *Botrytis cinerea* on cucumber

Figure 2. *Sclerotinia sclerotiorum* on cabbage.

Various methods developed to monitor fungicide resistance are recommended by FRAC, with one commonly used method being the colony radial growth assay (Smith et al. 1991). Plugs of mycelia are placed on agar amended with fungicide and the radial growth of the mycelia is measured and compared with the growth on unamended agar. For *Botrytis* and *Sclerotinia*, organisms were challenged by discriminatory doses of fungicides in amended agar to determine levels of resistance. Discriminatory doses of fungicides are based on EC_{50} values and are used to monitor resistant populations. The EC_{50} is the concentration of active ingredient that suppresses mycelial growth or spore germination to 50% of growth with no fungicide. Where no discriminatory doses were available, the fungi were evaluated for sensitivity by testing against a range of concentrations to determine the EC_{50} for each organism and chemical combination.

4.4.1.1. Carbendazim / benomyl

Benzimidazole fungicides have been widely used in vegetables, being very effective on a wide range of fungal diseases. However the main fungicide in this group, Benlate® (benomyl) was withdrawn from registration several years ago. Other fungicides in the same group, such as carbendazim (eg Bavistin®) are still registered for some crops, but not for most vegetables.

Populations of *Botrytis* resistant to benzimidazoles are generally stable and can persist for many years (Moorman & Lease 1992, Myresiotis et al. 2007), with significant loss of field control. Discriminatory doses have been determined previously for the benzimidazole Benlate®, with Northover (1988) used 1 mg/L as the discriminatory dose of benomyl, with radial growth of over 2 mm indicating resistance. The mycelial

growth of sensitive populations of *Botrytis* was reduced between 0.01 and 0.1 mg/L, with an EC_{50} of below 0.1 mg/L (Leroux et al. 1999, Northover & Matteoni, 1986). However the EC_{50} of moderately resistant populations was between 4 and 7 mg/L and of highly resistant populations was over 25 mg/L (Leroux et al. 1999). O'Brien (1992) recommended a discriminatory dose of 10 ppm (mg/L) a.i. benomyl, which would prevent spore germ tube growth in sensitive isolates.

Strains of *S. sclerotiorum* from alfalfa and canola sensitive to benomyl were inhibited at EC_{50} of <8 mg/L (Gossen *et al* 2001). Porter and Phipps (1985a) showed growth of *S. minor* isolated from peanuts to be inhibited when grown on agar amended with 1 and 2 mg/L benomyl.

4.4.1.2. Iprodione, procymidone

Dicarboximide resistant populations of *Botrytis* are generally unstable and are less fit than the sensitive populations. However *Botrytis* populations resistant to both benzimidazoles and dicarboximide fungicides are common and stable and can develop in the absence of fungicides (Yourman et al. 2001).

Iprodione (Rovral®) is registered to control both *Botrytis* and *Sclerotinia* in many vegetable crops, whereas procymidone (Sumisclex®) is only permitted for *Sclerotinia*. Resistant populations are known to occur for both fungi (Hubbard et al. 1997, Ma et al. 2009, Myresiotis et al. 2007). However there is conflicting evidence about the level of field control caused by dicarboximide resistance. While Hubbard et al. (1997) and Moorman and Lease (1992) found no evidence of increased disease levels being correlated with dicarboximide resistance, Panagiotakou and Malathrakis (1983) showed a decline in effectiveness when dicarboximide fungicides were used in greenhouse crops with resistant populations of *Botrytis*.

Several levels of dicarboximide resistance in *Botrytis* have been reported. Northover and Matteoni (1986) used discriminatory doses of 2 and 10 mg/L to separate low-level and high level resistant populations of *Botrytis*, using the criterion of radial growth on amended agar exceeding 20% of the growth on unamended agar. Beever and Brien (1983) used a more complex system, separating high and low level resistance by the levels of growth on the agar amended with discriminatory doses of 2 mg /L, 5 mg/L and 10 mg/L compared to the growth on unamended agar. Beever et al. (1989) recognised a further classification of ultra-low-level resistance, showing that populations within this new level of resistance were as competitive as the sensitive strains, but still less virulent.

Ma et al. (2009) reported that an EC_{50} of 2.5 mg/L could be used to distinguish dicarboximide resistant isolates of *S. sclerotiorum*. Porter and Phipps (1985a) reported growth of *S. minor* isolated from peanuts to be inhibited when grown on agar amended with 1 and 2 mg/L benomyl or >0.5 mg/L procymidone. Brenneman et al. (1987) used 2 mg/L iprodione and Hubbard and Subbarao (1994) 5 mg/L iprodione for detection of dicarboximide resistance in *S. minor.*

4.4.1.3. Boscalid

The oxathiin boscalid is a broad spectrum fungicide registered overseas to control *Botrytis cinerea, Sclerotinia* spp., *Alternaria* spp., *Monilinia* spp., powdery mildews and

other pathogens in fruit, vegetables and vines (Stammler & Speakeman 2006). In Australia, it is used under permit to control Botrytis neck rot in onions and *Sclerotinia* in beans, lettuce and Brassica and is registered for Botrytis bunch rot in grapevines.

Resistance to boscalid has been reported in *Botrytis* isolates from kiwi fruit (Bardas et al. 2010), stored apples (Kim & Xiao 2010) and grapevines (Fillinger et al. 2008). It is reported to be caused by alterations in the respiratory succinate dehydrogenase (Avenot et al. 2008).

Several papers have reported the sensitivity of *B. cinerea* or *S. sclerotiorum* to boscalid, with varying EC_{50} or ED_{50} values (Table 1). The mean EC_{50} values for *Botrytis* from the microplate tests appeared to be significantly lower than those found with mycelial growth tests on amended agar; however Leroch et al. (2010) used a comparatively high discriminatory dose of 5 ug/ml with a microplate test.

Table 1. EC_{50} or ED_{50} values for isolates sensitive to boscalid.

Citation	**Country**	***Pathogen***	**Method**	**Mean EC_{50} ug/ml**
Myresiotis et al. 2008	Greece	*Botrytis cinerea*	mycelial growth (agar)	2.14
Zhang et al. 2007	China	*Botrytis cinerea*	mycelial growth (agar)	1.07
			conidial germination	0.42
Stammler and Speakeman 2006	Germany	*Botrytis cinerea*	mycelial growth (microplate)	0.07*
Stammler et al. 2007	Germany	*Sclerotinia sclerotiorum*	mycelial growth (microplate)	0.01*

* Described as ED_{50}

4.4.1.4. Azoxystrobin

Azoxystrobin belongs to the strobilurin class of fungicides, derived from the wood-rotting mushroom fungi *Strobilurus tenacellus*. The mode of action of the strobilurins, or quinone outside inhibitors (QoI) fungicide group, involves affecting mitochondrial respiration by binding to the Qo site of the cytochrome *b*. Because of the site-specific mode of action, strobilurins have a relatively high resistance risk, and reduced sensitivity has been shown to develop rapidly (Bartlett et al. 2002). Resistance to azoxystrobin has been reported to *Botrytis cinerea* in citrus and strawberry (Ishii et al. 2009).

In vitro sensitivity of *Botrytis* to azoxystrobin has been evaluated either by spore germination (Myresiotus et al. 2008) or mycelial plug assay (Ishii et al. 2009). While azoxystrobin is not registered for Botrytis grey mould in vegetables, it is used for other diseases, and may have an impact on the sensitivity of the fungus if it is present when the product is applied. *S. sclerotiorum* sensitivity to boscalid and iprodione has been evaluated using the mycelial plug assay (Liu et al. 2009), however little has been reported on sensitivity to azoxystrobin. As azoxystrobin now is permitted for Sclerotinia rot on a range of vegetables, base line data should be generated.

Using *in vitro* evaluations of the strobilurins requires the addition of salicylhydroxamic acid (SHAM) to inhibit an alternative respiration pathway that operates in fungi (Myresiotis et al. 2008, Olaya & Koller, 1999).

4.4.1.5. Tebuconazole

Tebuconazole is a sterol demethylation-inhibiting (DMI) fungicide belonging to the triazole class. Used on grapes, cereals, stone fruits, peanuts, bananas and other fruits, it has a broad spectrum of antifungal activity, including *Sclerotinia* spp. and *Botrytis cinerea.* It is currently used control Sclerotinia rot on several vegetable crops.

Resistance in field isolates of *Mycosphaerella* in cereal (Leroux & Walker 2011) and *Penicillium expansum* in apple (Karaoglanidis et al. 2011) has been detected using mycelia growth on amended agar.

Some *in vitro* testing of *Sclerotinia* sensitivity to tebuconazole has been undertaken during fungicide screening investigations with *S. sclerotiorum* (Gwiazdowski & Jajor 2005) and *S. homoeocarpa* (Ok et al. 2011), but no resistance has been reported.

4.4.1.6. Fenhexamid

Fenhexamid belongs to the hydroxyanilide class of sterol biosynthesis inhibitors, which inhibit mycelial growth and spore tube elongation (Fillinger et al. 2008). It is used in many crops to control *Botrytis*. In Australia, it is registered for *B. cinerea* on grapes and strawberries and permitted for use on capsicum, lettuce and herbs. Testing on lettuce showed it was not effective at controlling *Sclerotinia* (Matheron & Porchas 2004).

Leroux et al. (2002) found three difference phenotypes of resistant strains of *Botrytis* with *in vitro* tests. The manufacturer Bayer (Lechaise pers comm. 2010) in their sensitivity testing used either a mycelial growth assay in a microplate plate with a discriminatory dose of 0.2 µg/ml, or a germ tube growth assay on amended agar with a discriminatory dose of 4 µg/ml. The former detects all three phenotypes, and the latter only one phenotype. However this latter phenotype is the only one to be detected in commercial monitoring and to have potential impact on field efficacy (Albertini & Leroux 2004). The tests used and the discriminatory doses chosen by other investigators to classify *Botrytis* as resistant to fenhexamid is variable. Baroffio et al. (2003) and Myresiotis (2007) both used an ED_{50} >= 0.1 µg /ml with a mycelial plate growth assay, whereas Leroch et al. (2010) used 5 µg/ml with a microplate plate test.

Resistance to fenhexamid has been detected in *Botrytis* from several crops, including cucumber (Myresiotis 2007), grapes (Baroffio et al. 2003) and strawberry and raspberry (Weber 2010).

4.4.1.7. Cyprodinil, fludioxonil

The anilinopyrimidine class of fungicides, containing cyprodinil and pyrimethanil, inhibit both germ tube and mycelial growth (Baroffio et al. 2003). They are used to manage *Botrytis* is a wide range of crops, including vegetables. Cyprodinil is formulated as a combined product with fludioxonil, a phenylpyrrole, which inhibits germination and alters germ tubes (Baroffio et al. 2003) and is sold as Switch®.

The FRAC recommendations are to use a discriminatory dose of 2.5 mg/L pyrimethanil with the mycelial growth test (Birchmore & Forster 1996). While investigations have shown sensitive isolates are inhibited at low EC_{50} values of 0.03 µg/ml cyprodinil (Baroffio et al. 2003, Myresiotis et al. 2007) or 0.1 – 0.7 µg/ml pyrimethanil (Moyano et al. 2003, Myresiotis et al. 2007), Birchmore & Forster (1996) found the resistant reference strain had an EC_{50} of 6.5 – 7 mg/L.

While pyrimethanil is currently permitted for use on some vegetables, the use of the combined product (e.g. Switch ®) reduces the probability of resistant populations developing. A discriminatory dose of ED_{50} >= 0.1 µg/ml was reported for undertaken *in vitro* testing of fludioxonil on *Botrytis* from vineyards (Baroffio et al. 2003).

Resistance to both the anilinopyrimidines and fludioxonil has been reported on *Botrytis* from grapes (Baroffio et al. 2003) and tomato (Korolev et al. 2009), however resistance to fludioxonil is more common in other pathogens such as *Penicillium* from apple (Li & Xiao 2008) and *Fusarium* on potato (Gachango et al. 2011).

4.4.1.8. Fluopyram

Fluopyram is a fungicide in the new pyridylethylamides chemical class, a succinate dehydrogenase inhibitor (SDI) which is active against a broad range of fungi (Fought et al. 2009). Boscalid is also a SDI fungicide, however so far there has been no indication of cross resistance with powdery mildew or *Corynespora cassiicola* in cucumber (Ishii et al. 2011) or *Alternaria alternata* in pistachio (Avenot & Michailides 2010). Avenot and Michailides (2010) suggested that the lack of cross-resistance of fluopyram could be from the higher activity of this fungicide, and that the binding site may be slightly different.

Testing for sensitivity to fluopyram was undertaken by mycelial growth tests, using EC_{50} values of <0.25 µg/L for *A. alternata* (Avenot & Michailides 2010) and <0.62 µg/L for *C. cassiicola* (Ishii et al. 2011).

4.4.2. Fungicide sensitivity of downy mildew and white blister

Studies in the U.K. have shown that downy mildew (*Bremia lactucae*) in lettuce (Fig. 3) rapidly developed resistance to phenylamide fungicides (such as metalaxyl) (Crute et al. 1987) and had spread to most lettuce production regions of the country within a two year period (Crute & Harrison 1988). Due to the obligate parasitic nature of this fungus, resistance testing involved inoculating cotyledons growing in fungicide solution with spore suspension before rating the percentage of seedlings bearing sporophores (Crute et al. 1987). Similar methods were used by Reuveni et al. (1980) in Israel for metalaxyl resistance in the downy mildew of curcurbits, *Pseudoperonospora cubensi*, and Brown et al. (2004) in California for fosetyl-aluminium and maneb fungicides. Cooke et al. (1996) assessed floating leaf discs in their resistance survey against phenylamides with *Phytophthora infestans* of potatoes.

Metalaxyl is the most active of the phenylamide fungicides, with inhibition of sensitive *B. lactucae* sporulation at 0.1 µg/ml, whereas resistant isolates grow with high level of

metalaxyl, producing sporulation at 100 µg/ml (Crute et al. 1987). Brown et al. (2004) used 50 µg/ml metalayxl as the discriminatory dose.

Sensitivity to dimethomorph has been studied in populations of *Phytophthora infestans* (Stein & Kirk 2004), and sensitivity to phosphorous acid (a phosphonate) with *Phytophthora capsici* (Bower & Coffey 1985) and other *Phytophthora* and *Pythium* species (Fenn & Coffey 1984), but neither of these fungicides appears to have been tested with downy mildew or white blister.

Definitive studies on the sensitivity of white blister (Fig. 4) have not been reported. Petkowski et al. (2005) suggested that reduced control with metalaxyl-M in trials was possibly due to resistance, but no further testing appears to have been undertaken. Lui and Rimmer (1992) showed that white blister developed resistance after five consecutive sprays of a systemic fungicide.

Figure 3. Downy mildew on lettuce

Figure 4. White blister on broccoli

4.4.3. Copper tolerance in bacteria

Copper resistance was first detected in the 1980s however it was noted that the presence of resistant populations did not always lead to failure of control, so resistance may have been present much earlier (Cooksey 1990). In addition, copper resistance was found to vary slightly with the formulation of the copper used, with some formulations when applied as a mix with other fungicides providing field control where resistance strains were detected (Scheck & Pscheidt 1998).

Copper resistance or tolerance in bacteria can be assessed by growing the bacteria on media supplemented with copper (Goto et al. 1994, Scheck et al. 1996) or by placing treated paper discs onto cultures and measuring zones of inhibition (Adaskaveg & Hine 1985).

Copper resistance has been detected in many of the plant pathogenic bacteria, with most occurring in the pseudomonads (Cooksey 1990, Goto et al. 1994). In Australia, copper resistance was detected in *Pseudomonas syringae* pv. *tomato* (Tesoriero et al. 1997), with the suggestion that it was introduced in the early 1990s with infected seed. Resistance has also been found in many of the xanthomonad group of bacteria, with

reports on peppers (Adaskaveg & Hine 1985, Cooksey 1990), carrots (Parks & Crowe 1998) and grapes (Marques et al. 2009).

Figure 5. Black rot on cabbage caused by *Xanthomonas campestris pv. campestris*

4.5 Fungicides for use on vegetables in Australia

Diseases in vegetable crops are usually managed by fungicide applications. A number of fungicides are either permitted or registered to use for management of these diseases in Australia (Tables 2 & 3), requiring knowledge of sensitivity testing to provide effective monitoring of resistant populations.

Table 2. Products registered for control of diseases caused by Botrytis or Sclerotinia in Australia March 2011 (Source: P. Dal Santo (AgAware). For full details: APVMA - www.**apvma**.gov.au*)*.

ACTIVE	CROP	Pathogen
azoxystrobin	Tomatoes, beans, lettuce	*Sclerotinia*
boscalid	Beans - Green, brassica, lettuce	*Sclerotinia*
boscalid + iprodione*# + chlorothalonil	Onions	*Botrytis*
carbendazim*#	Onions - Post-harvest	*Botrytis, Sclerotinia*
chlorothalonil	Beetroot, Spring Onions, Chervil, Globe artichokes - Globe, Broad Beans, Capsicum, Cucumbers, Endive, Snow peas, sugar peas, radishes, silverbeet, spinach, tomato	*Botrytis*
chlorothalonil + pyrimethanil*	Chicory (Leaves), Endive, Silverbeet (Chard), Spinach	*Botrytis*
copper	Beans, Chervil, galangal, rocket	*Botrytis*
copper + mancozeb + iprodione# + others	Vegetables - Leafy - Brassica	*Sclerotinia*
dimethomorph + mancozeb	Shallots, spring onions	*Botrytis*
fenhexamid*	Capsicums, chervil, lettuce, mizuna, rocket	*Botrytis*
guazatine	Tomatoes - Post-harvest	*Botrytis*
iprodione*	Brussels Sprouts,	*Botrytis*
iprodione#	Celery, celeriac, chilli,	*Sclerotinia*
iprodione*#	Lettuce, tomatoes, beetroot, Chinese brassica, curled mustard, Indian mustard, kale, mibuna, mizuna, mustard greens, rutabaga greens, turnip greens, leafy brassica	*Botrytis, Sclerotinia*
mancozeb	Cucumbers, peas, legumes, broad beans	*Botrytis*
procymidone*#	Capsicums, chervil, chilli, galangal, mizuna, rocket	*Sclerotinia*
pyrimethanil*	Capsicums, peas, snow peas, tomatoes	*Botrytis*
tebuconazole	Beetroot, chicory, endive, radishes, silverbeet, spinach, lettuce	*Sclerotinia*
thiram	Celery, lettuce	*Botrytis*

* resistance reported to *Botrytis,* not necessarily on the crop with registered use.
\# resistance reported to *Sclerotinia*, not necessarily on the crop with registered use.

Table 3. Products registered for control of downy mildew of lettuce and white blister of Brassica in Australia, March 2011 (Source: P. Dal Santo (AgAware). For full details: APVMA - www.**apvma**.gov.au*)*.

Fungicide	**Disease**
azoxystrobin	white blister
chlorothalonil	white blister
copper as ammonium acetate	lettuce downy mildew
copper as cuprous oxide	lettuce downy mildew, white blister
copper as hydroxide	lettuce downy mildew, white blister
copper as hydroxide + metalaxyl-M	white blister
copper as oxychloride	lettuce downy mildew, white blister
copper as sulphate (tribasic)	lettuce downy mildew
dimethomorph	lettuce downy mildew
dimethomorph + mancozeb	white blister
mancozeb	lettuce downy mildew, white blister
mancozeb+metalaxyl-M	lettuce downy mildew, white blister
metiram	lettuce downy mildew
phosphorous acid	lettuce downy mildew
propineb	lettuce downy mildew
propineb+oxadixyl	lettuce downy mildew
pyraclostrobin	white blister

A range of copper formulations are registered or have permits for use on vegetable crops in Australia, however each formulation may have a difference disease/crop combination use. These include:

- copper (Cu) as copper octanoate
- copper (Cu) present as copper ammonium acetate
- copper (Cu) present as copper ammonium complex
- copper (Cu) present as copper oxychloride
- copper (Cu) present as copper/cupric hydroxide
- copper (Cu) present as cuprous oxide
- copper (Cu) present as tribasic copper sulphate

5. FUNGICIDE RESISTANCE IN GREY MOULD AND SCLEROTINIA ROT

5.1 Introduction

Grey mould, caused by *Botrytis cinerea*, the anamorph of *Botryotinia fuckeliana*, is one of the most important diseases of crops worldwide. The fungus attacks a wide range of plant species, including many vegetables (Koike et al. 2007, Sun et al. 2010). Diseases caused by *Sclerotinia sclerotiorum, S. minor* and *Sclerotium rolsfsii* also result in serious yield losses in a wide range of vegetable crops (Persley et al. 2010). The regular application of fungicides is one method of control for diseases caused by *Botrytis* or *Sclerotinia* (Gullino 1992, Patterson & Gorgan 1985, Steadman 1979, Yunis & Elad 1991). However there is a limited range of products registered or with permits in Australia for use on vegetables (Tables 2 & 3). Development of fungicide resistance has been reported for many of these fungicides to *Botrytis* (Jarvis 1992, Myresiotis *et al* 2007, Sun *et al* 2010, Yunis & Elad 1991, Zang et al. 2007, 2009), however resistance within *Sclerotinia* populations are less common (Gosson et al. 2001, Hubbard et al. 1997, Ma et al. 2009). While the reported resistance is often on crops other than the ones registered, these fungi are not host specific, so there is potential for the resistant populations to move between crops.

This project component was undertaken to determine whether fungicide resistance populations of *Botrytis cinerea* and *Sclerotinia* spp. occur in vegetable crops within Australia. In addition base-line sensitivity data were developed for fungicides that were potential candidates for future use, so any shifts in sensitivity can be detected. The methods used for testing resistance were chosen where possible from the Fungicide Resistance Action Committee (FRAC) standards (www.frac.info), using discriminatory doses of fungicides in amended agar to determine levels of resistance. Where no discriminatory doses were published, or no FRAC standard was available, organisms were tested against a range of concentrations of each chemical to determine the base-line sensitivity from the dose response curve.

5.2 Materials and Methods

5.2.1. Fungal collection

Isolates of *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *S. minor* were obtained either from infected plant material or culture collections.

Plant material was placed into trays lined with wet "chux" wipes covered with paper towel and sealed in a plastic bag to create near 100% humidity. Fresh sporulating fungal growth or sclerotia were transferred with sterile forceps to potato dextrose agar (PDA) and incubated at room temperature (~22^{o}C).

Isolates from culture collections, received either as agar slopes or as dried sclerotia, were re-cultured onto PDA and incubated at room temperature (~22^{o}C).

5.2.2. Storage of isolates

Botrytis cultures were stored in water or on swabs. *Sclerotinia* cultures were stored either as sclerotes or on Whatman filter paper.

Water storage: Squares of agar (1 cm x 1 cm) with mycelial growth were cut from artificial media and approximately 15 squares were added to 10 ml of sterile distilled water in sterile McCartney bottles. Bottles were stored in the dark at 4^{o}C.

Swabs: Single use sterile swabs were used to collect spores from actively sporulating plates of *Botrytis*. Swabs were stored in the dark at 4^{o}C for up to 12 months.

Sclerotes: Dry sclerotia from mature *Sclerotinia* cultures were dried in a desiccator for one week in sterile McCartney bottles before storing at room temperature.

Whatman filter paper: PDA plates were inoculated with an agar plug from actively growing culture, then surrounded with squares of sterile Whatman filter paper (1 cm x 1 cm). Once the plates fully matured, the colonised filter paper squares were removed and placed in sterile envelopes and dried in a desiccator for 1 week. The envelopes were stored in a sealed airtight container with desiccant at 20^{o}C.

5.2.3. Amended agar preparation

Agar media was autoclaved and cooled to 70˚C, before adding various rates of commercial formulations of fungicides (Table 4). Potato dextrose agar (PDA) was used as the base media for fungicides except fenhexamid (tap water agar TWA) and cyprodinil (synthetic media, Hilber & Schuepp 1996). Streptomycin sulphate was included to manage bacterial contamination and SHAM was added at 100 mg/L to any agar amended with azoxystrobin.

Table 4. Active ingredient and manufacturer of fungicides tested

Active ingredient	Product	Manufacturer
azoxystrobin 500g/kg	Amistar WG	Syngenta
benomyl 500g/kg	Benlate	DuPont
boscalid 500g/kg	Filan	Nufarm
cyprodinil 500g/kg	Chorus	Syngenta
fenhexamid 500g/L	Teldor 500 SC	Bayer
fludioxonil 230g/L	Scholar 230 SC	Syngenta
fluopyram 500g/L	Luna 500 SC	Bayer
iprodione 500g/L	Rovral Aquaflo	Bayer
procymidone 500g/L	Sumisclex 500	Sumitomo
tebuconazole 430g/L	Folicur 430 SC	Bayer

5.2.4. Inoculation techniques

Culture retrieval: When required for inoculation, bottles or plates were removed from storage and allowed to reach room temperature prior to use. The stored sclerotia, filter paper squares or agar plugs in water were removed under sterile conditions and placed onto PDA and incubated at room temperature (~22^{o}C) for 5 days before use. When required for spore suspensions, plugs of agar with actively growing *Botrytis* mycelia were placed onto V8 agar and incubated at room temperature for 2 to 4 weeks.

Mycelial inoculation: A mycelial plug (6 mm diameter) was cut from the margin of an actively growing 5 day old culture and placed inverted onto the fungicide amended media.

Spore inoculation: Spores from mature *Botrytis* colonies grown on V8 plates were examined microscopically to ensure the absence of contaminants. Approximately 10 mL of sterile RO water was applied to the surface of the culture, then gently agitated with a swirling motion using a firm craft brush. The resulting spore suspension was sieved through fine muslin to remove hyphal matter and diluted to 1x10^{5} spores/mL using a Neubauer haemocytometer. 500 µL of this spore suspension was spread across amended and control plates.

Incubation: Inoculated plates were incubated at room temperature (~22^{o}C) under laboratory lighting conditions. For mycelial growth assays and resistance tests with existing discriminatory doses, plates were incubated for 48 hours. For assessment of spore germination, plates were incubated for 24 hours.

5.2.5. Fungicide sensitivity testing

Isolates of *B. cinerea, S. sclerotiorum* and *S. minor* were tested against a range of fungicides using the concentrations and methods outlined in Table 5. Discriminatory concentrations reported in literature were used where available.

Table 5. Active ingredient, concentration and method used to determine fungicide sensitivity

Fungicide	Organism	Concentration (mg a.i./L)	Inoc. method	Determination of resistance
benomyl	*Botrytis, S. minor, S. sclerotiorum*	0, 1, 10	Mycelial	growth >2mm on 2 discriminatory concentrations[A]
iprodione	*Botrytis, S. minor, S. sclerotiorum*	0, 2, 5	Mycelial	growth >2mm on 2 discriminatory concentrations[B]
procymidone	*Botrytis, S. minor, S. sclerotiorum*	0, 2, 5	Mycelial	growth >2mm on 2 discriminatory concentrations[B]
cyprodinil	*Botrytis, S. minor, S. sclerotiorum*	0, 0.05, 0.5	Mycelial	growth >2mm on 2 discriminatory concentrations[C]
fludioxonil	*Botrytis, S. minor, S. sclerotiorum*	0, 0.1, 0.5	Mycelial	growth >2mm on 2 discriminatory concentrations[D]
fenhexamid	*Botrytis*	0, 0.4	Spore	germ tube elongation on 1 discriminatory concentration[E]
boscalid	*Botrytis, S. minor, S. sclerotiorum*	0, 0.01, 0.05, 5, 50, 100	Mycelial	EC_{50}
azoxystrobin	*Botrytis, S. minor, S. sclerotiorum*	0, 0.01, 0.05, 0.1, 0.5, 1, 10	Mycelial	EC_{50}
tebuconazole	*S. sclerotiorum, S. minor*	0, 0.01, 0.05, 0.1, 0.5, 1, 10	Mycelial	EC_{50}
fluopyram	*Botrytis, S. sclerotiorum*	0, 0.01, 0.05, 0.5, 5, 50, 100	Mycelial	EC_{50}

[A] Northover, 1988
[B] Beever and Brien, 1983
[C] Baroffio et al. 2003, Birchmore & Forster 1996
[D] Baroffio et al. 2003
[E] Lechaise pers comm. 2010

5.2.5.9. Testing with discriminatory concentrations

Mycelial plugs of *B. cinerea, S. sclerotiorum* and *S. minor* isolates were placed as previously described onto unamended media or media amended with 2 concentrations of either benomyl, iprodione, cyprodinil or fludioxonil (Tables 4, 5). Each plate was inoculated with up to 10 isolates with two replicate plates and included a known sensitive isolate, and where possible a known resistant isolate. After two days incubation, the mean radial growth was calculated from two measurements made at right angles. The test was considered invalid if there was no growth on unamended PDA, radial growth was observed from the known susceptible isolate, or radial growth was not observed from the known resistant isolate where used. Resistance was indicated if the radial growth on amended PDA was >2mm.

Isolates of *B. cinerea* were inoculated by adding spores as previously described onto unamended TWA or TWA amended with fenhexamid (Table 5). Plates were examined microscopically after 24 hours for germ tube elongation.

5.2.5.10. Base line sensitivity

Sensitivity to boscalid, azoxystrobin or tebuconazole was evaluated by placing mycelial plugs of isolates as previously described onto unamended agar and agar amended with a range of concentrations (Tables 4, 5). The EC_{50} (concentration of the fungicide causing a 50% reduction in the growth rate compared to an unamended control) was determined for each isolate using probit analysis (GenStat 12.1 software).

The variation factor was calculated as the EC_{50} value of the least sensitive / EC_{50} of the most sensitive isolates (Kim & Xiao 2010).

5.3 Results and discussion

5.3.1. Fungal collection.

Fifty isolates of *Botrytis cinerea*, 77 *Sclerotinia sclerotiorum* and 24 *Sclerotinia minor* were collected from plant material and culture collections through Australia (Tables 6, 7). The host range included a wide range of vegetables. Isolates were also obtained from hosts other than vegetables, however as these were from vegetable growing areas and as these fungi are not host specific, they were included in the testing. The history of fungicide use was unknown, and no failure of control had been reported.

Table 6. Origin of isolates

	Botrytis cinerea	***Sclerotinia sclerotiorum***	***Sclerotinia minor***	**Total**
New South Wales	10	18	1	**29**
Queensland	-	11	2	**13**
South Australia	28	16	5	**49**
Tasmania	6	19	6	**31**
Victoria	1	7	9	**17**
Western Australia	5	8	2	**15**
Total	**50**	**79**	**25**	**154**

Table 7. Hosts of isolates

	Botrytis cinerea	***Sclerotinia sclerotiorum***	***Sclerotinia minor***	**Total**
Bean	-	27	3	**30**
Brassica	2	11	-	**13**
Capsicum	2	-	-	**2**
Cucurbit	9	12	-	**21**
Herbs	3	3	-	**6**
Lettuce	7	14	18	**39**
Tomato	11	-	-	**11**
Other vegetable	2	10	1	**13**
Other non vegetable	14	2	3	**19**
Total	**50**	**79**	**25**	**154**

5.3.2. Benzimidazole (benomyl)

Some of the isolates were unable to be tested due to contamination of the original cultures in storage. 34 of the 48 *Botrytis* isolates tested were from vegetables (Table 8), with the non vegetable crops grown near or in rotation with vegetables being pyrethrum, tea, rhubarb and strawberry. Only two of the *S. sclerotiorum* isolates tested were not from vegetables (canola and mint) and three of the *S. minor* isolates were sourced from pyrethrum.

Of the 48 *Botrytis* isolates tested for sensitivity to benzimidazoles, 20 (43.6%) had over 2 mm growth on PDA amended with 1 g/L benomyl and were classified as resistant (Table 9). 14 of these also had >2 mm growth on PDA amended with 10 g/L benomyl (data not shown). All the Tasmanian isolates tested were resistant, however they were all from non vegetable crops. This high level of resistance is most likely from previous use of the fungicide, as it is no longer registered for use in these vegetable crops. As benzimidazole resistance is stable and long lived, these results show that even if this group of fungicides were available for use it would be of limited value in managing diseases caused by *Botrytis*.

Resistance to benzimidazoles was also detected within the 76 *Sclerotinia* isolates tested, with over 2mm radial growth observed in 7 (9.2%) of the *S. sclerotiorum* isolates with PDA amended with 10 mg/L benomyl (Table 9). There was also a further 12 (15.8%) isolates that showed >2 mm radial growth on PDA amended with 1 mg/L benomyl (data not shown). Gossen et al. (2001) found the sensitive isolates were inhibited at EC_{50} of <8 mg/L benomyl, so while these 12 isolates showed a reduction in inhibition, there still may be some level of control achieved. Western Australia was the only state with no resistance or reduction in inhibition detected to *S. sclerotiorum*.

High levels of resistance were detected in the 25 isolates of *S. minor*, with 16 of the isolates growing in the presence of PDA amended with 1 mg/L benomyl (Table 8, 9). Ten of the 18 isolates from lettuce were resistant, where this fungicide had been commonly used (Table 8).

Table 8. Hosts of isolates sensitive and resistant to benzimidazoles (benomyl). This group of fungicides are not registered for use in any of these crops

	Botrytis cinerea		*Sclerotinia sclerotiorum*		*Sclerotinia minor*	
	No. isolates	**No. resistant***	**No. isolates**	**No. resistant#**	**No. isolates**	**No. resistant***
Bean	-	-	27	5	3	2
Brassica	2	2	10	0	-	-
Capsicum	2	0	-	0	-	-
Cucurbit	9	1	11	1	-	-
Herbs	2	0	3	0	-	-
Lettuce	6	1	13	1	18	10
Tomato	11	6	-	0	-	-
Other vegetable	2	2	10	0	1	1
Total vegetable	**34**	**12**	**74**	**7**	**22**	**13**
Non vegetable	14	8	2	0	3	3

* radial growth of over 2mm observed on PDA amended with 1 mg/L benomyl.
# radial growth of over 2mm observed on PDA amended with 10 mg/L benomyl.

Table 9. Origin of isolates sensitive and resistant to benzimidazoles (benomyl).

	Botrytis cinerea		*Sclerotinia sclerotiorum*		*Sclerotinia minor*	
	No. isolates	**No. resistant***	**No. isolates**	**No. resistant#**	**No. isolates**	**No. resistant***
New South Wales	8	6	16	0	1	0
Queensland	-	-	11	3	2	1
South Australia	27	5	15	1	5	3
Tasmania	6	6	19	2	6	5
Victoria	2	-	7	1	9	6
Western Australia	5	3	8	0	2	1
Total	**48**	**20**	**76**	**7**	**25**	**16**

* radial growth of over 2mm observed on PDA amended with 1 mg/L benomyl.
# radial growth of over 2mm observed on PDA amended with 10 mg/L benomyl.

5.3.3. Dicarboximide (iprodione, procymidone)

Resistance to the dicarboximide group was detected in 38% of the *Botrytis* isolates sourced from vegetable crops (Table 10). All isolates from Tasmania were resistant (Table 11). While these were all from non vegetable crops, *B. cinerea* is not host specific, and spread of the resistance populations into vegetables grown nearby could occur. Dicarboximide resistant populations are not as fit as the susceptible populations (Fourie & Holz 2003, Raposo et al. 2000), therefore by minimising selection pressure with reduced use of the fungicide, the resistant populations should also be reduced. However research on *Botrytis* isolated from greenhouse crops in the USA reported no change in fitness levels of the populations (Moorman & Lease 1992), indicating that reduction in resistant populations cannot be relied on as part of a management strategy.

Resistance of both *S. sclerotiorum* and *S. minor* populations in vegetables was lower than *Botrytis*, with 7% and 10% respectively of the isolates growing on the amended agar (Table 10). Populations of *Sclerotinia* resistant to iprodione may also have reduced fitness, with sclerotia from *S. minor* dicarboximide resistant populations found to be less virulent and less able to survive than sclerotia from susceptible populations (Hubbard et al. 1997).

These results indicate that with correct use and following recommendations for resistance management, iprodione will still have a significant role to play in fungicide programs for management of diseases caused by *Botrytis* and *Sclerotinia*.

Table 10. Hosts of isolates sensitive or with low level (2 mg/L iprodione) or high level (5 mg/L iprodione) resistance to dicarboximides.

	Botrytis cinerea			***Sclerotinia sclerotiorum***			***Sclerotinia minor***		
	No. isolates	**No. resistant**		**No. isolates**	**No. resistant**		**No. isolates**	**No. resistant**	
		low	**high**		**low**	**high**		**low**	**high**
Bean	-	-	0	27	0	0	3	0	0
Brassica#	2	0	2	10	0	0	-	-	-
Capsicum	2	0	0	-	0	0	-	-	-
Cucurbit	9	0	1	11	1	0	-	-	-
Herbs	2	0	1	3	1	0	-	-	-
Lettuce#	6	1	2	13	1	1	17	2	0
Tomato#	11	2	2	-	0	0	-	-	-
Other vegetable#	2	0	2	10	1	0	1	0	0
Total vegetable	**34**	**3**	**10**	**74**	**4**	**1**	**21**	**2**	**0**
Non vegetable	14	5	5	2	0	0	3	1	0

#registered for use with iprodione (Rovral®)

Table 11. Origin of isolates sensitive or with low level (2 mg/L iprodione) or high level (5 mg/L iprodione) resistance to dicarboximides.

	Botrytis cinerea			***Sclerotinia sclerotiorum***			***Sclerotinia minor***		
	No. isolates	**No. resistant**		**No. isolates**	**No. resistant**		**No. isolates**	**No. resistant**	
		low	**high**		**low**	**high**		**low**	**high**
New South Wales	8	3	2	16	3	0	1	0	0
Queensland	-	-	-	11	0	0	2	0	0
South Australia	27	2	5	15	1	0	5	2	0
Tasmania	6	3	3	19	0	0	6	1	0
Victoria	2	0	2	7	0	0	9	0	0
Western Australia	5	0	3	8	0	1	2	0	0
Total	**48**	**8**	**15**	**76**	**4**	**1**	**25**	**3**	**0**

Ten *Botrytis* isolates and 12 *S. sclerotiorum* isolates were also tested for sensitivity to procymidone. Cross resistance within *Botrytis* populations is known to occur between the three main chemicals in the dicarboximide group, iprodione, procymidone and vinclozolin (Beever & Byrde 1982). However in these experiments, while all the *Botrytis* isolates resistant to iprodione were also resistant to procymidone, the isolates susceptible to iprodione were variable in their resistance to procymidone (Table 12). In comparison, all *S. sclerotiorum* isolates tested were sensitive to procymidone, irrespective of their sensitivity to iprodione. The mode of action of the dicarboximides is not clear (Beever & Byrde 1982, Pappas & Fisher 1979), and it is possible there are some differences in activity against mycelial growth between the different active ingredients. Thus sensitivity to these group of fungicides should be tested against the correct active ingredient, and cross resistance should not be assumed.

Table 12. Comparative sensitivity of Botrytis and S. sclerotiorum isolates to iprodione and procymidone.

Sensitivity to iprodione	***Botrytis***				***S. sclerotiorum***		
	No. isolates tested	No isolates resistant to procymidone			No. isolates tested	No. isolates resistant to procymidone	
		Sens.	Low res.	High res.		Sens.	Res.
Sensitive	5	3	1	1	9	9	0
Low resistance	2	1	1	0	2	2	0
High resistance	3	0	0	3	1	1	0

5.3.4. Anilinopyrimidine (cyprodinil)

Cyprodinil was used as the representative fungicide from the anilinopyrimidine group. While pyrimethanil (eg Scala ®) is the registered product for vegetables, cross resistance/sensitivity exists between the fungicides (Hilber & Schüepp 1996, Myresiotis et al. 2007).

Of the 48 *Botrytis* isolates tested against cyprodinil, only three isolates from tomato and one from cucumber were sensitive to the lowest concentration of 0.05 g a.i. /L. At 0.5 g a.i. /L, 66% *Botrytis* isolates were sensitive to cyprodinil (Table 13, 14). Reduced sensitivity to anilinopyrimidines has been reported in *Botrytis* from grapes in Australia (Sergeeva et al. 2002) from vegetables in Spain (Moyano et al. 2004) and Greece (Myresiotis et al. 2007). While Moyano et al. (2004) found only low levels of resistance, with 10% of the 40 isolates tested resistance to pyrimethanil, Myresiotis et al. (2007) had higher levels, with 31 (56%) of the 55 isolates resistant to cyprodinil. However all three papers used significantly different concentrations to determine resistance, with pyrimethanil at 2.5 mg/L (Sergeeva et al. 2002), 0.7 mg/L (Moyano et al. 2004) and 0.1 mg /L (Myresiotis et al. 2007) and cyprodinil at 0.03 mg/L (Myresiotis et al. 2007).

Reduced sensitivity to high levels of cyprodinil is of concern, however further monitoring is needed to determine whether reduced field efficacy of *Botrytis* to cyprodinil exists in vegetable crops.

S. sclerotiorum was less sensitive to cyprodinil, as 88% of the isolates grew on the higher rate of 0.5 g a.i. /L (Table 13, 14). None of the 25 isolates of *S. minor* were sensitive to either rate of cyprodinil. However as none of the anilinopyrimidines are registered for use with *Sclerotinia*, this lack of sensitivity is of little immediate concern to the vegetable industry.

Table 13. Origin of isolates with mycelial growth after 48 hours on agar amended with either 0.05 or 0.5 mg/L cyprodinil.

	Botrytis cinerea			***Sclerotinia sclerotiorum***		
	No. isolates	**No. with growth**		**No. isolates**	**No. with growth**	
		0.05 mg/L	**0.5 mg/L**		**0.05 mg/L**	**0.5 mg/L**
Bean	-	-	-	27	27	23
Brassica	2	2	0	10	10	8
Capsicum#	2	2	0	-	-	-
Cucurbit	8	7	4	10	10	10
Herbs	2	2	1	3	3	3
Lettuce	5	5	4	13	13	11
Tomato#	8	5	3	-	-	-
Other vegetable#	2	2	1	10	10	9
Total vegetable	**29**	**25**	**11**	**73**	**73**	**64**
Non vegetable	13	12	6	2	2	2

#registered for use with pyrimethanil

Table 14. Source of isolates with mycelial growth after 48 hours on agar amended with either 0.05 or 0.5 mg/L cyprodinil.

	Botrytis cinerea			*Sclerotinia sclerotiorum*		
	No. isolates	**No. with growth**		**No. isolates**	**No. with growth**	
		0.05 mg/L	**0.5 mg/L**		**0.05 mg/L**	**0.5 mg/L**
New South Wales	9	8	5	16	16	16
Queensland	-	-	-	11	11	9
South Australia	22	19	4	14	14	14
Tasmania	6	5	3	19	19	16
Victoria	2	2	0	7	7	6
Western Australia	5	4	3	8	8	5
Total	**44**	**38**	**15**	**75**	**75**	**66**

5.3.5. Phenylpyrrole (fludioxonil)

Isolates of *S. sclerotiorum* and *S. minor* tested were sensitive to fludioxonil at both 0.1 and 0.5 mg a.i./L. Only three *Botrytis* isolates from vegetable were not sensitive at the 0.1 mg/L rate, and two at the 0.5 mg/L rate (Table 15).

Fludioxonil is not registered for use in vegetables, however as it is used as a mixed formulation with cyprodinil in the product Switch®, which has potential as an effective product for management of both *Botrytis* and *Sclerotinia.* It has registration overseas for a wide range of diseases on many crops, including powdery mildew and diseases caused by *Colletotrichum, Cercospora, Alternaria, Stemphylium* and *Phoma.*

Table 15. Host of isolates with mycelial growth after 48 hours on agar amended with 0.1 or 0.5 mg/L fludioxonil (not registered for any of the crops).

	Botrytis cinerea			*Sclerotinia sclerotiorum*		
	No. isolates	**No. with growth**		**No. isolates**	**No. with growth**	
		0.1 mg/L	**0.5 mg/L**		**0.1 mg/L**	**0.5 mg/L**
Bean	-	-	-	27	0	0
Brassica	2	0	0	10	0	0
Capsicum	2	0	0	-	-	-
Cucurbit	8	1	0	10	0	0
Herbs	2	0	0	3	0	0
Lettuce	5	1	1	13	0	0
Tomato	8	1	1	-	-	-
Other vegetable	2	0	0	10	0	0
Total vegetable	**29**	**3**	**2**	**73**	**0**	**0**
Non vegetable	13	2	2	2	0	0

Table 16. Source of isolates with mycelial growth after 48 hours on agar amended with either 0.1 or 0.5 mg/L fludioxonil.

	Botrytis cinerea			*Sclerotinia sclerotiorum*		
	No. isolates	**No. with growth**		**No. isolates**	**No. with growth**	
		0.1 mg/L	**0.5 mg/L**		**0.1 mg/L**	**0.5 mg/L**
New South Wales	9	1	1	16	0	0
Queensland	-	-	-	11	0	0
South Australia	22	2	2	14	0	0
Tasmania	6	1	0	19	0	0
Victoria	2	1	1	7	0	0
Western Australia	5	0	0	8	0	0
Total	**44**	**5**	**4**	**75**	**0**	**0**

5.3.6. Hydroxyanilide (fenhexamid)

Germ tube elongation was not detected in any of the 32 isolates of *Botrytis* tested, indicating that all isolates were sensitive to fenhexamid at 0.4 mg/L.

5.3.7. Oxathiin (boscalid)

A total of 48 *Botrytis* isolates were tested for sensitivity to boscalid. Of these three had EC_{50} values over 10 mg/L (22, 38 and 74), with one still having <50% inhibition at 100 mg/L (Fig. 6). These were considered resistant isolates and were not included in the base line data. Zhang et al (2007) found resistant mutants to be able to grow on 10 mg/L, and Kim and Xiao (2010) used a discriminatory dose of 5 mg/L. Two of the isolates originated from SA (cucumber, herb) and one from NSW (lettuce). Boscalid is registered/permitted for *Sclerotinia* on lettuce, beans and brassicas. However it is also registered for use on early blight (*Alternaria solani*) on tomato, potato, eggplant and capsicum, which could affect the sensitivity to *Botrytis* and *Sclerotinia* if they are present at the time of application.

The individual EC_{50} values of the remaining 45 isolates ranged from 0.04 to 2.74 mg/L, with a mean of 0.79 mg/L. These EC_{50} values were lower than those found by both Zhang et al (2007) and Myresiotis et al (2008). The frequency distribution of the EC_{50} values was unimodal (Fig. 7), with the highest frequency of 0.1-0.2 mg/L. The frequency of EC_{50} values was random for each crop, however SA and NSW had the greatest range, with WA and Victoria both having lower EC_{50} values (Fig. 7). The variation factor of the sensitive isolates was 68.5, and with all isolates increased to 1850.

Mycelial growth of only one isolate was completely inhibited at the highest concentration of 100 mg/L, with inhibition of the other sensitive isolates ranging from 62 to 98%. Inhibition of the resistant isolates ranged from 44 to 60%. Kim and Xiao (2010) noted that mycelial growth of *Botrytis cinerea* was not completely inhibited at 25 mg/L, even with the sensitive isolates. They also observed that some isolates with high

EC_{50} values with the mycelial growth assay were sensitive when tests for conidial germination. Therefore further testing should be undertaken to determine whether the isolates categorised as resistant *in vitro* would result in lowered efficacy of the product *in planta*.

The EC_{50} values of the 63 *S. sclerotiorum* isolates tested ranged from 0.01 to 2.8 mg/L, with a mean of 0.23 mg/L (Fig. 8). This was slightly higher than the mean of 0.01 mg/L reported by Stammler et al. (2007), but lower than the mean of 0.042 mg/L reported by Liu et al. (2009) The frequency distribution was unimodal (Fig. 9), with again SA and NSW having the widest spread of EC_{50} values.

The EC_{50} values of the 24 *S. minor* isolates tested ranged from 0.01 to 3.5 mg/L, with a mean of 0.26 mg/L (Fig 10). The frequency distribution was unimodal (Fig. 11). All crops had a range of EC50 values, however Victoria had the greatest range with the lowest and the highest EC_{50} values both sourced from lettuce from Victoria.

Similar to *Botrytis*, none of the *Sclerotinia* isolates had complete inhibition of mycelial growth at 100 mg/L, with *S. sclerotiorum* ranging from 54- 96% and *S. minor* from 79 – 96%.

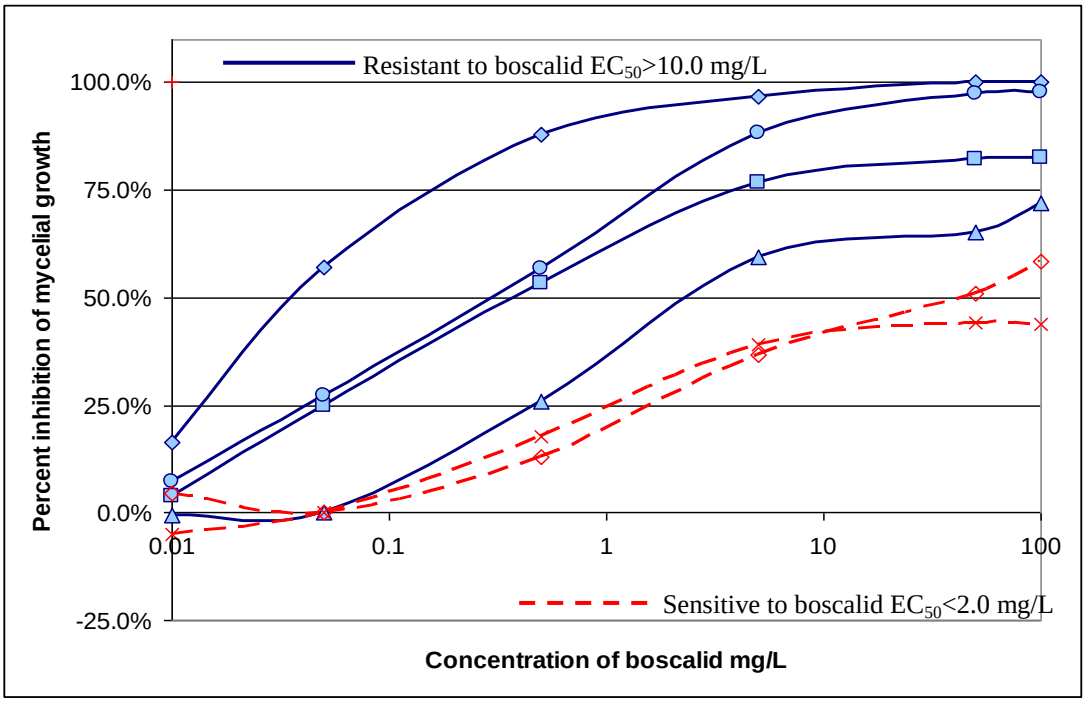

Figure 6. Dose response curves of *Botrytis cinerea* to boscalid. The solid blue lines are sensitive isolates with EC_{50} values < 2.0 mg/L. The dashed red lines are resistant isolates with EC_{50} values >10 mg/L.

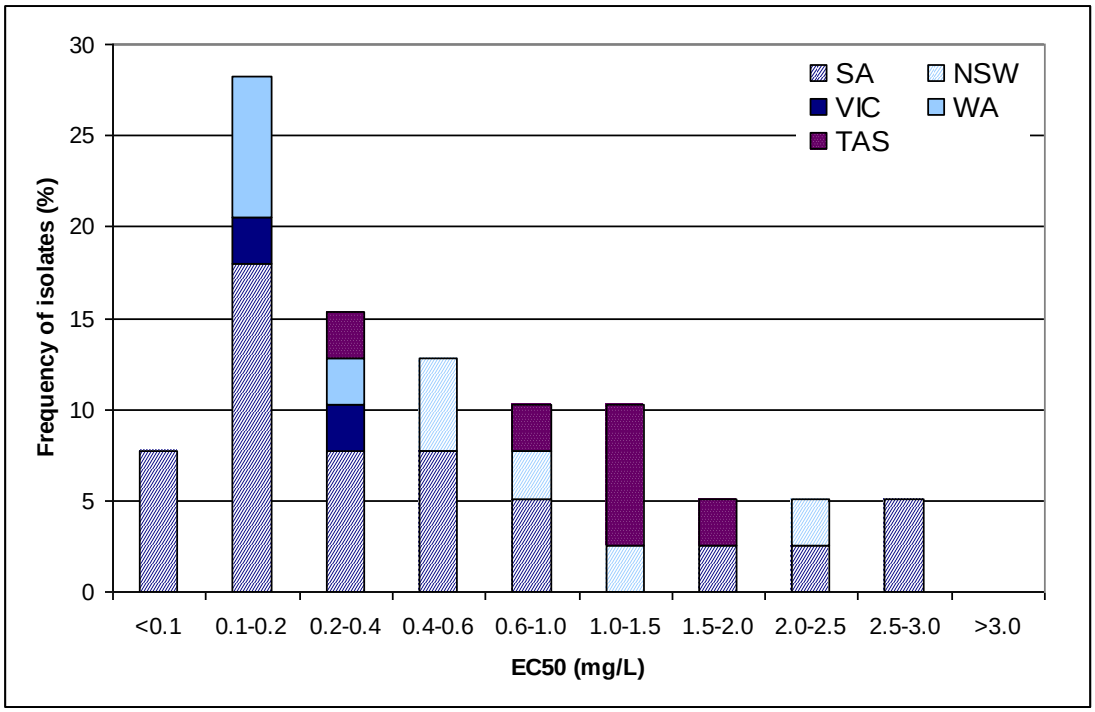

Figure 7. Frequency distribution of EC_{50} of 45 *Botrytis cinerea* isolates sensitive to boscalid based on the inhibition of mycelial growth.

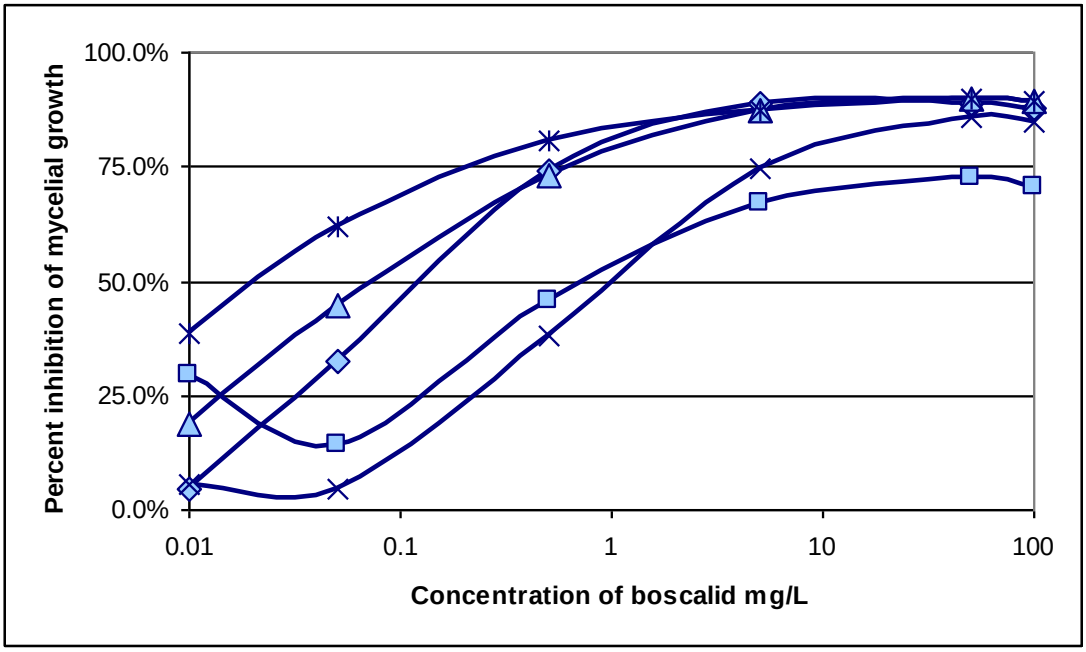

Figure 8. Dose response curves of *Sclerotinia sclerotiorum* to boscalid. EC_{50} values from 0.015 to 0.96 mg/L.

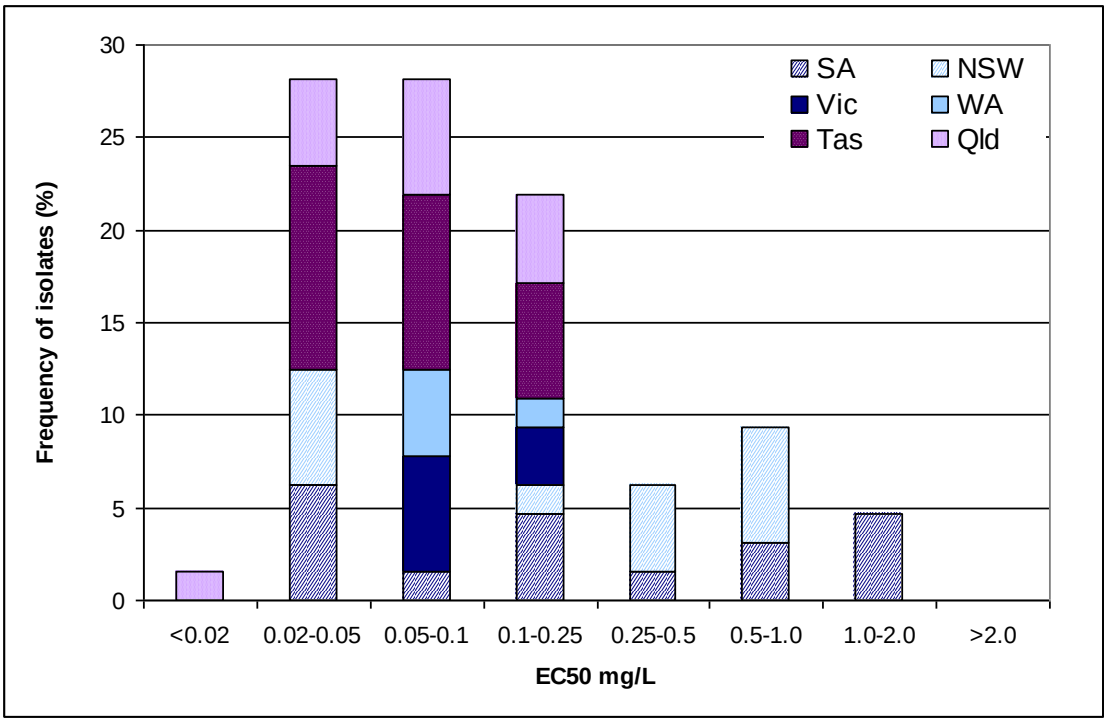

Figure 9. Frequency distribution of *Sclerotinia sclerotiorum* isolates sensitive to boscalid based on the inhibition of mycelial growth.

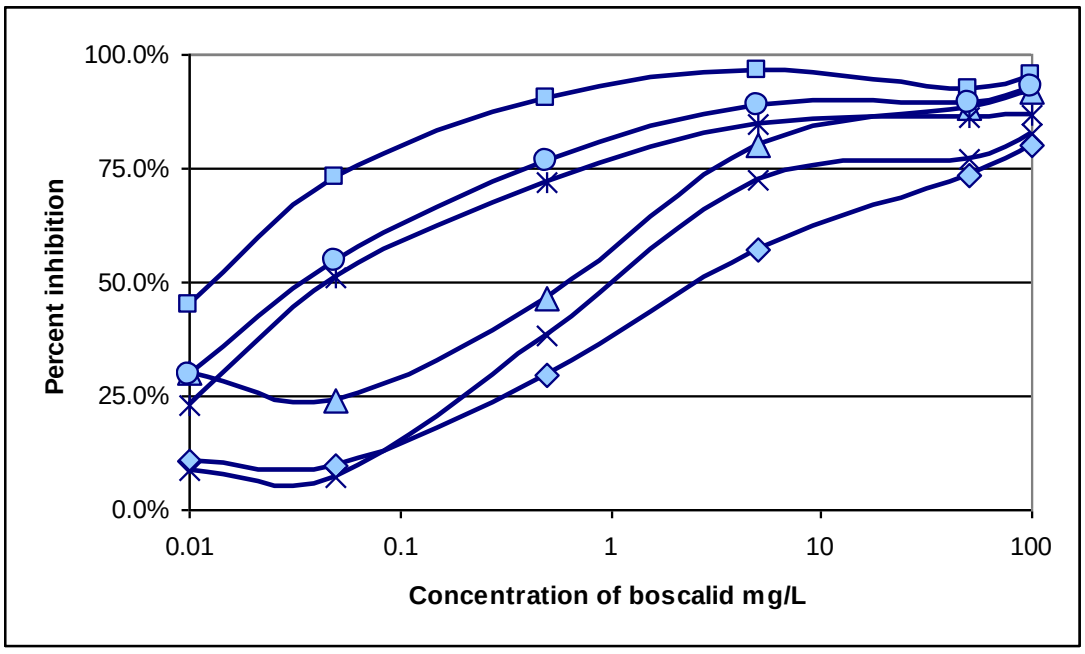

Figure 10. Dose response curves of *Sclerotinia minor* to boscalid. EC_{50} values from 0.01 to 3.5 mg/L.

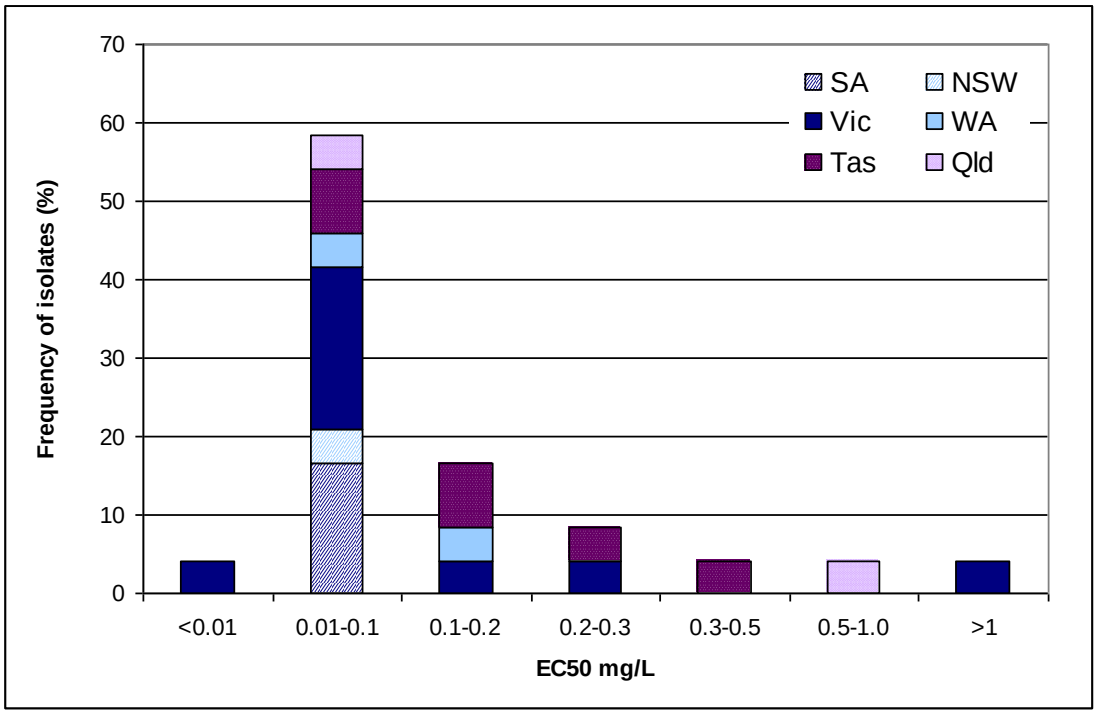

Figure 11. Frequency distribution of *Sclerotinia minor* isolates sensitive to boscalid based on the inhibition of mycelial growth.

5.3.8. Strobilurin (azoxystrobin)

Of the 38 *Botrytis* isolates tested for sensitivity to azoxystrobin, four had EC_{50} values over 10 mg/L with less than 25% inhibition at 10 mg/L (Fig.12). These were considered resistant isolates, and were not included in the base line frequency data. Two of the resistant isolates originated from WA (cucumber, potato), one from NSW (lettuce) and one from SA (potato). Azoxystrobin is registered/permitted for use tomatoes, beans and lettuce to control *Sclerotinia*, and on Brassicas for white blister. However it is also registered/permitted on many vegetable crops for powdery mildew, downy mildew and diseases caused by *Alternaria* (e.g. early blight), which could impact on the sensitivity of *Botrytis* and *Sclerotinia* if they are present at the time of application.

The EC_{50} values of the remaining 34 isolates ranged from 0.025 to 2.0 mg/L, with a mean of 0.2 mg/L and a variation factor of 80. The frequency of EC_{50} values was unimodal and random for each state (Fig. 13). Lettuce had a slightly higher EC_{50} range than the other crops (data not shown.)

The EC_{50} values of the 72 *S. sclerotiorum* isolates tested ranged from 0.006 to 0.3 mg/L, with a mean of 0.06 mg/L and a variation factor of 50. The EC_{50} values of the 24 *S. minor* isolates tested ranges from 0.006 to 0.35 mg/L, with a mean of 0.04 mg/L and a variation factor f 58.3. For both fungi there was a similar range of values between the states (Figs 14, 15) and crops (data not shown).

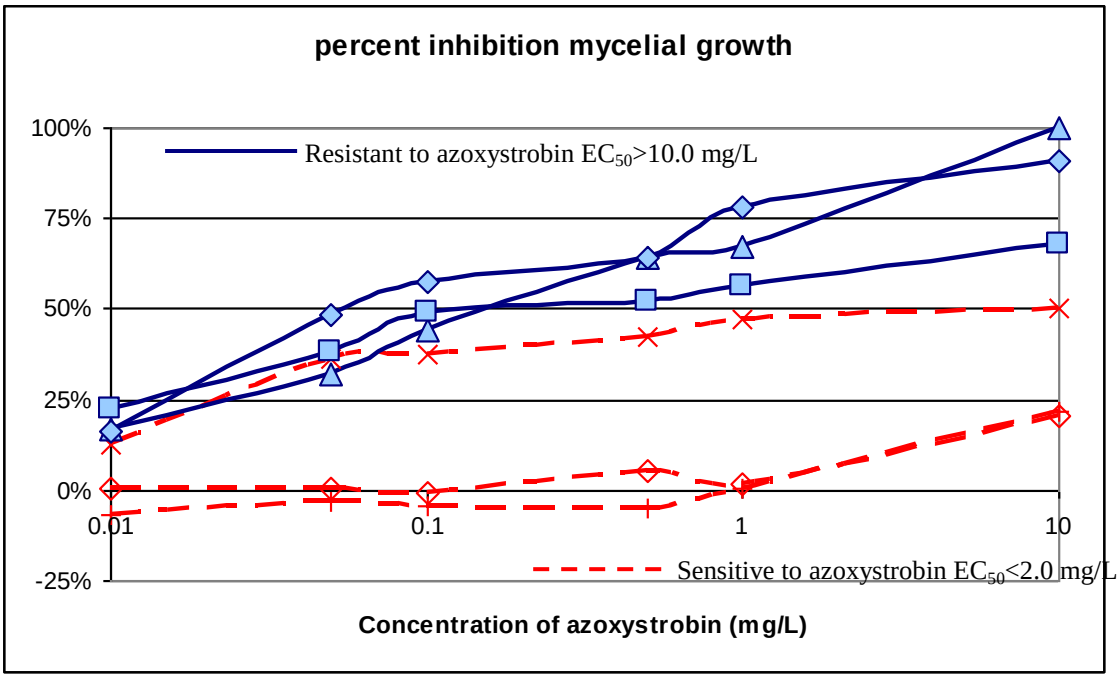

Figure 12. Dose response curves of *Botrytis cinerea* to azoxystrobin. The solid blue lines are sensitive isolates with EC_{50} values < 2.0 mg/L. The dashed red lines are resistant isolates with EC_{50} values >10 mg/L.

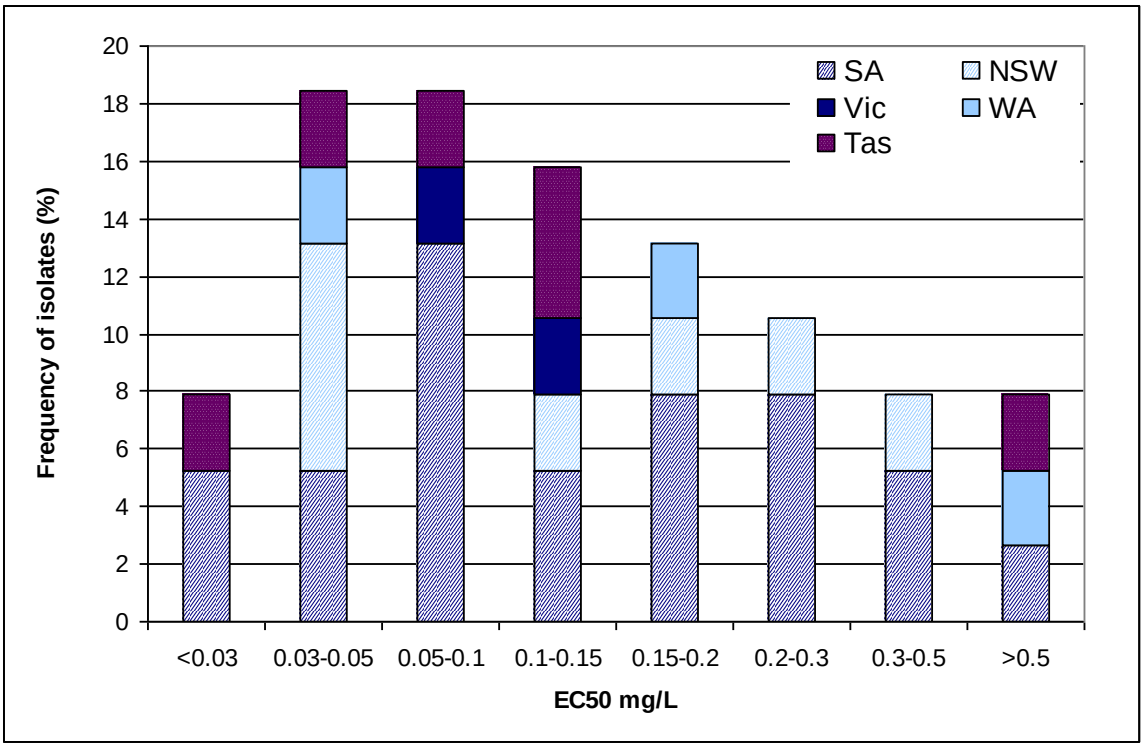

Figure 13. Frequency distribution of *Botrytis cinerea* isolates sensitive to azoxystrobin based on the inhibition of mycelial growth.

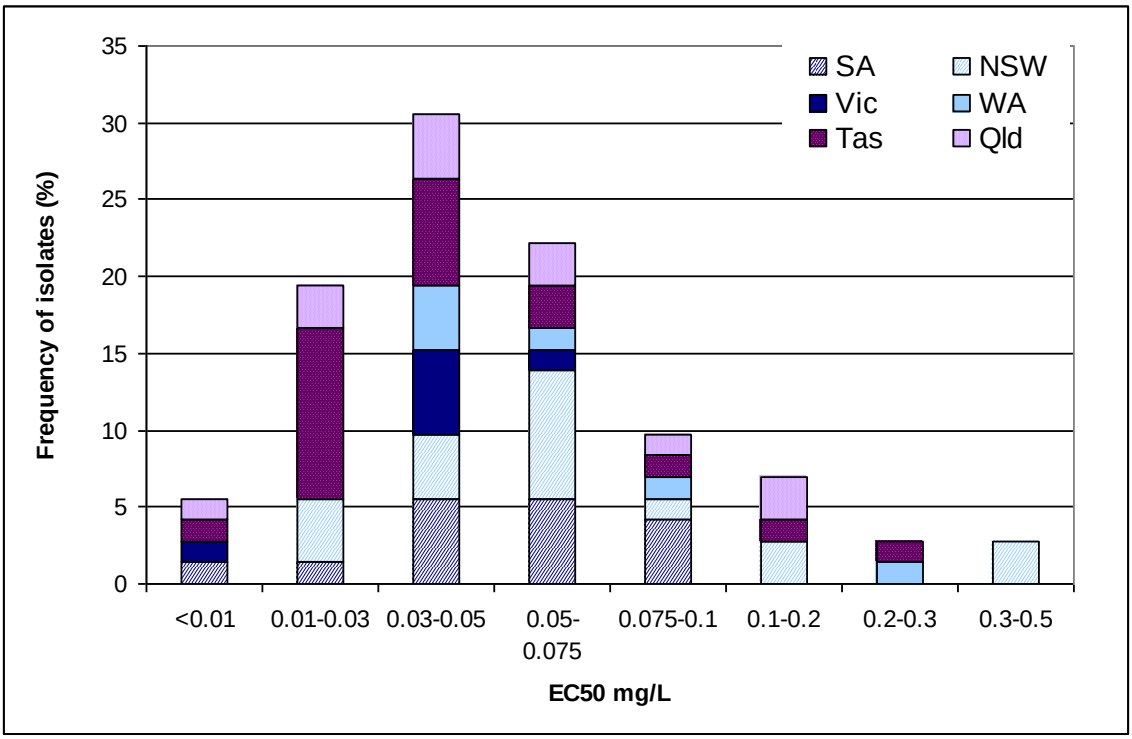

Figure 14. Frequency distribution of *Sclerotinia sclerotiorum* isolates sensitive to azoxystrobin based on the inhibition of mycelial growth.

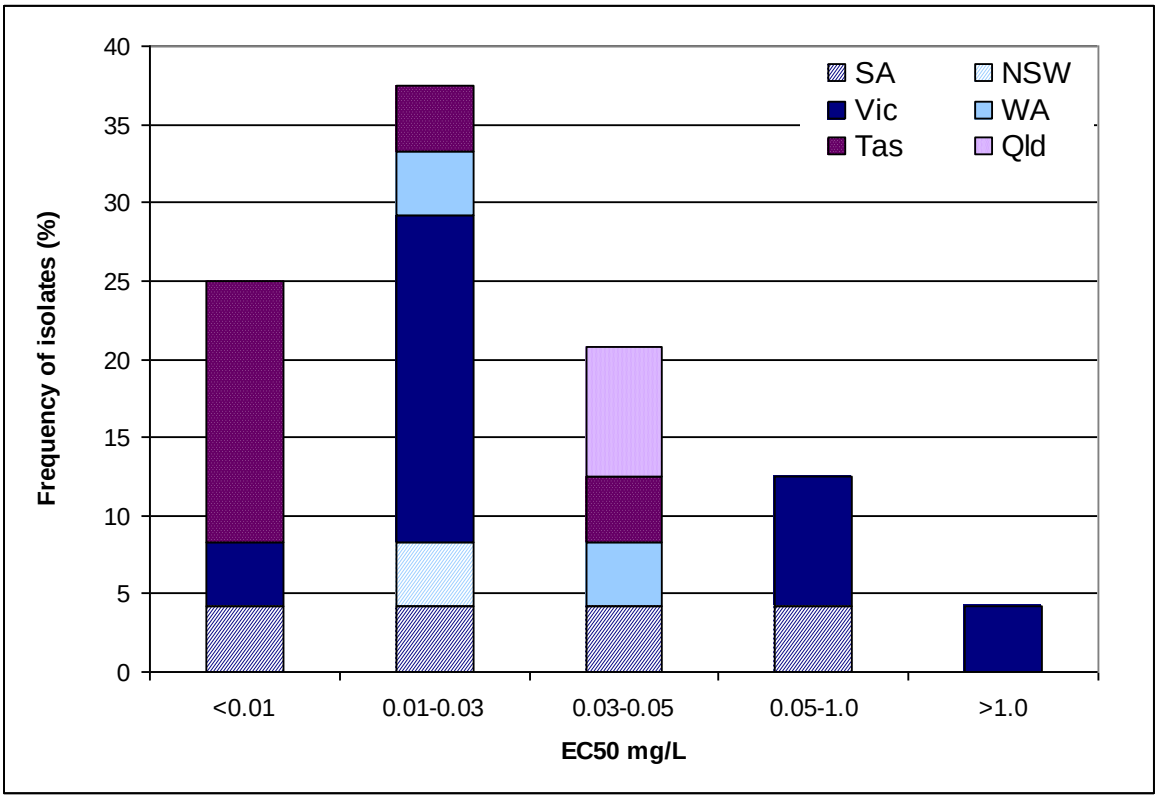

Figure 15. Frequency distribution of *Sclerotinia minor* isolates sensitive to azoxystrobin based on the inhibition of mycelial growth.

5.3.9. DMI – triazole (tebuconazole)

A total of 71 *S. sclerotiorum* and 24 *S. minor* isolates were tested for sensitivity to tebuconazole. The EC_{50} values for *S. sclerotiorum* ranged from 0.0036 mg/L to 0.75, with a mean of 0.16 mg/L and a variation factor of 207. The EC_{50} values for *S. minor* ranged from 0.014 to 0.147 mg/L, with a mean of 0.066 mg/L and a variation factor of 10.5. For both fungi there was a similar range of values between the states (Fig. 16, 17) and crops (data not shown). The exception was *S. sclerotiorum* from lettuce, or sourced from Victoria where the frequency was highest in the lower EC_{50} values.

All *S. Sclerotinia* isolates were sensitive, however the inhibition at 10 mg/L ranged from 86 to 99%, with 9% of the isolates having some mycelial growth at 10 mg/L. The mycelial growth of all *S. minor* isolates was completely inhibited at 10 mg/L and 42% had complete inhibition at 1 mg/L.

Tebuconazole is registered/permitted to control *Sclerotinia* on a range of vegetables.

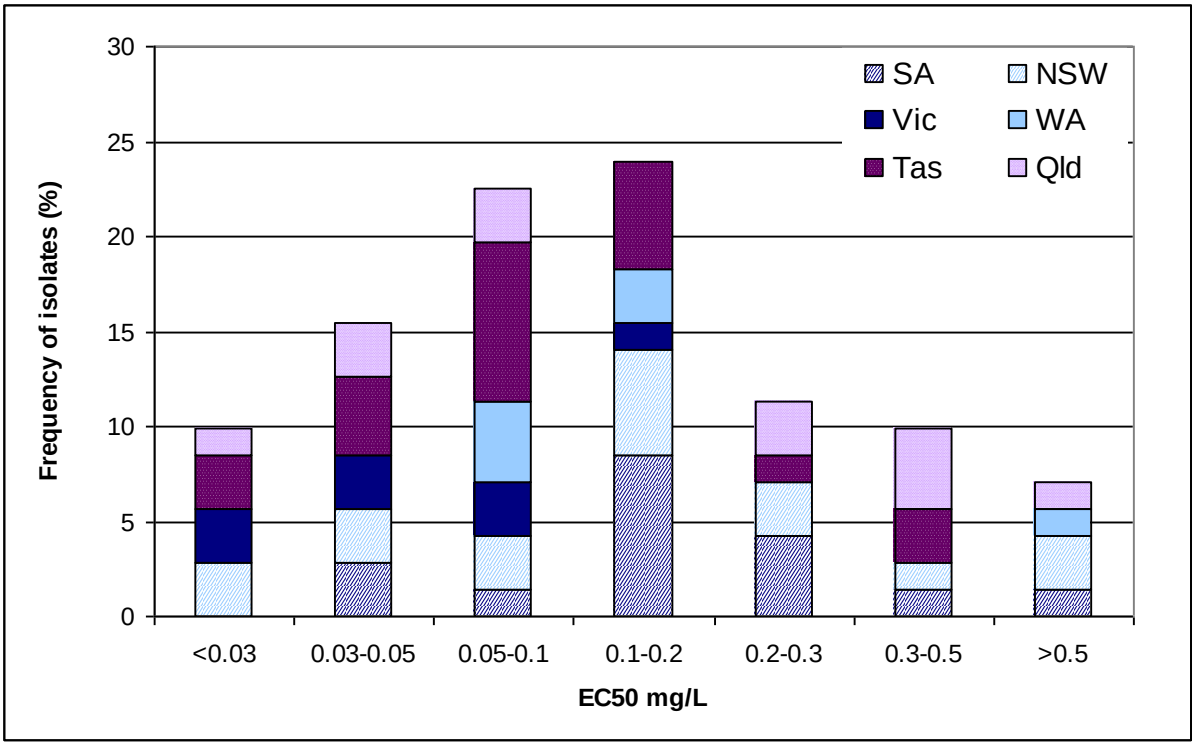

Figure 16. Frequency distribution of *Sclerotinia sclerotiorum* isolates sensitive to tebuconazole based on the inhibition of mycelial growth.

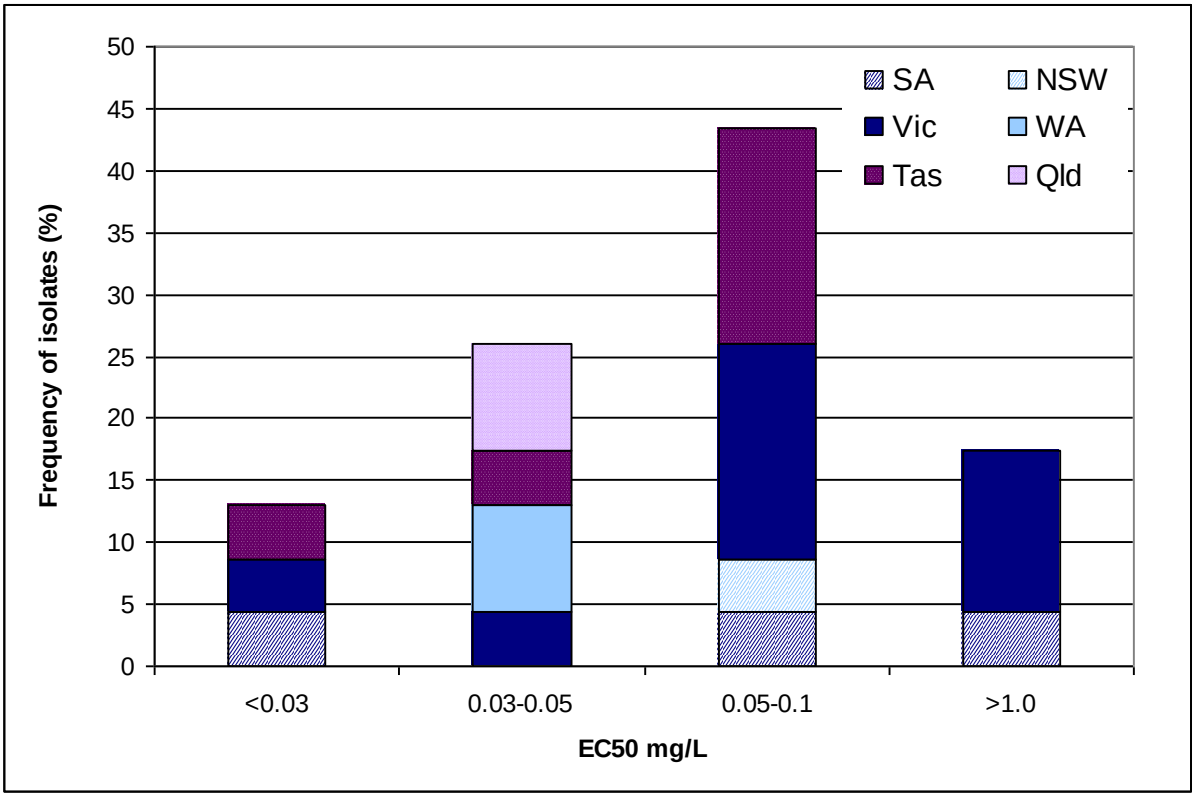

Figure 17. Frequency distribution of *Sclerotinia minor* isolates sensitive to tebuconazole based on the inhibition of mycelial growth.

5.3.10. Fluopyram

The sensitivity to fluopyram of three of the four *Botrytis* isolates was similar, however one was more sensitive at the higher concentrations (Fig 18). All had similar EC_{50} values, ranging from 1.1 to 1.8 mg/L.

The sensitivity of the two *Sclerotinia sclerotiorum* isolates was variable (Table 17), with EC_{50} values of 0.24 and 3.4 mg/L.

Fluopyram and boscalid are both SDI fungicides, however neither Ishii et al. (2011) or Avenot & Michailides (2010) found evidence of cross resistance in either powdery mildew and *Corynespora cassiicola* in cucumber or *Alternaria alternata* of pistachio respectively. These results suggest that there is also no cross resistance with *Botrytis cinerea*, however the Sclerotinia isolate with the lower sensitivity to boscalid also had the lower sensitivity to fluopyram (Table 17). More isolates would need to be tested to confirm cross resistance in *Sclerotinia*.

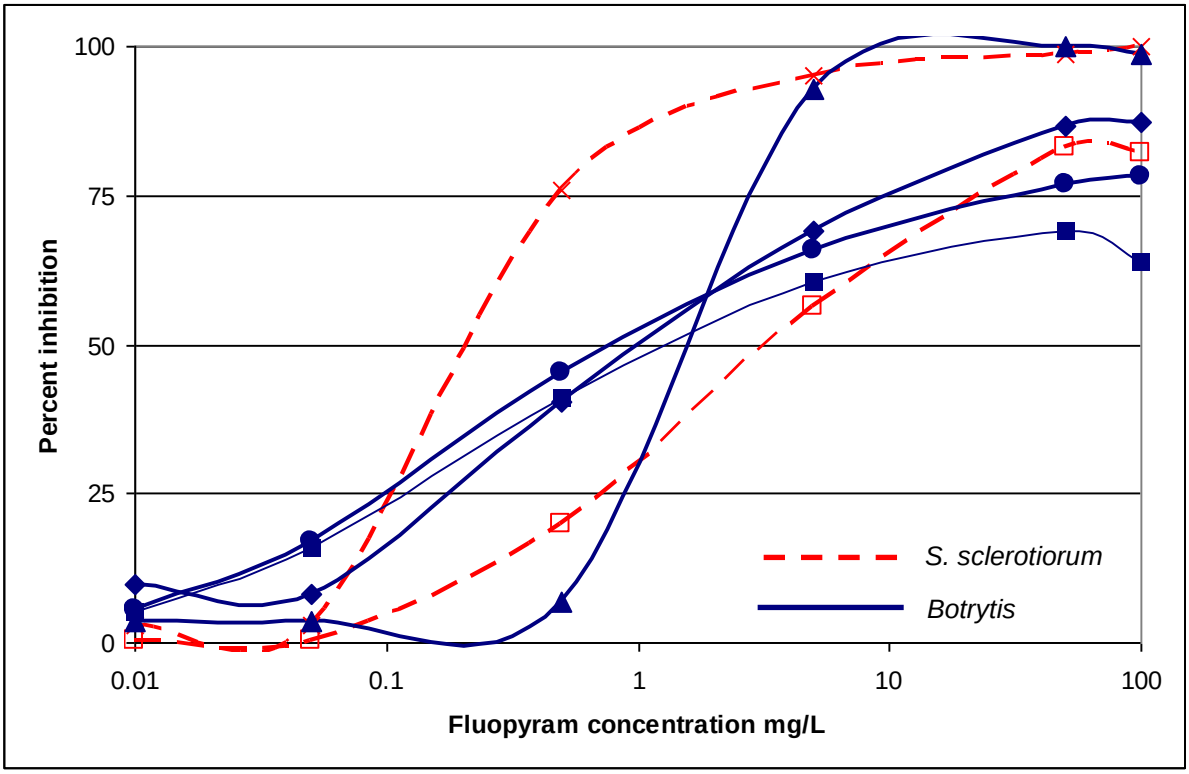

Figure 18. Dose response curves of *Botrytis cinerea* (solid blue lines) and *Sclerotinia sclerotiorum* (dashed red lines) to fluopyram.

Table 17. Sensitivity of *Sclerotinia sclerotiorum* and *Botrytis cinerea* isolates to boscalid and fluopyram.

Isolate	**EC_{50} mg/L**	
	boscalid	**fluopyram**
S. sclerotiorum	0.5	3.4
	0.015	0.24
Botrytis cinerea	22.7	1.8
	38.2	1.1
	2.5	1.75
	0.14	1.4

5.4 Conclusion

Overall this study has shown that fungicide resistant populations exist in the vegetable industry, albeit at low levels for many of the fungicides. For some of the samples, details of previous spray programs and control achieved were not available. For others, no loss of control had been reported. However the presence of a resistant population indicates that the adherences to fungicide resistant management strategies is vital to keep resistant populations at a low level and maintain field sensitivity of the pathogen for the fungicide. The availability of a wide range of fungicide groups is critical to provide the alternative fungicides necessary for these strategies to be effective.

6. FUNGICIDE RESISTANCE IN LETTUCE DOWNY MILDEW AND WHITE BLISTER OF BRASSICAS

6.1 Introduction

Studies in the U.K. have previously shown *Bremia lactucae*, the cause of lettuce downy mildew (Fig. 3), rapidly developed resistance to phenylamide fungicides such as metalaxyl (Crute et al. 1987). Metalaxyl is the most active of the phenylamide fungicides, with inhibition of sensitive *B. lactucae* sporulation at 0.1 µg/ml, whereas resistant isolates grow with high level of metalaxyl, producing sporulation at 100 µg/ml (Crute et al. 1987). Brown et al. (2004) used 50 µg/ml metalayxl as the discriminatory dose.

No previous Australian studies have documented chemical resistance issues in *Albugo candida*, the cause of brassica white blister. Petkowski et al. (2005) suggested that reduced control with metalaxyl-M in trials was possibly due to resistance, but no testing was done to confirm it.

This study was undertaken to monitor the resistance status of *Bremia lactucae* isolates to metalaxyl and *Albugo candida* isolates to chemicals from the strobilurin class and copper as copper hydroxide.

6.2 Materials and methods

Due to the obligate parasitic nature of this straminopilous fungus resistance testing involved inoculating cotyledons growing in fungicide solution with spore suspension before rating the percentage of seedlings bearing sporophores (Crute et al. 1987).

6.2.1. Sample handling

Infected plant material was received from the field and either used as a source of inocula immediately after receipt, or small pieces of sporulating infected material were frozen in sterile distilled water at -80^{o}C and used at a later date. Sporulation was induced if required by placing infected plant material into humidified chambers and incubating overnight in the dark at room temperature.

6.2.2. Inoculum preparation

Infected plant material, either fresh or defrosted, was placed into a 50 mL Falcon tube with 10 mL sterilised H_20, shaken to release spores into suspension and left for 30 m. Plant material was discarded and the spore concentration estimated using a haemocytometer and diluted to obtain a final concentration between 10^5 and 10^6/mL. If not required immediately, spore suspensions were frozen at -80^{o}C until use.

6.2.3. Preparation of seedlings for testing

Preliminary screening experiments were conducted to determine plant cultivars that would be successfully infected with pathogenic isolates and display typical disease symptoms within an appropriate time-frame.

Lettuce seed cv. *Cobham Green* or were planted into small plastic containers with small amount of sterilised commercial seedling mix), 10 seeds per container, watered with half strength liquid fertiliser (Thrive®) and incubated at 25^{o}C. Plants were sprayed with fungicide, or sterile distilled water, 24 hours before inoculation approximately 4 to 5 mL per container.

Broccoli seed cv. *Tiburon* was planted into small plastic containers with small amount of sterilised commercial seedling mix, 10 seeds per container in small plastic containers with dome lids, watered with half strength liquid fertiliser and incubated at 15^{o}C under ultra violet light planted (Figure 19). Plants were sprayed with fungicide, or sterile distilled water, 24 hours before inoculation, approximately 4 to 5 mL per container.

6.2.4. Inoculation

Plants were inoculated at cotyledon stage plus 2 true leaves (approximately 2 to 4 weeks after sowing) with approximately 4 mL spore suspension per container, left with lids off to dry and then incubated in a growth cabinet under lights at 15^{o}C. Expression of disease symptoms was observed between 7 and 14 days after inoculation for downy mildew and approximately 15 to 30 days for white blister.

6.2.5. Fungicide preparation

Fungicides were mixed with water at the rate outlined in Table 18 and applied to plants 24 hours before inoculation using 4 to 5 mL per tub of ten plants. Where resistance was observed, the downy mildew isolates were tested again at twice the recommended rate.

Table 18. Fungicides used in resistance testing of downy mildew and white blister pathogens

Chemical	**Active ingredient**	**Amount of product / 100mL of water**
Apron®	metalaxyl 350 g/kg	28.5 µL
Ridomil Gold MZ WG	metalaxyl 40g/kg + mancozeb 640g/kg	250 mg
Copper oxychloride 500WP	copper oxychloride 500g/kg	25 mg
Mancozeb 750DF	Mancozeb 750g/kg	200 mg
Flint 500WG®	Trifloxystrobin 500g/kg	10 mg
Amistar 250SC®	Azoxystrobin 250g/L	20 mg
Cabrio®	Pyraclostrobin 250g/L	50 µL
Kocide®	copper hydroxide 300g/kg	125 mg

6.2.6. Statistical analysis

The data was analysed as a series of Chi-squared tables, using the "Fisher Exact Test" to determine whether the fungicide had any effect on proportion of successfully infected plants compared with water treated controls. Where data were not significantly different, the fungicide was deemed to have had no effect thereby inferring that it was

resistant to the fungicide. In contrast, it was concluded that there was no fungicide resistance issue where there were highly significant differences in the mean proportion of fungicide treated versus untreated plants.

Figure 19. Brassica seedling assays for determining chemical resistance to white blister

6.3 Results and discussion

6.3.1. Sensitivity of Bremia lactucae isolates on lettuce seedlings

Seven of the 19 isolates of *Bremia lactucae* were shown to be resistant to metalaxyl (Table 19).

Table 19. Metalaxyl resistance status of lettuce downy mildew isolates of *Bremia lactucae*

Isolate Number	Year of collection	Location	Metalaxyl Rate	Resistance*
09/57	2009	NSW	standard	+
125	2006	Vic	standard	-
09/226	2009	Vic	standard	+
			double	+
09/122	2009	NSW	standard	+
			double	+
121	2006	Qld	standard	-
			double	-
181	2008	NSW	standard	+
			double	+
91	2006	Vic	standard	-
09/252	2009	NSW	standard	-
122	2006	Vic	standard	-
136	2006	Vic	standard	+
			double	+
09/266	2009	Vic	standard	+
			double	+
220	2009	Vic	standard	+
240	2009	Vic	standard	+
			double	+
233	2009	Vic	standard	-
09/268	2009	Vic	standard	-
238	2009	Vic	standard	-
240	2009	Vic	standard	-
077	2005	NSW	standard	-
237	2009	Vic	standard	-

* + = resistant, - = sensitive.

As downy mildew was not resistant to mancozeb or copper, the combination of these products with metalaxyl may still provide protection against downy mildew where resistance to metalaxyl exists in the field.

6.3.2. Sensitivity of Albugo candida isolates to strobilurins and copper

Due to drought conditions throughout eastern Australia only five isolates were obtained and tested against three strobilurin fungicides (azoxystrobin, pyraclostrobin and trifloxystrobin), and copper hydroxide. Disease expression was evident in control after 15 days but no disease was observed on any plants treated with fungicide. Final assessment was made at 25 days and this confirmed no disease symptoms on any of the treated plants.

One isolate (09/636) of *Albugo candida* showed reduced sensitivity to pyraclostrobin, further testing of the isolate revealed this resistance is very low. Results are summarised in Table 20.

Table 20. Sensitivity of five *Albugo candida* isolates to strobilurins and copper

Isolate Number	Location	azoxystrobin	pyraclostrobin	trifloxystrobin	Copper hydroxide
09/267	Vic	-*	-	-	-
09/269	Vic	-	-	-	-
SA001	SA	-	-	-	-
09/941	NSW	-	-	-	-
09/636	NSW	-	+/-*	-	-

*- = sensitive to chemical, +/- = partial resistance

It should be noted that resistance to strobilurins may have been underestimated due to the low number of isolates tested in this study. Further studies should focus upon testing more isolates from a wider geographical range. Resistance can develop and spread rapidly and the recent La Nina weather pattern has brought wetter conditions to Eastern Australia that has necessitated an increased frequency in the application of these chemicals.

7. BACTERIAL TOLERANCE TO COPPER

7.1 Introduction

Phytopathogenic bacteria cause economic losses in a wide range of Australian vegetable crops, particularly during wet or humid conditions (Tesoriero & Milligan 1991, Persley et al. 2010). Copper (*Cu*) is routinely applied to many crops as a fungicide and bactericide. *Cu* -based products are the only registered chemical controls available in Australia for outbreaks of bacterial diseases of vegetables and many other crops.

Effectiveness of *Cu* sprays depends upon three key factors: the chemical form of *Cu* in the product (Scheck & Pscheidt 1998); the need for thorough coverage when applied (Agrios 1988); and the susceptibility of the target bacteria (Cooksey 1990).

Cu resistance in phytopathogenic bacteria was first described in the 1980s (Cooksey 1990) but had likely arisen earlier since it was demonstrated in isolates of *Xanthomonas axonopodis* pv. *vesicatoria* from capsicums that had been collected over a decade earlier. It is unlike fungicide resistance that often evolves through genetic alteration of target sites in the fungus. Plasmid-mediated resistance to heavy metals has been demonstrated in a number of bacteria (Silver & Misra 1988). *Cu* resistance in bacteria is often akin to antibiotic resistance where copper is bound, inactivated or excreted from the target cell (Davies & Smith 1978).

Plasmid-mediated resistance to copper has been demonstrated in several bacteria including the following phytopathogens: *Pseudomonas syringae* pv. *tomato* (Bender & Cooksey 1985), the cause of tomato bacterial speck; *Xanthomonas axonopodis* pv. *vesicatoria*, the cause of a leaf spot on capsicums (Stall et al. 1986); *Pseudomonas syringae* pv.*syringae* from cherries (Sundin et al. 1989) and *Xanthomonas campestris* pv. *jugulandis* from bacterial blight of walnuts (Lee et al. 1993).

Only two previous studies have documented high tolerance to copper in Australia. Tesoriero et al. (1997) confirmed a widespread occurrence in *P. syringae* pv. *tomato* isolates from NSW and Victoria. Martin et al. (2004) found that 28% of Queensland isolates of *X. axonopodis* pv. *vesicatoria* from capsicums and chilli exhibited tolerance to 1mM *Cu.*

Cu concentration thresholds reported for determination of isolates as resistant, intermediate or sensitive has varied with different species and studies; *P. syringae* isolates exhibiting a tolerance ≥0.32 mM $CuSO_4$ were deemed to be resistant by Scheck et al. (1996), whereas Tesoriero *et al*. (1997) classified *P. syringae* pv. *tomato* isolates growing on <0.6 mM, 0.6-1.2 and >1.2 mM $CuSO_4$ to be sensitive, tolerant (intermediate) and resistant, respectively. *Cu* resistance was defined as tolerance to ≥0.8 mM $CuSO_4$ for *X. axonopodis* pv. *vesicatoria* (Gore & O'Garro 1999).

The purpose of this study was to broaden our knowledge of the incidence and distribution of *Cu* tolerance in bacterial populations from a range of Australian vegetable crops.

7.2 Materials and methods

Cu tolerance assays were performed using a method adapted from those described for *Pseudomonas syringae* (Scheck *et al*. 1996; Andersen *et al.* 1991; Tesoriero *et al*. 1997). Casitone yeast extract-glycerol agar (CYEG) was prepared and the pH adjusted with NaOH to compensate for the drop in pH that occurred on addition of copper sulphate, giving a final pH of 6.5-7. A 50 mg/mL (approximately 200 mM) stock solution of $CuSO_4.5H_2O$, prepared in sdH_2O and filter-sterilised, was added to the cooled agar prior to pouring. Four final concentrations copper-amended media were used: 0, 0.1, 0.5, 0.75 and 1 mM.

A collection of 99 bacterial isolates were assembled and assessed for *Cu* tolerance (Table 21). *Clavibacter michiganensis* subsp *michiganensis (Cmm)* isolates were included in this study as they can also affect capsicums.

Bacterial suspensions were prepared in 0.85% NaCl containing 0.02% Tween 20, from 48 hr cultures on King medium B, to an OD_{590} of 0.22-0.28. Aliquots (10 µL) were spotted onto the copper-amended media. *Cu* resistant *Pseudomonas syringae* pv. *tomato* (isolate 2161= PT23, provided by D. Cooksey, USA) was included as a positive control. Growth on the plates was scored following 2 and 6 days incubation at 25°C (Figure 20). The upper *Cu* tolerance was determined for each isolate from triplicate aliquots on separate media plates and from three independent experiments.

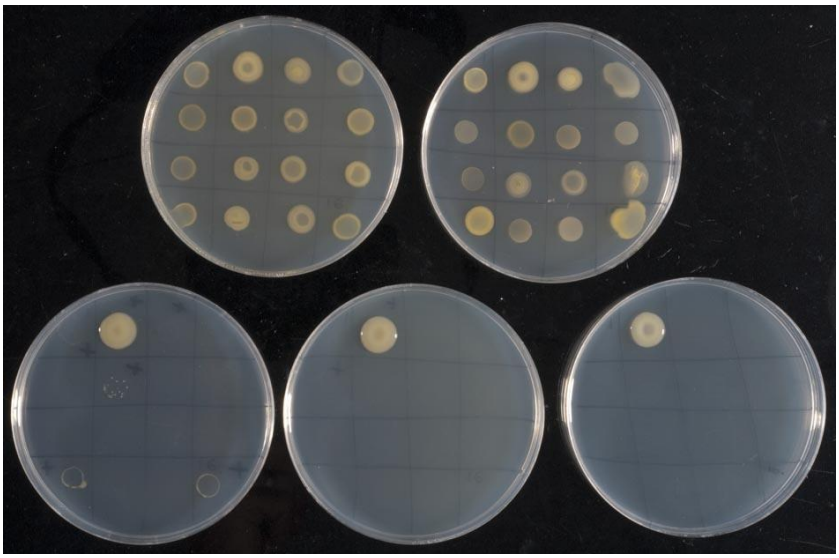

Figure 20. Copper tolerance screening assay with bacterial isolates on CYEG media amended with increasing copper concentrations. Top left: 0 *Cu*; top right 0.1mM *Cu*; bottom left 0.5mM *Cu*; bottom centre 0.75mM *Cu*; bottom right 1mM *Cu*.

Table 21. Bacterial isolates screened for tolerance to Copper

Species/Pathogen	Isolate Number	Specimen Number	Host	Location	Date of Isolation
Acidovorax sp.	2334	05/208	Watermelon	NSW	2005
Acidovorax konjaci	4230	06/556	Cucumber	NSW	2006
Acidovorax konjaci	4246	03/713	Cucumber	NSW	
Acidovorax avenae pv. *citrulli*	4231	05/208	Watermelon	NSW	2005
Acidovorax avenae pv. *citrulli*	4250	DAR74308	Watermelon	Qld	
Acidovorax vallerianellae	4246	04/142	Corn salad	NSW	2005
Ralstonia solanacearum	4032		Potato	Qld	2005
Ralstonia solanacearum	4221	DAR77786	Blueberry	NSW	2010
Pseudomonas syringae pv. *tomato*	2162	PT23	Tomato	USA	1990
Pseudomonas syringae	4181	09/776	Parsley	NSW	2009
Pseudomonas sp.	4232	06/582-9	Thai Basil	NSW	2006
Pseudomonas sp.	4255	10/422	Leek	NSW	2010
Pseudomonas cichorii	4180	09/681	Basil	NSW	2009
Pseudomonas cichorii	4182	09/812(2)	Lettuce	Vic	2009
Pseudomonas cichorii	4183	09/812(1)	Lettuce	Vic	2009
Pseudomonas syringae pv. *porri*	4244	10/326	Leek	NSW	2010
Pseudomonas syringae	1993	03/636	Tomato	NSW	2003
Pseudomonas syringae	1998	04/085	Tomato	NSW	2004
Pseudomonas syringae	2062	07/562	Silverbeet	NSW	2007
Pseudomonas syringae	2071	07/556	Chinese Cabbage	NSW	2007
Pseudomonas syringae	2075	06/561	Parsley	NSW	2006
Pseudomonas syringae	2076	06/852-9	Thai Basil	NSW	2006
Pseudomonas syringae	2077	07/733	Basil	NSW	2007
Pseudomonas syringae	2080	07/545	Silverbeet	NSW	2007
Pseudomonas syringae	2109	06/396	Daikon Radish	NSW	2006
Pseudomonas syringae	2123	03/439	Cucumber	NSW	2003
Pseudomonas syringae pv. *syringae*	4102		Celery	Vic	2008

Species/Pathogen	Isolate Number	Specimen Number	Host	Location	Date of Isolation
Pseudomonas syringae pv. *lachrymans*	4108	09/487	Cucumber	NSW	2009
Xanthomonas campestris	4109	09/214	Eggplant	NSW	2009
Xanthomonas campestris pv. *campestris*	4241	10/293	Red Cabbage	WA	2010
Xanthomonas campestris pv. *campestris*	4243	10/309	Cabbage	NSW	2010
Xanthomonas campestris pv.*vitiens*	4220	10/143	Lettuce	NSW	2010
Clavibacter michiganesis	1984	J1578	Tomato	Qld	2005
	1985	J1677	Tomato	Qld	2005
	1987	4011	Tomato	Qld	2005
	1988	4018	Tomato	Qld	2005
	1994	04/019	Tomato	NSW	2004
		04/068			
	1995	DAR76145	Tomato	NSW	2004
	2000	04/529	Tomato	NSW	2004
	2002	06/875	Tomato	NSW	2006
	2004	06/890	Tomato	NSW	2006
	2007	07/163	Tomato	NSW	2007
	2008	07/192	Tomato	NSW	2007
	2009	07/193	Tomato	NSW	2007
	2010	07/194-2	Tomato	NSW	2007
	2012	07/231	Tomato	WA	2007
	2013	07/231-2	Tomato	WA	2007
	2020	07/911	Tomato	NSW	2007
	2023	07/917	Tomato	NSW	2007
	2027	07/922	Tomato	NSW	2007
	2045	J1578(2)	Tomato	Qld	2008
	2047	04/438B	Tomato	Vic	2004
	2048	04/388	Tomato	NSW	2004
	4009	08/684	Tomato	SA	2008

Species/Pathogen	Isolate Number	Specimen Number	Host	Location	Date of Isolation
Clavibacter michiganesis	4055	08/845	Tomato	Tas	2008
	4056	08/924	Tomato	Vic	2008
	4066	LMG2891	Tomato	Hungary	1963
	4067	LMG3679	Tomato	Kenya	1945
	4068	LMG3680	Tomato	Australia	1933
	4069	LMG3681	Tomato	U.K.	1956
	4070	LMG3683	Tomato	Sicily	1956
	4071	LMG3685	Tomato	U.S.	1939
	4072	LMG3686	Tomato	Zimbabwe	1960
	4073	LMG3687	Tomato	Italy	1961
	4074	LMG3689	Tomato	Zambia	1962
	4075	LMG3690	Tomato	Channel Islands U.K.	1962
	4076	LMG3694	Tomato	South Africa	1967
	4077	LMG3695	Tomato	Romania	1970
	4078	LMG3696	Tomato	Belgium	1967
	4079	LMG5597	Tomato	New Zealand	1961
	4080	LMG5602	Tomato	New Zealand	1967
	4081	LMG5603	Tomato	New Zealand	1967
	4082	LMG5604	Tomato	New Zealand	1968
	4083	LMG5605	Tomato	Tonga	1968
	4085	LMG5610	Tomato	Brazil	1964
	4086	LMG5616	Tomato	U.S.	1983
	4087	LMG5643	Tomato	Canada	1982
	4088	LMG5644	Tomato	Canada	1982
	4089	LMG5726	Tomato	Bulgaria	1983
	4090	LMG5727	Tomato	Bulgaria	1983
	4091	LMG7333T	Tomato	Hungary	1957

Species/Pathogen	Isolate Number	Specimen Number	Host	Location	Date of Isolation
Clavibacter michiganesis	4092	09/281	Tomato	NSW	2009
	4094	09/212	Tomato	NSW	2009
	4111	09/703	Tomato	WA	2009
	4179	09/684	Tomato	NSW	2009
	4193	09/790-A3	Tomato	NSW	2009
	4197	09/841	Tomato	Vic	2009
	4245	10/334	Tomato	NSW	2010
	4256	10/422	Tomato	NSW	2010
	4257	10/494	Tomato	NSW	2010
	4258	10/494B	Tomato	NSW	2010
	4259	10/494C	Tomato	NSW	2010
	4260	07/581	Tomato	SA	2007
	4261	07/911	Tomato	NSW	2007
	4262	10/627	Tomato	Vic	2010
	4272	11/111	Tomato	SA	2011
	4273	11/122G	Tomato	NSW	2011

7.3 Results and discussion

Relative *Cu* tolerance of bacterial isolates is presented in Figures 21 and 22. A wide range in *Cu* tolerance was found. Five isolates from NSW and Tasmania were strongly resistant and capable of growth on 1mM *Cu*-amended media. The positive control test organism, *P. syringae* pv. *tomato* (#2162) which has plasmid-mediated *Cu* resistance, tolerated 0.75 mM Cu but not 1mM Cu-amended media. One each of *P. syringae* isolates from cucumber and leek were capable of growth on 1mM *Cu*-amended media. A further *P. syringae* isolate from leek tolerated 0.75 mM *Cu* as could two isolates of *P. cichorii* from lettuce. Two *Acidovorax* isolates from vegetable seedlings also tolerated 0.75 mM *Cu*-amended media. Further *P. syringae* isolates from celery, parsley, silver beet and tomato had upper *Cu* tolerance levels of 0.5 mM. The remaining bacterial isolates from a wide host range were sensitive to *Cu* and had an upper tolerance level of 0.1 mM. *Xanthmonas campestris* pv. *campestris* isolates from brassicas were sensitive to *Cu*. This is consistent with results from a previous study of black rot disease in Australian brassica crops (Berg et al. 2004).

Sixty-two percent (40/65) of the *Cmm* isolates grew on 0.5 mM *Cu*-amended media. Of these more than half (23) tolerated 0.75mM *Cu*, and a further 3 isolates had an upper tolerance level of 1mM *Cu*-amended media. *Cu* resistance has not previously reported in *Cmm* isolates. This has some implications for tomato growers, particularly greenhouse growers who are using IPM. Better control of *Cu*-resistant bacteria can be achieved by tank-mixing *Cu* with a dithiocarbamate fungicide. However, chemicals from that class disrupt certain beneficial insects and mites. One alternative might be to tank-mix *Cu* with iron chloride (Lee et al. 1993, Scheck & Pscheidt 1998). Zevenhuizen et al. (1979) determined that the only accurate predictor for the efficacy of copper products was the amount of free cupric ions (Cu^{2+}) in solution. Moreover, they found that metallic copper content did not correlate with product efficacy. It appears that the availability of soluble copper for bacterial toxicity is influenced by chemical interactions once it has been applied and other chemicals in the product formulation. Arman & Wain (1958) showed that *Cu* forms complexes with organic compounds that leach from plants. Some of this bound *Cu* is unavailable for further reactions and therefore does not affect bacteria. Equilibrium establishes between the complexed and free Cu^{2+} giving rise to an effective concentration of Cu^{2+}.

The results from this study will assist the Australian vegetable industry to better manage bacterial diseases. In particular, growers will be able to judge risks of *Cu* resistance for certain host and pathogen combinations. The chemical industry will also be able to respond and improve efficacy of copper-based products. Field assessment of tank-mixing *Cu* with iron chloride could be the subject to future studies. It is initially required to confirm its efficacy to Australian crops affected by *Cu* -tolerant bacteria. Efficacy data may also be required to support registration of a formulated product.

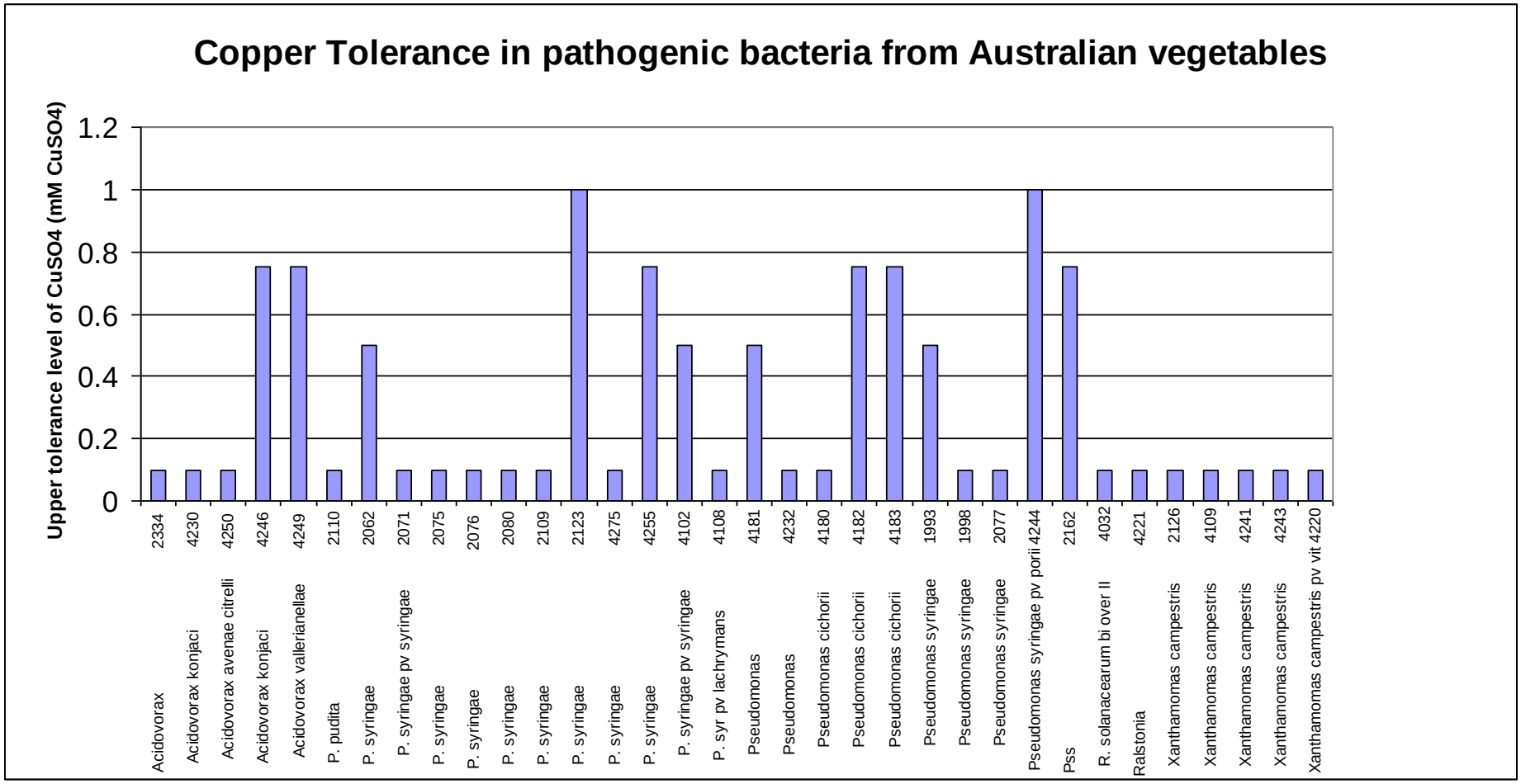

Figure 21. Tolerance levels to *Cu* of 34 isolates of pathogenic bacteria from Australian vegetable crops

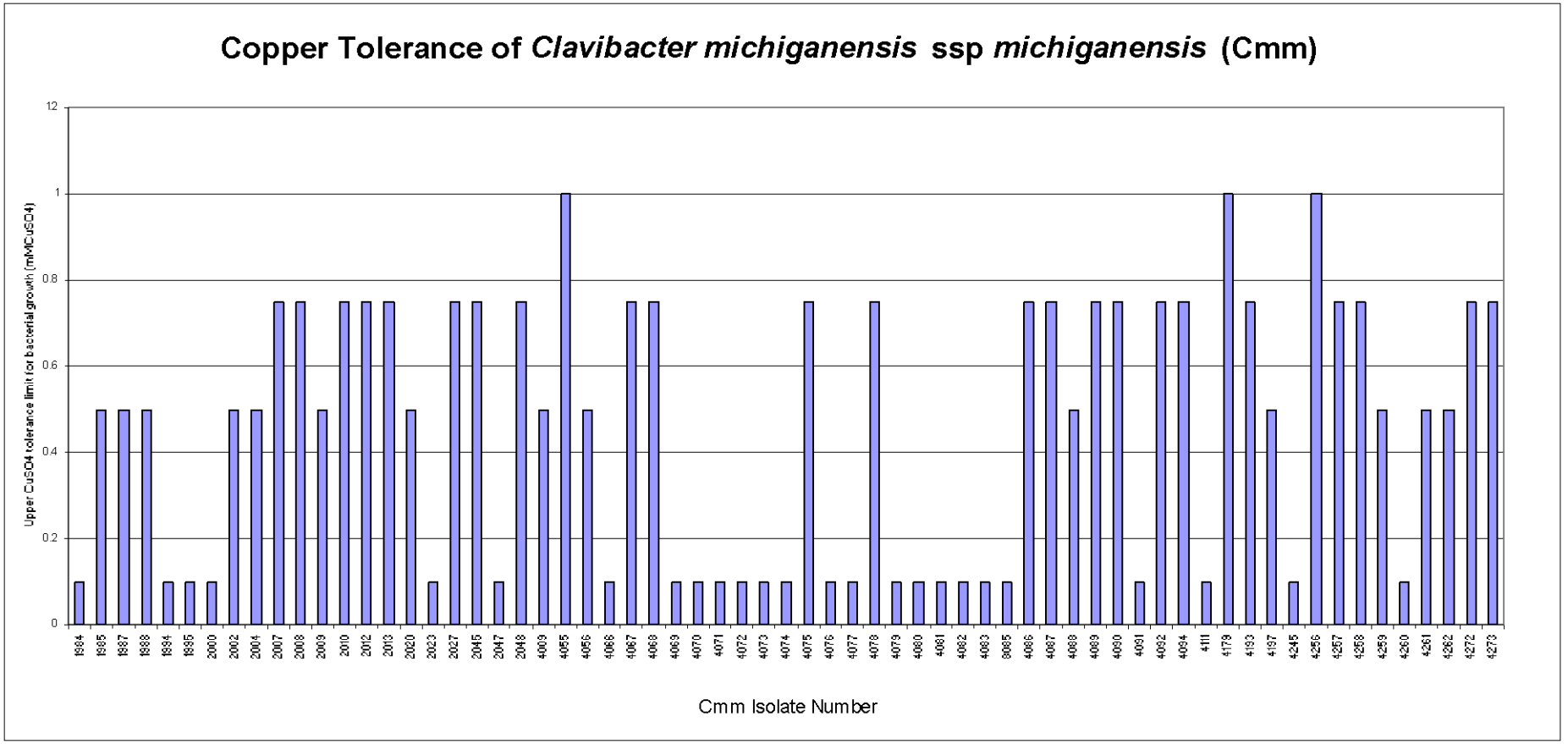

Figure 22. Tolerance levels to *Cu* of 65 Australian and overseas reference isolates of the bacterium, *Clavibacter michiganesis* from tomato.

8. OUTPUTS AND OUTCOMES

Information about the project and results of the research has been presented orally at industry conferences and grower meetings.

- Vegetable pathology meetings with program participants, HAL and Industry in 2008, 2009 and 2010.
- "Innovation and R&D Showcase" session at the Vegetable Industry Conference Melbourne 2009
- Australian Hydroponics and Greenhouse Conference in Sydney 2009 ("Improving the control of bacterial canker of tomato in Australia")
- Protected Cropping Association Conference in Adelaide 2011 ("Managing bacterial canker in Australia").
- Vegetable workshop: Foliage diseases at Cranbourne Victoria March 2008
- Soil borne Diseases Workshop, Victoria 2010.
- National Horticulture Conference, Lorne Victoria, September 2011
- Grower workshop Wannaroo WA Sept 2011.
- Grower workshop Coffs Harbour NSW October 2011
- Grower workshop Virginia SA June 2009
- Grower workshop Sydney NSW October 2011

Posters have been developed and presented at:

- Australian Hydroponics and Greenhouse Conference 2009 (Appendix 11.1)
- Vegetable Industry Conference Melbourne 2009
- Werribee field day 2009
- National Horticulture Conference, Lorne Victoria, September 2011 "*Resistance exists to fungicides and copper in vegetables"* (Appendix 11.2) (also on line http://www.hin.com.au/Associations/DFA/Resources/Resistance-exists-to-fungicides-and-copper-in-vege)

Posters of the Vegetable IPM Diseases program were bound into a booklet and handed out at the Vegetable Industry Conference, Werribee field day and grower meetings (Appendix 11.3).

Results have been presented in grower magazines and newsletters:

- Vegetable Industry Annual reports 2008, 2009, 2010.
- Asian and World Foods News letter 2011

Mrs Barbara Hall attended the International Congress of Plant Pathology in Italy, and presented a separate report on the outcomes and knowledge gained from that trip (Appendix 11.4).

Results of this research have been incorporated into:

- VG07109: development of effective pesticide strategies compatible with IPM Management used on farm.
- VG07110: Best practice production models (lettuce, Brassica). http://www.hin.com.au/Resources/Manual-Brassica-Ute-Guide, http://www.dpi.qld.gov.au/26_20351.htm (Appendix 11.5)

Two articles are currently in production, and a third planned. These will be submitted to Scientific journals, including Australasian Plant Pathology.

- Copper tolerance in bacteria
- Fungicide resistance of *Botrytis cinerea* from vegetable crops in Australia.
- Fungicide resistance of *Sclerotinia sclerotiorum* from vegetable crops in Australia.

Werribee field day 2009.

9. FUTURE RESEARCH RECOMMENDATIONS

- Expand testing to determine whether the *in vitro* resistance detected in some fungicide groups would translate to reduced field efficacy.

- Expand the testing of fluopyram to confirm cross resistance in *Sclerotinia*.

- Obtain baseline data for new fungicides for a range of pathogens prior to registration or before they are widely used.

- Continue resistance testing for existing and additional pathogens, including the mildews, *Colletotrichum* and *Alternaria*.

- Develop molecular tests for rapid diagnosis of resistance, particularly for downy mildews and white blister which cannot be cultured and therefore require testing by *in planta* assays that are laborious and time-consuming.

- Determine survival and threshold levels of resistant populations that correspond to field resistance.

- Sensitivity testing to the dicarboximide group of fungicides should be undertaken using the fungicide under question, as there may not be cross resistance between the different active ingredients for all fungi.

- Field assessment of tank-mixing copper with iron chloride could be the subject to future studies. It is initially required to confirm its efficacy to Australian crops affected by Cu resistant bacteria. Efficacy data may also be required to support registration of a formulated product.

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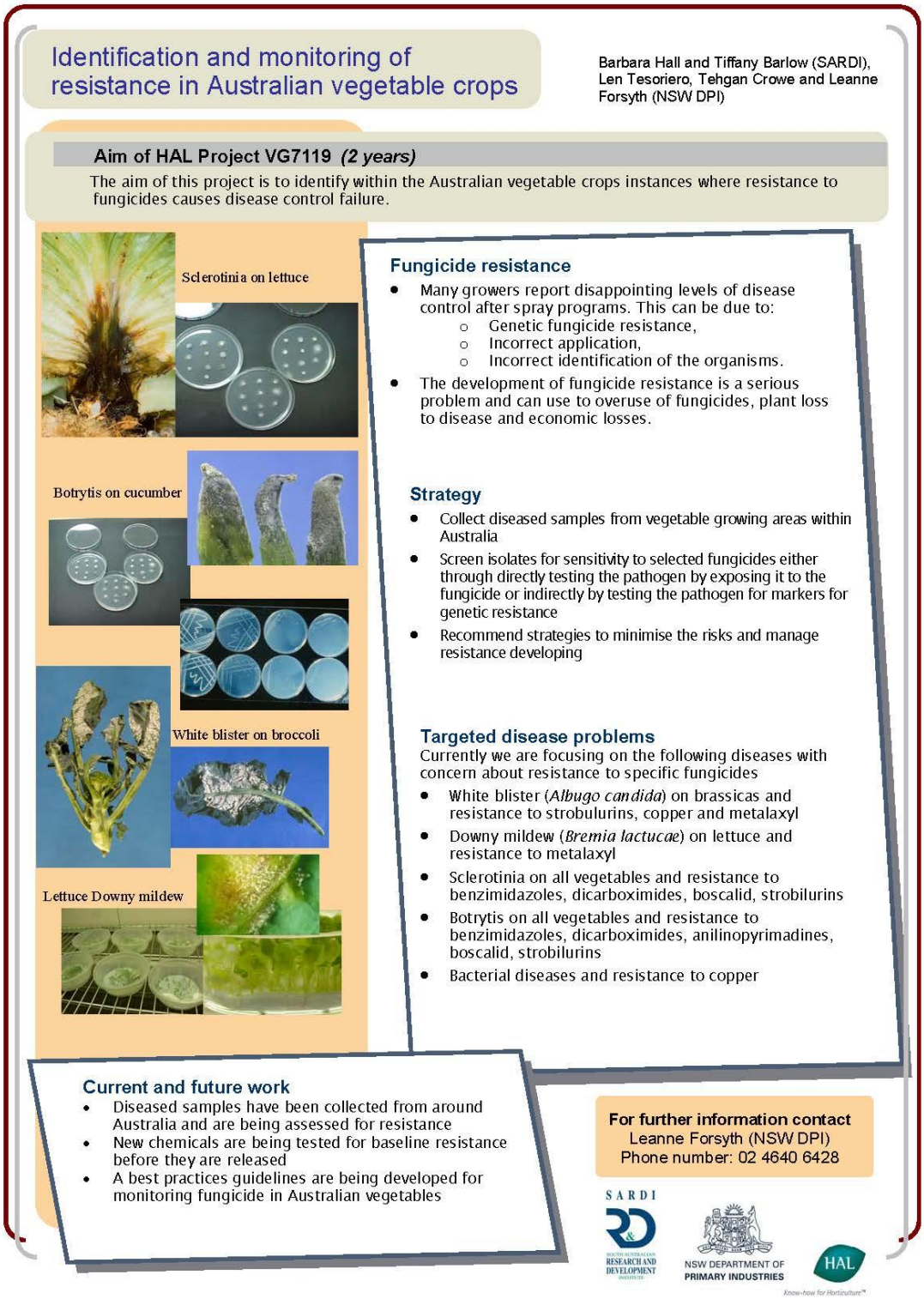
11. APPENDICES.

11.1 Poster of project overview



11.2 Poster presented at Lorne

RESISTANCE TO FUNGICIDES AND COPPER IN VEGETABLES

Barbara Hall[1], Len Tesoriero[2], Leanne Forsyth[2], Tiffany Barlow[1]

1 South Australian Research and Development Institute, Plant Research Centre, GPO Box 397, Adelaide SA 5001
2 NSW DPI Elizabeth Macarthur Agricultural Institute, Private Bag 4008 Narellan NSW 2567

Control of many vegetable diseases relies on the use of fungicides and copper bactericides. However with exposure to these chemicals, populations of resistant pathogenic fungi and bacteria can develop. Isolates of *Botrytis cinerea, Sclerotinia sclerotiorum, S. minor* and phytopathogenic bacteria from five genera were collected from vegetables throughout Australia and tested for resistance to a range of chemicals. Isolates of *B. cinerea* and *Sclerotinia* spp. were resistant to a range of fungicides from the benzimidazole, dicarboximide, anilinopyrimidine and strobilurin groups. Tolerance to copper products was detected in certain isolates from the genera *Pseudomonas, Xanthomonas* and *Clavibacter.* The detection of strong copper tolerance in several isolates of *Clavibacter michiganensis* subsp. *michiganensis,* the cause of bacterial canker in tomato, has not been previously reported. This paper will report on results of the fungicide resistance screening.

Resistance exists to fungicides and copper in vegetables

Barbara Hall, Tiffany Barlow: South Australian Research and Development Institute, GPO Box 397 Adelaide SA 5001
Len Tesoriero, Leanne Forsyth: NSW DPI Elizabeth Macarthur Agricultural Institute, Private Bag 4008 Narellan NSW 2567

SARDI
SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

Fungicide resistance was evaluated on 50 isolates of *Botrytis cinerea*, 77 of *Sclerotinia sclerotiorum* and 24 of *S. minor* from vegetable crops throughout Australia.

	Resistance detected		
	S. sclerotiorum	*S. minor*	*B. cinerea*
Azoxystrobin	×	×	✓
Boscalid	×	×	✓
Carbendazim	✓	✓	✓
Cyprodinil	✓	–	✓
Fenhexamid	–	–	×
Iprodione	✓	✓	✓
Procymidone	✓	–	✓
Tebuconazole	×	×	–

Low levels of fungicide resistance detected in *Sclerotinia sclerotiorum*, *S. minor* and *Botrytis cinerea*

99 bacteria isolates from vegetables were tested on agar amended with 0, 0.1, 0.5, 0.75 and 1mM copper.

Bacteria	Disease	No. isolates tolerant >0.1mM (no. tested)
Acidovorax spp.	Cucurbit fruit blotch	2 (5)
Clavibacter michiganensis	Tomato canker	41 (65)
Pseudomonas spp.	Various leaf rots	10 (22)
Ralstonia	Bacterial wilt	0 (2)
Xanthamonas campestris	Black rot	0 (5)

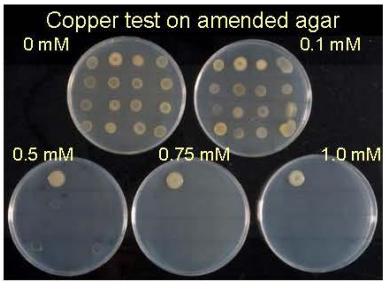
Some bacteria can tolerate high levels of copper

Following recommended resistance management strategies will enable continued use of these fungicides in Australia.


Botrytis cinerea on cucumber


Sclerotinia sclerotiorum on cabbage


Black rot on cabbage



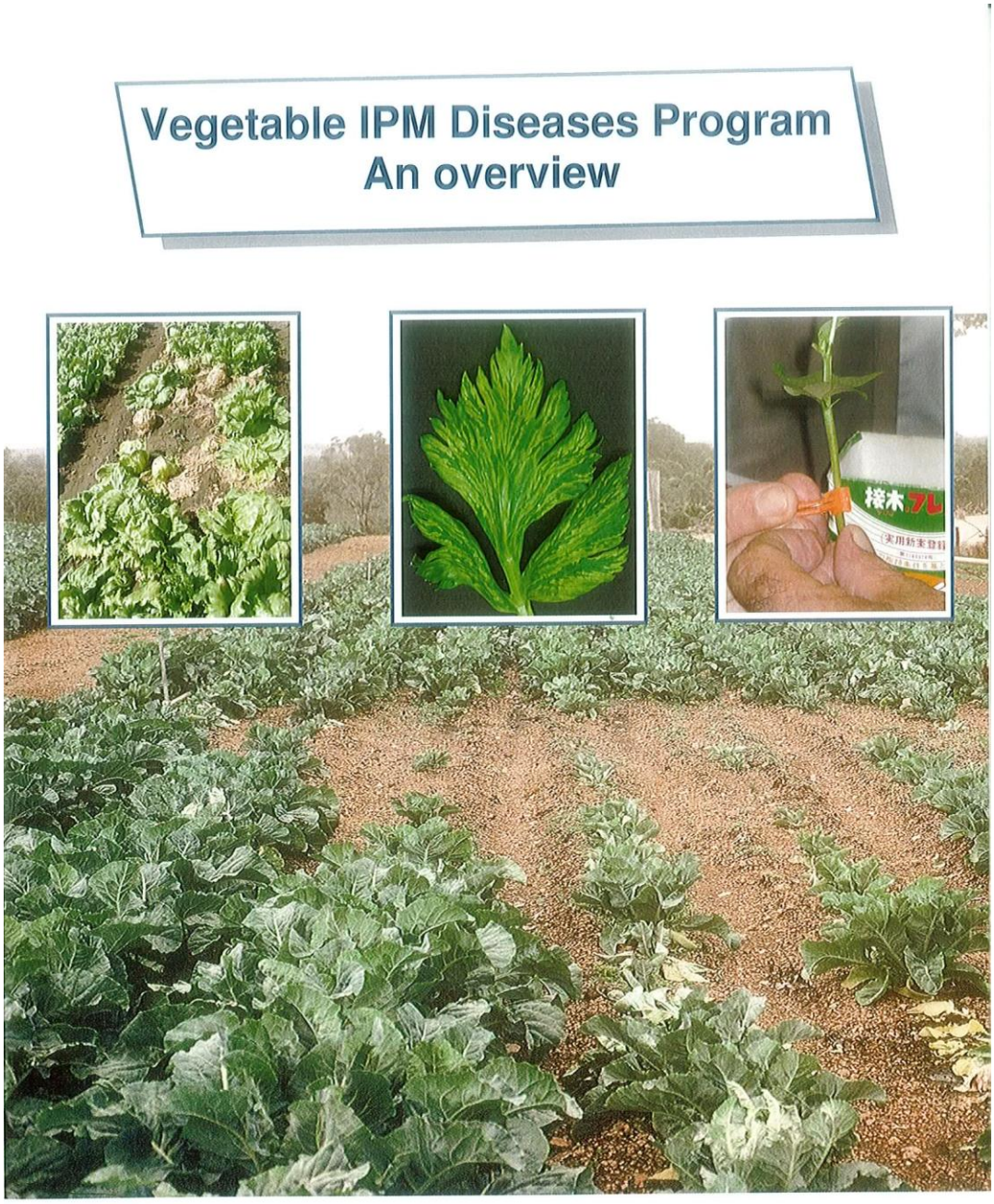
HAL Horticulture Australia

This project was part of a National collaborative project with NSW DPI facilitated by HAL and funded by the National Vegetable Levy. The Australian Government provides matched funding for all HAL's R&D activities.

NSW DEPARTMENT OF PRIMARY INDUSTRIES

Government of South Australia

11.3 IPM Diseases booklet.



11.4 Report on attendance at IPPC, Italy

HAL MILESTONE 107 REPORT VG07119

9th International Congress of Plant Pathology

by Barbara H. Hall
South Australian Research and Development Institute
November 2008

HORTICULTURE AUSTRALIA LIMITED

Milestone Report 107 November 2008

Project Title: **Identification and monitoring of resistance in vegetable crops in Australia**

HAL Project Number: VG07119

Research Organisation: South Australian Research and Development Institute
GPO Box 397, ADELAIDE SA 5001

Project Leader: Leanne Forsyth
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Key Personnel: Barbara Hall
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This report presents information on new fungicides and fungicide resistance gained from the attendance by Mrs Barbara Hall at the 9th International Congress of Plant Pathology.

CONTENTS

INDUSTRY SUMMARY

The 9th International Congress of Plant Pathology was held in Torino, Italy, 24-29th August 2008, attracting approximately 1400 participants from all over the world. With the theme "*Healthy and safe food for everybody* for the Congress", the keynote sessions were developed to emphasise the role of plant pathology in food safety and food security.

Over 1800 abstracts of both invited and offered papers have been published in the International Journal of the Italian Society for Plant Pathology, *Journal of Plant Pathology* (2008), 90 (2, Supplement) August 2008.

Two sessions were held on fungicide resistance, with paper discussing development of resistance in various crops with various fungicides. However the main topic of concern to all was the anticipated reduction in the number of active ingredients available to growers in the EU how this would impact on current resistance management strategies.

The main outcome of the congress was the exchange of scientific knowledge in an appropriate forum. The congress provided an ideal opportunity for plant pathologists from around the world to network, present their research, discuss common interests and develop collaborations.

9TH INTERNATIONAL CONGRESS OF PLANT PATHOLOGY.

The 9th International Congress of Plant Pathology was held in Torino, Italy, 24-29th August 2008, attracting approximately 1400 participants from all over the world. With the theme "*Healthy and safe food for everybody* for the Congress", the five keynote sessions were developed to emphasise the role of plant pathology in food safety and food security. Topics included: Role of plant pathology in food safety and food security; Host-pathogen interactions and molecular plant pathology; Diseases of Mediterranean crops and forests; Recent developments in disease management; and Knowledge and technology transfer. These were complemented by a plenary session on Global food security, a session celebrating the Centenary of the American Phytopathological Society and over 1800 posters and papers presented in the fifty concurrent sessions.

The abstracts have been published in the International Journal of the Italian Society for Plant Pathology, *Journal of Plant Pathology* (2008), 90 (2, Supplement) August 2008.

Three sessions presented information on chemical control, new fungicides and fungicide resistance.

- A keynote session with 3 invited speakers was held on "Recent developments in disease management".
- A concurrent session on "Concepts in Chemical control", with 4 invited speakers, 3 short papers and 22 posters.
- An evening session on "Resistance risk among new fungicides", with 10 invited speakers.

Many of the same speakers presented the same information at both the evening and concurrent sessions, therefore the following is a collation of the information presented at all sessions.

- Dr Andy Leadbeater, Syngenta discussed the challenges of chemical control. He emphasised that sustainable use of fungicides was the key to maintain their effectiveness long term. This was facilitated by the Fungicide Resistance Action Committee (FRAC), who plays a vital role in the design and support of strategies to manage resistance. However EPPO legislation is planned that will limit the number of chemical active ingredients registered in the EU to less that 200 from the current levels of over 400. This will have significant implications for resistance management, and may cause problems with residues from exported products from Australia.
- Proff Doug Gubler, University of California, USA presented evidence for Botrytis resistance for captan and fenhexamid in strawberries (USA). Every application increased resistance, and while it dropped off at the end on the season, there were still detectable levels at the beginning f next season. Increasing the chemistry in the rotation reduced the levels of resistance, with 4 chemistries better than 2. Comment was passed in discussion that resistance is inheritable, and if not inheritable it should not be considered resistance. Therefore is the Captan effect actual resistance??
- Dr Hideo Ishii, National Institute for Agro-Environmental Sciences, Japan discussed resistance in Japan to QoI, including the difficulties in molecular detection of QoI fungicide resistance, as instability can occur in the pathway. Cross resistance between

QoI fungicides is variable, particularly with the new generation of fungicides developed. He also presented information about the resistance to benzimidazoles and boscalid with *Corynespora cassiicola* leaf spot of cucumber. Boscalid was tested with YBA media at 10ppm a.i.

- Dr Andy Leadbeater, Syngenta, discussed the resistance risk of QoI fungicides. QoI resistance first detected in 1998 in *Bumeria graminis.* The "STAR" group were formed in FRAC (Fungicide Resistance Action Committee) to develop management strategies. There are 2 mutations with QoI resistance – G143A which can be fast or slow, but is always strong, and F129L which is fast but moderate (Reference Sierotzki et all 2000, 2007). Not all pathogens have demonstrated resistance to the QoI – eg rust (Reference Grasso et al 2006).
- Dr M.A. de Waard, Wageningen University, The Netherlands, presented information on the role of drug transporters in fungicides resistance. Over expression of drug transporters may protect plants against the azole fungicides group, and can result in resistance to chemically unrelated fungicides. Inhibitors of these drug transporter activity have potential for a range of new disease control agents.
- Dr Bart Fraaije, Rothamsted Research, UK presented information on resistance strategies with DMI's. Variants exist in the genetic changes with DMI's, therefore using a mix of triazole fungicides may help with minimising resistance development. The mutations are differentially selected by different members of the azole classes in the field. Mechanisms leading to reduced sensitivity to DMI's are different depending on the species of fungus. Three known mechanisms are mutation of the cyp51 gene, over expression of the cyp51 gene, or efflux pumps decreasing fungicide concentrations in cells. In some species the mechanisms is unknown (information presented by Mehl *et al*., Germany).
- Dr Haramoto, Japan, provided an evaluation of resistcne risk for a novel fungicides Cyflufenamid. This is a new fungicide for powdery mildew on barley, and a leaf assay or pot test was used to determine the EC50 levels. Baseline data has been undertaken, and all isolates tested were sensitive over 3mg a.i. After 6 applications in ne pot test, there was no change in sensitivity. In field applications of 2 per year for 3 years, also no change in sensitivity. There is no evidence of cross resistance to kreoxyl methyl. The resistance management strategies are same as all others – max 2 applications per year in a preventative mix with other fungicides.
- Dr James Adaskaveg, University of California, USA presented information on the resistance risk of stone fruit diseases in California. Resistance of *Monilinia* to the anilinopyrimidines was detected in 2007. The cause of resistance development in tree fruit was from misuse of the fungicide with poor application methods. The introduction of only one registered single site effective fungicides lead to significant overuse.
- Dr Angelo Garibaldi, Agroinnove, Italy presented data on the resistance status in Italian viticulture. Downy mildew: resistance to azoxystrobin (tested with leaf disc assay) was reported in 2001 in Northern Italy. 90% of downy mildew tested are also resistant to cymoxanil. Metalaxyl and dimethomorph are still sensitive at this stage. Powdery mildew: DMI resistance has been detected but no loss of efficacy reported (excised leaf assay). Grey mould: benzimidazole resistance has disappeared with no use, boscalid has reduced sensitivity, dicarboximides are stable at low levels, and anilinopyrimadines have reduced sensitivity. Work by Toffolatti et al (Italy) showed

that QoI resistance to downy mildew progressively decreased after a 12 month suspension of use, but a strong increase was found when applications resumed.

- Dr Ulrich Gisis, Syngenta Crop protection, Switzerland discussed risk assessment of resistance development. Two types of resistance exist, polygenic with a gradual shift in sensitivity (eg DMI's), or monogenic with a large change – they are either sensitive or resistant (eg QoI's). (References Cohen et al 2007, Rubin et al *Plant Disease* 2008). Zhu et al (China) undertook a risk assessment of *Phytophthora melonis* developing resistance to flumorph, as new CAA fungicides developed in China, defining the risk as moderate.

Some posters presented information on reports of fungicides resistance to various crops.

- Czechoslovakia: 216 isolates of cucurbit powdery mildew tested by modified leaf disc assay were sensitive to fenarimol. All were resistant or tolerant to benzimidazoles. Dinocap showed decreasing efficacy.
- India: Oomycete resistance to metalaxyl. Resistance detected in *Phytophthora infestans, P. parasitica* and *Pseudoperenopspora cubensis* (cucurbit downy mildew).
- Japan: *Pseudocercospora vitis* (grapevine leaf blight) resistant to strobilurins. Isolates also resistant to benzimidazoles.
- China: Resistance of *Fusarium fujikuroi* (Rice bakanae disease) to prochloraz.
- China: resistance of *Sclerotinia sclerotiorum* of canola to carbendazim and dimethachlon.

Molecular probes have been developed for determining resistance.

- Japan: Taqman probe assays for Melanin biosynthesis inhibitors and strobilurins on *Magnaporthe grisea* isolates (rice blast).
- Greece: DMI resistance to *Cercospora beticola* of sugarbeet.
- Japan: Mutations in benzimidazole resistant *Fusarium asiaticum* (head blight of cereals).
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Several posters presented information on characterization of resistance and fitness of resistant strains.

- Greece: fitness of anilinopyrimidine resistance strains of *Botrytis cinerea*. Results variable, but suggest that development of resistance did not affect the fitness of the isolates.
- Switzerland: evolution of the CYP51 based sensitivity in *Mycosphearella graminicola*. Arose locally and wind dispersed. Recombinant mutants may have higher resistance.
- Italy: resistance of *Botrytis cinerea* to benzimidazoles showed different mechanisms with variable resistance from low to high.
- China: metalaxyl resistant mutants of *Phytophthora boehmeriae* (cotton blight) shown to have good fitness.

- Switzerland: Mixtures of azoles could react synergistically against *Mycosphaerella graminicola* isolates with reduced sensitivity to DMI's.
- Japan: mutants of oxolinic acid resistance of *Burkholderia glumae* (bacterial grain rot of rice) with GyrA83 substitution retain the ability to survive on rice plants and are the common cause of resistance for field isolates.
- China: Resistance of *Fusarium fujikuroi* (Rice bakanae disease) to prochloraz potentially related to the cyp51 mRNA gene.

OUTCOMES

Collaboration / Networking

Informal meetings and discussions took place throughout the trip and as a result links were established with researchers and industry personnel from around the world. In particular, links with researchers developing techniques to detect resistance in both current and novel fungicides.

Knowledge

- The EPPO has plans to limit the number of chemical active ingredients registered in the EU to less that 200. Depending whether the MRL's are also altered for the banned chemicals, this may have significant implications with residues from exported products from Australia.
- Discussions of techniques for detecting resistance and development of molecular probes will be useful for testing resistance in Australia.
- Knowledge of risk assessments of fitness will enable more effective determination of suitable products for minor registration.

Communication.

- The information collected during this trip will be passed onto growers and industry personnel through information nights, growers meetings and HAL research meetings.

RECOMMENDATIONS

- That HAL continue to fund attendances at conferences to establish and maintain personal contact with international researchers. Collaborative research projects can strengthen the knowledge base and assist in development of future projects and collaborations.
- That HAL provide the opportunity for the minor use chemical program and similar to monitor the EPPO plans for reduction in active ingredients, and assess how this will impact on the Australian Vegetable industry.

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11.5 Best practice IPM booklets