Hort Innovation

Final Report

Biological Control of Botrytis cinerea in Strawberry

Project leader:

Dean A Metcalf

Delivery partner:

Metcalf BioControl

Project code:

BS12007

Project:

Biological Control of Botrytis cinereal in Strawberry BS12007

Disclaimer:

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Level 8
1 Chifley Square
Sydney NSW 2000

Telephone: (02) 8295 2300

www.horticulture.com.au

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Summary

Grey Mould disease in strawberries is caused by the fungus *Botrytis cinerea* which establishes latent infections in developing flowers which appear as decays as the fruit ripen. The objective of the project was to develop a biological control agent for *Botrytis cinerea* (grey mould) in strawberries. A variety of micro organisms were collected from different strawberry growing environments around Australia which had naturally colonised strawberry leaves and were tested for the ability to attack *B. cinerea* in culture and on strawberry leaves. From thirty four candidates the most promising nine were selected for field trials. A prototype spore powder was prepared containing each of the microbes which contained around one billion spores per gram.

Field trials in Queensland and Tasmania demonstrated that *Trichoderma* spp. were very capable of colonising the ovary of strawberry flowers and a corresponding reduction in latent *B. cinerea* infection was noted. This reduction in latent *B. cinerea* infection is the first step in reducing the impact of the disease. From these two trials the list of test microbes was reduced to the best four candidates which were all *Trichoderma* spp TSQ6 & TSQ7 collected in Queensland, and TST6 and TST7 collected in Tasmania.

In the second Queensland experiment the latent flower infection in untreated controls was 86.5% which provided a robust disease challenge, but *Trichoderma* colonisation significantly reduced this latent infection and all biological control agents provided statistically significant reductions in disease. Isolate TSQ6 provided the best reduction from 30.41% to 10.35%. A blend of the four isolates provided reduction to13.78% (P=0.05).

Disease incidence was low in the Tasmanian and Victorian field trials using the best candidates. In the Tasmanian trial the level of latent infection was reduced from 8.5% to 0-2% by different agents. In the Victorian trial, *Trichoderma* was not as active as in some others at colonising the flower ovary (this may be a varietal issue) but disease incidence was reduced from 2.36% to 0.97% (P=0.05) by the *Trichoderma* blend.

Trials investigated whether biological control agents could reduce the number of sclerotia which survive the winter to infect the crop in spring, but in our trials virtually all of the sclerotia decayed within three months without adding a biological control agent to the soil.

Some different rates of application were tested and it was found that 100g of spore powder per hectare was enough to reduce the level of latent *B. cinerea* infection in fruit by around 80% and 200g reduced this to 90%. The 100g rate seems the best in terms of benefit for cost of application.

It is difficult to integrate biological control into a crop program without consideration of chemical inputs. It was found that there was no inhibition of *Trichoderma* from being oversprayed with sulphur, copper oxychloride, copper hydroxide, or potassium bicarbonate, which provide some powdery mildew control options for times when biological control is used. Myclobutanil and trifloxystrobin inhibited the *Trichoderma* population by about one third.

A prototype spore formulation (which has been named "Strawbotizer") has been prepared which is ready for commercial trials. Results suggest the formulation will reduce losses to *B. cinerea* by around 65% if used at 100g/Ha on a weekly basis with only compatible chemicals. Growers would need to trial the formulation for a month to assess the full effect on the disease as first protected flowers ripen into fruit.

Keywords

<Botrytis cinerea,Trichoderma, biocontrol>

Introduction

Grey mould is caused by the fungus *Botrytis cinerea*. Strawberry growers have always struggled to manage this disease using chemical fungicides and an environmentally friendly alternative control is urgently needed, the disease has evolved resistance to most groups of chemical fungicides. The project aims to develop a biological control agent for *B. cinerea* in strawberries using a microbe which is a natural predator of *B. cinerea* and is adapted to growing in the strawberry crop environment to suppress the disease.

Botrytis cinerea is a major disease of Australia's strawberry industry and is considered internationally to be the most serious fruit rot of strawberries. The industry produces $308 million of fruit per annum (Industry Strategic Plan 2009-13). *B. cinerea* is common in all production climates within Australia. The disease can infect strawberry flowers to create latent infections which appear as the fruit ripen, or may directly penetrate the skin of ripening fruit. The disease may manifest in the field, or fruit can decay after packing which damages consumer appeal. *B. cinerea* readily invades any wounds in the skin including abrasions made by insects and produces large numbers of airborne spores within a few days which spread to nearby fruit, stems or leaves. In cool wet and humid weather the disease is a particular problem and can cripple production if not managed. The disease forms sclerotia in infected fruit which survive to release spores in the following season which perpetuates the disease cycle.

The project will collect and screen *Trichoderma* spp. that are indigenous to strawberry plantations and are able to grow on strawberry flowers or on the surface of fruit and foliage, and are able to attack *B. cinerea.*

Methodology

A detailed report has been compiled containing a literature review, methods and results.

In summary, the stages of the project were:

(a) Collect and screen microbes that are indigenous to strawberry plantations for ability to suppress *B. cinerea* in-vitro.

(b) Conduct assays to determine whether each microbe is better suited to protect flowers from being infected (creating latent infections that appear as fruit ripen), or better at protecting against infection of the fruit skin) ideally a final protective formulation might include microbes that can perform both of these tasks.

(c) Conduct assays to determine which isolates will reduce the survival of *B. cinerea* sclerotia (which reduces disease carryover between seasons).

(d) Conduct replicated field trial plot experiments of the most promising twelve isolates in Queensland in August 2014 and Tasmania in November 2014.

(e) Conduct field trials of the most promising four isolates in Queensland (August 2015) Tasmania (November 2015) & Victoria (January 2016).

(f) Conduct experiments to determine the ideal rate of application and compatibility with the foliar fungicides used in the strawberry industry.

Outputs

Following reviews of technical literature and commercial practice. candidate microbes were successfully identified and laboratory and field trials have been conducted.

A range of extension activities have been conducted including:

- Article in Strawberries Australia Magazine.
- Presentation to Queensland growers near Caboolture in June 2015.
- Presentation to Victorian Strawberry Growers at Wandin in Nov 2015.
- Presentation at Berry Quest 2015.
- Presentations at the Soil Care conferences in Lismore and Cairns.

On going collaborations between Metcalf BioControl and industry representatives which have been highly successful.

The project has successfully developed an experimental prototype bio-fungicide which can now be trialed under commercial conditions.

Outcomes

The principal outcome of the work is that the prototype biofungicide has been successfully developed by the methods described in the attached appendix. This is the achievement and original goal of BS12007.

The ultimate desired outcome is to see the widespread adoption of the prototype within the strawberry industry. Now that the biofungicide has been developed evaluation on a larger commercial scale can begin.

Evaluation and Discussion

Experience in biological control has shown that the selection of a biological control agent requires a micro organism that it adapted to growth and survival on the host plant and in the climate where the crop is grown, and that microbe needs to be capable of inhibiting the growth of the pathogen. For this reason a variety of micro organisms were collected from different environments and which naturally colonised strawberry leaves were tested for the ability to cause mycelial lysis in culture. Quite a large proportion of the flower samples collected contained latent *B. cinerea* inoculum, demonstrating the widespread latent nature of the disease. More than half of the collected isolates were able to cause lysis of *B. cinerea* mycelium in culture, which was promising, but also set a high standard for selecting only the best. *B. cinerea* is a captive target in these assays, and assays on plant tissue are a harder test.

An interesting array of mycelial interactions were seen. In some cases physical coiling was noted, in some cases distortion of growth was seen suggesting antibiotic production, and in some cases *B. cinerea* hyphae dissolved in the pathway of antagonist hyphae suggesting secretion of extracellular chitinases.

The microbes (which were mainly *Trichoderma* or *Gliocladium* spp) were tested for ability to restrict lesions on planta and inhibit sporulation. Using this method the microbes capable of invading *B. cinerea* lesions and attacking the disease were selected. Using the results of multiple leaf assays 34 candidates were reduced to nine best candidates for the first round of field trials.

Before going in to field trials it was necessary to verify that each candidate could be mass cultivated. Prototype spore powders containing around 10^9 CFU per gram were prepared.

It is useful to know not only the effects of each biological control agent on disease incidence, but also to know how successful the biological control agent is in colonising the " infection court " (site of infection). A semi selective medium for *Trichoderma* spp. named RASP (Metcalf *et al*., 2003) was used to monitor *Trichoderma* colonisation. By chance, *B. cinerea* is one of very few other fungi that can be detected on this medium. *B. cinerea* does not so much grow on the medium as sporulate in tissue segments placed in the medium. The ability to detect both *Trichoderma* spp. and *B. cinerea* on a single sample was a very valuable tool for monitoring population dynamics and was used throughout the series of field trials.

In the first field trial at Palm View Qld, nine isolates and *Trichoderma harzianum* Td81b were tested. It was necessary to monitor the progress of fruit development to determine the duration before the first treated fruit would ripen. It was found that the ripening duration was 4-5 weeks. This is important for both trial work and commercial use. It is not possible to judge the full effect of the biological control agent without applying for a month, so that the effect on latent infection within ripening fruit can be seen. The strawberry flower is infact a cluster of dozens of ovules (as marked by "achenes" or seeds on the ripe fruit). For a flower sample to show *B. cinerea* sporulation, only one of these would need to contain a latent infection. Similarly, if *Trichoderma* is found to be established within surface sterilised fruit, it need only have colonised a single ovary of the dozens present. However, the test does provide a generalistic indication of population dynamics

In the first Queensland experiment the effect on latent infection of applying biological control agents was

pronounced as can be seen in Figure 12 and Table 7, where 97% of fruit contain latent infection in the untreated controls and this is reduced, often by more than half in treated fruit where *Trichoderma* is detected is as much a 100% of surface sterilised fruit. There were 15.32% infected fruit in untreated controls and four treatments had one third as many infected fruit (not statistically significant, but something to work with).

The first trial in Tasmania tested ten isolates in a similar field experiment. Highly significant reductions in latent infection were recorded. Though the rate of application was the same as the Queensland experiment the percentage of fruit in which *Trichoderma* colonised the ovary (as measured by incubation on RASP medium), was about half of the Qld experiment. The variety and climate are different. The level of infection in the untreated controls was 13.34% The lowest percentage of infection in the trial was TSQ6 at 2.77%

In the second Queensland experiment the treatment list was reduced to the best candidates. Based on the two previous field experiments and the screening assays the isolates selected were *Trichoderma* TSQ6, TSQ7, TST6 and TST7. The latent flower infection in untreated controls was 86.5% which provided a robust disease challenge, but Trichoderma colonisation significantly reduced this latent infection and all biological control agents provided statistically significant reductions in disease. Isolate TSQ6 provided the best reduction from 30.41% to 10.35%. The *Trichoderma* blend provided reduction to13.78%

The second field trial using the best candidates in Tasmania was inconclusive due to a hot and dry season and no infection, despite latent infections in 8.5% of fruit. Samples on RASP medium suggested that latent infection was reduced to 0 -2% by *Trichoderma* treatments.

The field trial at Coldstream in Victoria tested the best candidates. Flower colonisation by *Trichoderma* was lower among these treatments than in other experiments and no significant reductions in latent infection were demonstrated. Disease incidence was low at 2.36%, but there were statistically significant reductions in infection provided by TST7 (to 1.0%), TSQ7 (to 1.17%) and the *Trichoderma* blend (to 0.97%). *Trichoderma* spp. seemed to behave a little differently in colonisation in the Victorian experiment and it may raise the interaction of *Trichoderma* races with strawberry varieties.

Sclerotia are generally regarded as an important source of inoculum for *B. cinerea*, as they are known to over winter and produce conidia in spring. In our experiment in parasitism of sclerotia there was no difference in survival of sclerotia between untreated control and *Trichoderma* treated sclerotia. The soil used in the experiment seems to have contained the microbes necessary for sclerotial parasitism without amendment of additional biological control agents. This may be generally the pathway of decay for buried *B. cinerea* sclerotia, and it would be worthwhile to know whether the same result would be obtained if the sclerotia were incubated in the open air.

The rate of application experiment showed that the 100g rate of spore powder application was enough to reduce the level of latent infection in fruit by around 80% and 200g reduced this to 90%. Application of 500g reduced latent infection to around 2% The 100g rate seems to be the optimum from a cost:benefit perspective.

Recommendations

The prototype spore powder "Strawbotizer" has shown potential to suppress the disease and is suitable for trials under commercial conditions.

Scientific Refereed Publications

<Provide a list of all **refereed scientific publications** published during the project that can be attributed or partly-attributed to the project. Use the format described below for journal publications, books and chapters in a book. Enter 'None to report' if there are no refereed scientific publications to report. Other publications such as magazine articles should be included in the Outputs.

Journal article

Orange, V., Apple, G.S., Banana, L.F., 2013. The nutritional profile of fruit varieties in Australia. *Journal of Horticultural Research* **163**, 51–59.

Whole book

Lettuce, I., Tomato, B.R., 2014. The Base Elements of a Salad (second edition). Vegetable Publishing, Melbourne.

Chapter in a book or Paper in conference proceedings

Broccoli, G., Capsicum, R.G., 2013. Growing fruits and vegetables. In: Peach, J.S., Avocado, R.D. (Eds.), Introduction to Australian Horticulture. Horticulture Publishing, Sydney, pp. 281–304.

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Intellectual Property/Commercialisation

Metcalf Biocontrol and HIAL hold intellectual property in the prototype and Metcalf BioControl confirms its interest to proceed toward commercialization based on the success of this study.

References

Please refer to Appendix attached.

Acknowledgements

Thankyou to Jason Hingston (Strawberry Industry Development Officer) for participation in Victorian experiments and Dr Fiona Giblin (University of Sunshine Coast) for participation in Queensland experiments.

For trial sites we are grateful to Mr Brendon Hoyle (Strawberry Fields) and Mr Sam Violi (Grower at Coldstream and Strawberries Australia Past President).

For technical support thankyou to Karen Obod, Des Burnet and Gordon Burr.

Appendices

A detailed report on experiments is attached

BS12007: Biological control for *Botrytis cinerea* in strawberries.

I Summary

The objective of the project was to develop a biological control agent for *Botrytis cinerea* (grey mould) in strawberries. A variety of micro organisms were collected from different strawberry growing environments around Australia which had naturally colonised strawberry leaves and were tested for the ability to attack *B. cinerea* in culture and on strawberry leaves. From thirty four candidates the most promising nine were selected for field trials. A prototype spore powder was prepared containing each of the microbes which contained around one billion spores per gram.

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A prototype spore formulation has been prepared which is ready for commercial trials. Results suggest the formulation will reduce losses to *B. cinerea* by around 65% if used at 100g/Ha on a weekly basis with only compatible chemicals. Growers would need to trial the formulation for a month to assess the full effect on the disease as first protected flowers ripen into fruit.

1.0. Introduction

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The project will collect and screen *Trichoderma* spp. that are indigenous to strawberry plantations and are able to grow on strawberry flowers or on the surface of fruit and foliage, and are able to attack *B. cinerea.*

The stages of the project will be:

(a) Collect and screen microbes that are indigenous to strawberry plantations for ability to suppress *B. cinerea* in-vitro.

(b) Conduct assays to determine whether each microbe is better suited to protect flowers from being infected (creating latent infections that appear as fruit ripen), or better at protecting against infection of the fruit skin) ideally a final protective formulation might include microbes that can perform both of these tasks.

(c) Conduct assays to determine which isolates will reduce the survival of *B. cinerea* sclerotia (which reduces disease carryover between seasons).

(d) Conduct replicated field trial plot experiments of the most promising twelve isolates in Tasmania in March 2014 and in Queensland in August 2014.

(e) Conduct field trials of the most promising four isolates in Tasmania & Victoria in the March 2015 and Queensland in the around August 2015.

(f) Conduct an experiment to determine the ideal rate of application and compatibility with the foliar fungicides used in the strawberry industry (mostly those used for powdery mildew control, some incompatibility is to be expected, but on past experience in grapes it is easily possible to re-schedule spray timings to avoid incompatibilities).

2.0. Literature

Opportunities.

Botrytis cinerea is a sclerotial fungus or member of the *Sclerotiniaceae*, sharing similar cellular biology and life cycle features with other plant pathogens in the genera *Botrytinia*, *Sclerotinia* and *Sclerotium*. In strawberries the flowers are invaded and this sometimes results in a decay of the pedicel at that stage, but the decay can remain latent to cause a rapid decay when the fruit ripen (Hancock, 1999). In Florida, marketable yield of strawberries is decreased by 10-15% even with weekly fungicide applications (Legard et al., 2000). *B. cinerea* spores which land on grape flowers can penetrate the inflorescence via capscars (Cole, 1999) and styles (Holz, 2001). Eighty percent cap fall appears to coincide with maximum susceptibility of flowers (Warren *et al*, 1998), which seems to be a time when cap scars are open to invasion, and fresh pollen is available as a nutritional source to enhance conidial germination. *B. cinerea* can also spread between berries within bunches later in the season. *B. cinerea* infections of flowers are generally latent, and manifest themselves as grey mould when berries decay at veraison. *B. cinerea* can also penetrate the cheeks of berries to cause bunch rots.

Protection of flower parts using microbes which prevent infection by *B. cinerea* is a logical first step in development of biological control agents (Boff *et al*., 2001). Later season control of spread between berries in infected bunches might be obtained by microbes which can attack *B. cinerea* on the berry phylloplane. A third opportunity at biological control is prevention of sporulation on infected tissue which will especially reduce late season infection by airborne spores. A fourth biocontrol opportunity exists in the possibility of using fungi capable of parasitising resting *B. cinerea* sclerotia before they can germinate to produce the spores.

In addition to this literature summary, detailed literature reviews which are relevant to the present proposal have been compiled by the Chief Investigator on use of *Trichoderma* spp. in biological control (Metcalf, 1997), the role of chitinolytic enzymes in biological control (Metcalf, 1997) management of *Botrytis* spp. in *Allium* crops (Metcalf & Dennis, 1999), and an invited literature review was published on management of *Sclerotium cepivorum* including biological control strategies (Metcalf 2002).

Antagonists.

A variety of microbes have been reported to inhibit development of *Botrytis* on leaf surfaces, including leaf surface bacteria (Blakeman and Brodie, 1976) *Bacillus pumillus*, *Pseudomonas fluorescens* (Swadling & Jeffries, 1996), yeasts (Cook *et al*. 1997a), actinomycetes (Kemira, 1996), and fungi including *Trichoderma* (Shtienberg & Elad, 1997), *Epicoccum* (Zapp *et al.* 1995; Michailides & Elmer, 2000), Alternaria spp. (Michailides & Elmer, 2000), *Gliocladium* spp. (Sutton *et al*., 1997; Volkmann *et al*. 1999; Shafia *et al*., 2001) and *Ulocladium* spp. (Volkmann *et al*. 1999; Michailides & Elmer, 2000; Boff *et al*., 2001). *Gliocladium roseum* has been recently reclassified as *Clonostachys rosea* (Schroers et al., 1999). In roses, Yohalem (2003) demonstrated control using the fungus *Ulocladium atrum* and *Clonostachys rosea* which performed better than iprodione.

Screening techniques

Biological control agents need to be able to proliferate in the space where they are required to suppress the pathogen and screening techniques need to address this. In screening for antagonists of *B. cinerea* Cook *et al.* (1997) developed a technique for isolation of bacteria and yeasts which adhere to *B. cinerea* germ tubes. Alternate strategies include isolation of a large number of organisms from the environment where the biocontrol agent must work which are cut down to a few best candidates by a series of culture, bioassay, and pot experiments (Swadling & Jeffries, 1996). The proponents of the present project have used a similar approach successfully in screening for biocontrol agents of *Botrytis allii* in onions (Metcalf & Dennis, 1999), Botrytis cinerea in grapes (Metcalf, 2005) and *Sclerotinia minor* in pyrethrum (Metcalf *et al*., 2003).

Mechanisms involved in biological control

Modes of action of biological control agents against *B cinerea* are numerous and variable and the involvement of two or more mechanisms has been demonstrated in several systems. Mechanisms have been reviewed by Elad (1996). Some proposed mechanisms by which biocontrol agents inhibit *B. cinerea* include competition for nutrients and space (Elad, 1996) inhibition of extracellular pectolytic enzyme secretion (Zimand *et al*, 1996), secretion of extracellular enzymes which damage *B. cinerea*'s cell wall such as chitinases or β-1,3 glucanases (Lorito *et al*., 1993), proteases which digest the pathogens extracellular enzymes (Zimand *et al*, 1996), antibiotic secretions (Ghisalberti & Sivasithamparam, 1991), or even modification of the plant phyllosphere to create conditions less favourable for conidial germination due to modified surface wettability (Cooper, 1986).

Suppression of sporulation by colonisation of infected plant tissue by secondary invaders is a valid biocontrol strategy which may have no direct effects on the success of infection by *B. cinerea* (Kohl *et al*., 1995). *Ulocladium atrum* competes strongly for nutrients and space within necrotic tissues with *B. cinerea,* by becoming the dominant organism *U. atrum* also reduces the opportunity for *B. cinerea* to disperse further by sporulation (Kessel *et al.,* 1999). A similar approach was demonstrated using *Ulocladium* sp., *Epicoccum purpurascens*, and *Alternaria* sp. in kiwifruit (Michailides & Elmer, 2000) and greenhouse tomatoes (O'Neill *et al*., 1996).

Parasitism of resting *B. cinerea* sclerotia by biocontrol agents has not been investigated in significant detail as compared to protection of foliar parts, however it has been studied intensively for other fungal sclerotia such as *Sclerotinia sclerotiorum* (Gerlagh *et al*., 1996), *Sclerotium cepivorum* (Gerlagh *et al*., 1996; Metcalf & Dennis, 1999) and *Sclerotinia minor* (Metcalf *et al*., 2003). *B. cinerea* sclerotia appear to be less resistant to parasitism than some other sclerotial fungi (Gerlagh *et al*., 1996).

Sites of activity.

Antagonist preparations perform differently in protection of different plant parts. For example germination of *Gliocladium roseum* conidia was greater and more rapid on stamens than leaves (Yu & Sutton, 1998). In strawberry flowers neither *B. cinerea* or the antagonist *T. harzianum* were readily able to colonise flower stages where fresh pollen was not available (Hjelyord *et al*, 2001).

Inoculum density.

The effective inoculum density for application is related to environmental conditions (RH, leaf wetness, temperature and nutrient status) however it is possible to establish how many conidia need to be present to provide disease inhibition by testing different concentrations of the antagonist. If too many conidia are added to the plant surface there may be no activity due to autoinhibition of conidial germination (Yu & Sutton, 1998). Conversely, in another study in strawberry flowers, the presence of high numbers (above 10^7) of *T. harzianum* conidia was required to create a micro-environment inhibitory to *B. cinerea* germination, a proposed mechanism was sheer increase in respiration (Hjelyord *et al*., 2001).

Effects of environmental conditions and plant physiology.

There is danger in generalising on ecological requirements of *Trichoderma* spp. as individual isolates vary broadly in ecological requirements (Metcalf, 1997). The ecological requirements of *B. cinerea* and biological control agents need to be taken into account when planning an integrated disease control strategy which includes biological control. High relative humidity and free moisture on leaves are required for *B. cinerea* conidia to germinate (Yunis *et al*., 1990). Optimum temperatures for conidial germination and infection occur between 9 -21°C (min 2°C, max >25°C), but mycelial growth after penetration is somewhat protected from environmental temperature and humidity requirements. Sporulation occurs over a wide temperature range (Shteinberg & Elad, 1997).

The colonisation of leaf surfaces by *Trichoderma* spp. is altered by air temperature (difficult to establish a general relationship) and relative humidity (far more active when RH was above 90% (Elad & Kirshner, 1993). The relationships of plant nutrition to phylloplane colonisation are complex and are affected by the NPK balance of the fertiliser and vary between species of plants. The population fluctuates with time elapsed since the initial *Trichoderma* application and had not begun to decline at 15 to 18 days when air temperature was 25°C and humidity was 75 to 85% (Elad and Kirshner, 1993).

Light intensity can also be an important determinant of both *B. cinerea* and prospective biocontrol agent spore germination. Periods of low light intensity predispose plants to development of *both B. cinerea* infections. Activity of the biocontrol agent *Clonostachys rosea* (syn *G roseum*) is influenced by duration of periods of high light intensity. Stress to the host plant as induced by low light intensity may mediate the increased spore germination (Shafia *et al*., 2001).

Another measure which may be useful as well as Relative Humidity in understanding establishment of biological control agents in the phylloplane is the wetness duration (of leaves or floral parts). On raspberry leaves *Gliocladium roseum* conidia required around 16 hours for almost all conidia which did ultimately germinate to produce germ tubes. Once leaf wetness had occurred the level of biological control activity became related to temperature. Twenty five °C was the optimum germination temperature at which 75% of conidia germinated, whereas 18% germinated at 10°C. Suppression of *B. cinerea* sporulation was more than 80% effective during 32 hours of leaf wetness while the temperature was above 20°C, however below this temperature the level of inhibition diminished to be 30% on stamens at 10°C (Yu & Sutton, 1998).

Enhancement of biocontrol agent conidial germination.

Hjelyord *et al*. (2001) described a process for pre-priming conidia for germination, known as "nutrient activation". Conidia are incubated in shaken potato dextrose broth for 6 hours, collected by centrifugation and washed in sterile distilled water and stored at 4°C before use. When applied to flowers these "activated" conidia germinated more rapidly to reduce *B. cinerea* infection twice as effectively as quiescent conidia (Hjelyord *et al*., 2001). Loss of germination ability was a key factor in reduced level of control of *B. cinerea* by *Gliocladium roseum* in raspberry. Periods of decline in CFU were related to high temperature and irradiance (Yu & Sutton, 1999).

Effects of Chemical Management Practices.

Due to widespread resistance to many commercial fungicides it is desirable to be able to mix biocontrol preparations with these chemicals, or apply chemicals in alternating use patterns with biologicals, without loss of efficacy on the part of the biological control agent, to minimise the developement of further resistance, including resistance to the biological control agent. Successful integration of biological fungicides has been demonstrated in spray programs with diethofencarb, carbendazim, iprodione and vinclozolin (Elad *et al*., 1993).

Application of some fungicides can reduce the natural population of *Trichoderma* spp on the phylloplane. For example 0.5g/L iprodione reduced the natural *Trichoderma* population from 6.6×10^3 to 1.5×10^3 per *Ruscus* phylloclade. In this experiment *T. harzianum* (T39) appeared to be quite tolerant of iprodione (Elad & Kirshner, 1992).

Conclusion

To achieve successful disease management by biological control it is necessary to use a microbe which is adapted to both the crop and environmental niche where it must perform, and to understand it's environmental requirements and mode of action. There are four separate parts of the strawberry production cycle where biological control can adversely affect the disease and it is desirable to attempt

to exploit all of these niches to achieve an integrated biological control strategy within broader IPM strategies.

3.0. Isolate collection and screening:

The first step in the study was to find candidate biological control agents, and a survey was conducted of nine plantations including production areas in Tasmania, Victoria and Queensland. Locations are listed in Table 1.

To sample the foliage, the variety of strawberry and location was recorded on a snap seal plastic bag which was placed over the foliar organs without any contact with human hands and broken off within the bag (Figure 1). At each site thirty leaf and 30 flower samples were taken for each variety tested. The sample was stored in ice before transport to Metcalf Bio Control's laboratory in Tasmania.

Samples were incubated on RASP medium (Figure 2) which is described in Appendix 1 and is largely selective for *Trichoderma* and *Gliocladium* spp. Leaf samples were incubated by removing a $1cm^2$ section of foliage that was placed aseptically on rasp agar (2 per plate). Flowers were bisected and placed on opposite sides of an agar plate. Agar plates were incubated at 22°C for 10 days before colonies were identified and sub cultures taken onto Pectin Agar (Appendix 1).

Micro-organisms collected in the survey were assigned a unique identification code at the time of isolation and are listed in Table 2. After the isolation each culture was purified by sub culturing a single hyphal tip and master cultures prepared for all isolates which are stored in double sealed agar in a mite-proof chamber.

To test the ability of each potential biological control agent to suppress *B. cinerea* the first step was to conduct a series of culture antagonism experiments. In these, the test isolate and *B. cinerea* were both inoculated onto different sides of three replicate agar plates. The two fungi were incubated together at 22°C until the first hyphal tips met in the centre of the agar plate (usually this took around 48 hrs) and the interaction of the two colonies was monitored by inverting the culture plate on the stage of a light microscope, so that the fungi could be viewed through the base of the plate. Observations are collated in Table 2. Some *Trichoderma* or *Gliocladium* spp. had negligible effect on *B. cinerea* hyphae, but about two thirds exhibited some type of hyphal inhibition. No hyphal antagonism was noted from the *Alternaria* or *Epicoccum* isolates. Some different modes of action were noted among the test isolates including *Trichoderma* TSQ7 which had a mode of action strongly based on mechanical coiling. TSQ7 changed direction to grow along the wall of *B. cinerea* before branching and ramifying to wrap around *B. cinerea* hyphae which began to decay (Figure 3). *Gliocladium* GSV11 exhibited a different mode of action where hyphal contact was not necessary for lysis to occur. The hyphae of *B. cinerea* were seen to crumple and decay as the smaller hyphae of GSV11 grew nearby (Figure 4). It is likely that GSV11 secretes some type of antimicrobial compounds (antibiotics) as well as multiple chitinase isosymes.

Table 1: Locations from which strawberry foliage was sampled in the survey for biological control agents.

Grower / Business name	Region	Varieties sampled
Smith, Tickleberry	New Norfolk, Tas	Red Gauntlet
DM Jennings & Sons	Cygnet, Tas	Seascape
Country World Hydroponics	Wandin, Vic	Albion
Kevin Chapman/ Blue Hills Berries	Wandin, Vic	San Andreas Albion
Strawberries Victoria Breeding Garden	Wanding, Vic	Experimental Varieties
Jay Berries	Wandin, Vic	Unknown
Ray Daniels/ Sunray.	Wamuran Qld	Rubi Gem Splendour
Merve Schifke	Wamuran Qld	Fortune Festival
Andrew Kroll	Caboolture, Qld	Festival

Figure 1: Dean Metcalf (left) with strawberry IDO Jason Hingston collecting samples from the breeding garden at Wandin (Vic).

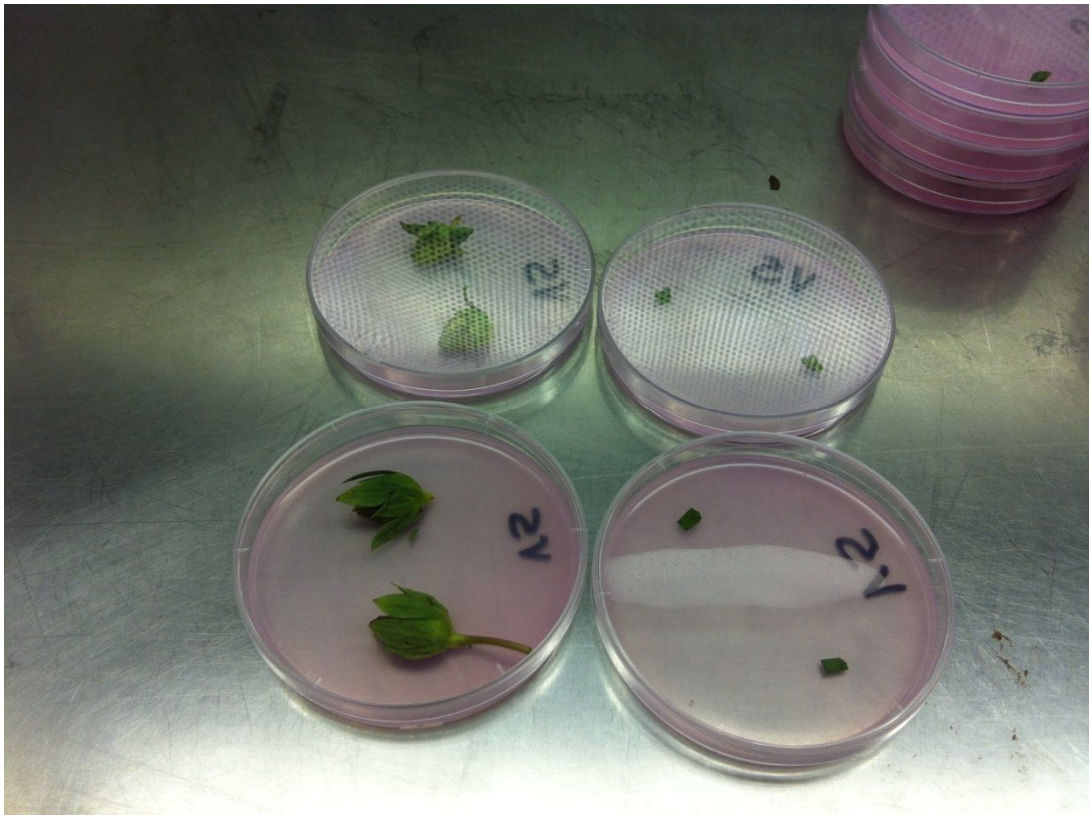

Figure 2: Strawberry foliage samples incubated on RASP medium.

Table 2: Microorganisms collected from strawberry plantations in Tasmania, Victoria and Queensland, together with notes on tests in dual culture with *Botrytis cinerea* (*Bc*).

Genus & isolate identification code*.	**Variety/ Grower/**	**Observation notes *Bc = Botrytis cinerea***	**Further evaluate?**
Isolates from Tasmania			
Trichoderma TST1	Unknown Jennings Calyx	24 hrs: *Bc* hyphae are browned and beginning to decay completely where colonies met. Lysis occurs in advance of contact. Little or no coiling was visible. Some mild stress signs on the *Trichoderma* hyphae.	Yes
Trichoderma TST2	Unknown Jennings Calyx	24hrs. Lysis of *Bc* hyphae and dissolution as the two fungi grew together.	Yes
Trichoderma TST3	Unknown Jennings Calyx	24hrs: TST3 formed an even growing front which moved through the *Bc* colony lysing hyphae and the infection cushions on the plate base about 3 longitudinal *Bc* cells length in advance of hyphae. . No physical contact required for dissolution.	Yes
Trichoderma TST6	Red Gauntlet Smith Calyx	24hrs: TST6 overgrew *Bc* hyphae without hesitation which became coagulated leading to cell death. Hyphal tip bursting seemed common, but not much dissolution at this point.	Yes
Trichoderma TST7	Red Gauntlet Smith Calyx	This isolate grew very sparsely on PA, but did radially expand as rapidly as other *Trichoderma*'s. The *Bc* hyphae dissolved in its path without physical contact even though hyphae were sparse. Did not observe any coiling.	Yes
Trichoderma TST8	Red Gauntlet Smith Calyx	12hrs. Browning and dissolution of *Bc* hyphae prior to physical contact as the colonies approach each other.	Yes
Gliocladium GST4	Red Gauntlet Smith Calyx	12hrs. Dissolution of *Bc* hyphae when the colonies met.	Yes
Gliocladium GST9	Red Gauntlet Smith Calyx	This isolate was very slow growing, but observed mycoparasitic coiling on *Bc* hyphae and dissolution with intense ramification on the *Bc* hyphae. May be too slow growing to be useful, but is a strong antagonist.	Maybe test further.
Gliocladium GST10	Red Gauntlet Smith Calyx	12hrs. This isolate caused lysis of *Bc* prior to hyphal contact, with unusual distortion and irregular branching of hyphae before vacuolisation and release of protoplasm. On contact GST10 was seen to turn at 90° to grow along the *Bc* hyphae and ramified on the cell wall.	Yes

Gliocladium GST11	Red Gauntlet Smith Calyx	24hrs. Minimal signs of browning of *Bc* when the colonies met. Not a strong antagonist.	No
Gliocladium GST12	Red Gauntlet Smith Calyx	This isolate was very slow growing, possibly same fungus as GST9. Observed *Bc* hyphae dissolve in the path of GST12 and Mycoparasitic coiling. 2nd test – Confirmed hyphal dissolution with some Mycoparasitic coiling. The isolate is very slow growing.	Maybe test further.
Isolates from Victoria			
Trichoderma TSV3	San Andreas Flowers Chapman	24hrs: Dissolution of *Bc* hyphae approx. 500 micrometres ahead of the advancing colony margin. Some distortion and irregular branching of *Bc* hyphae in zone ahead of dissolution.	Yes
Trichoderma TSV4	San Andreas Flowers Chapman	24hrs. No signs of lysis when the colonies met. Hyphae grew together.	No
Trichoderma TSV9	Breeding garden at Wandin Flowers	24hrs: The foremost tips of *Bc* were lysed as the two colonies met, but TSV9 did not appear to grow easily into the *Bc* colony. *Bc* hyphae seemed to be ramifying a few mm back into the colony and might have an opportunity to resist or form sclerotia	B grade
Gliocladium GSV5	Breeding garden at Wandin Flowers	24hrs. *Bc* encircled the *Gliocladium* colony which is slow growing. Some lysis of *Bc* visible at the colony margin. Possibly too slow growing to be useful.	Yes
Gliocladium GSV8	Breeding garden at Wandin Flowers	The *Gliocladium* isolate has been overrun by a *Trichoderma* spp. **Re- name culture TSV8** after this round of experiments. 24hrs: Lysis of hyphae without hyphal contact, although not strong lysis compared to some others in this study. Dissolved the infection cushions on base of plate.	Yes
Gliocladium GSV11	Breeding garden at Wandin Flowers	24 hrs: GSV11 is very slow growing and was rapidly encircled by the *Bc* colony. *Bc* hyphae turned brown in the leading colony edge and clearly died, but were slower to dissolve (took photo). This is a good biochemical antagonist but may be too slow growing.	Yes
Gliocladium GSV12	Breeding garden at Wandin	24hrs: The two hyphal fronts met. There was a low level of *Bc* lysis but the overall effect was only for the two colonies to hold their own ground. 48hrs. This isolate grew a lot more slowly than *Bc* in the assay and was encircled by *Bc* hyphae. It did exhibit some hyphal coiling on *Bc*, but may be too slow growing to be effective	No

Gliocladium GSV13	Unknown Jay Berries at Wandin Flowers	A few signs of browning of *Bc* hyphae with a clear zone effect at the forefront of *Gliocladium* colony where some *Bc* conidia had germinated. Very slow growing.	Maybe
Gliocladium GSV15	Unknown Jay Berries at Wandin Leaves.	24hrs. The two colonies mingled without any signs of lysis.	No
Alternaria ASV6	Albion Chapman Flowers	24hrs. No negative effects on *Bc* hyphae.	No
Epicoccum ESV2	San Andraeus Chapman leaves	24hrs. No negative effects on *Bc* hyphae.	No
Epicoccum ESV7	Breeding Garden at Wandin Leaves	24hrs. No negative effects on *Bc* hyphae.	No
Epicoccum ESV16	Jay Berries Wandin Leaves	24hrs. No negative effects on *Bc* hyphae.	No
Isolates from Queensland.			
Trichoderma TSQ1	Sunray Rubi Gem Leaves	24hrs. Lysis of *Bc* hyphae prior to any hyphal contact. Browning and death of *Bc*. Likely to be a diffusible principle.	Yes
Trichoderma TSQ2	Polyhouse Kroll Leaves	12hrs. After the colonies met hyphal antagonism against *Bc* was observed with mycoparasitic coiling.	Yes
Trichoderma TSQ3	Polyhouse Kroll Leaves	12hrs. No hyphal antagonism.	No
Trichoderma TSQ4	Fortune Schifke Leaves	24hrs. Hyphal coiling leads to vacuolisation, browning and death with ramification of *Trichoderma* on the *Bc* hyphae. Good antagonist.	Yes
Trichoderma TSQ5	Fortune Schifke Leaves	12hrs. *Bc* hyphae are browning and in a state of decay a few millimetres before they are reached by the *Trichoderma* hyphae. 24hrs. As the hyphal tips grew together hyphal coiling becomes visible. This is a strong antagonist (took photos).	Yes
Trichoderma TSQ6	Fortune Schifke Leaves	48hrs. Overgrowth of *Bc* colony with browning of hyphae and non-contact lysis (took photograph).	Yes
Trichoderma TSQ7	Fortune Schifke Leaves	24hrs. This isolate is a strong coiling antagonist. Ramified very strongly on *Bc* and altered growth direction to remain on *Bc* hyphae. Quite a large amount of coiling needed to occur before *Bc* hyphae were lysed, but they rapidly became swollen and vacuolated (took photo).	Yes
Trichoderma TSQ8	Fortune Schifke Leaves	12hrs. After the hyphae grew together the cells die in a one-by-one manner and lysis with coiling occurs. This is a good antagonist.	Yes

Trichoderma TSQ9	Festival Schifke Leaves	24hrs. No signs of lysis on hyphal contact.	No
Trichoderma TSQ22239	Collected by Apollo Gomez from Qld in 2013	24hrs. This isolate possibly has some sort of volatile toxin. As the *Trichoderma* culture grew *Bc* hyphae were visibly coagulated up to 2cm ahead of the advancing *Trichoderma* mycelium. The effect was stronger nearer to advancing front of *Trichoderma* hyphae.	Yes.

***Notes on identification codes:**

First letter = Genus of fungus: T (*Trichoderma*), G (*Gliocladium*), A (*Alternaria*), E (*Epicoccum*).
Second letter = Plant of origin – in this case always S (Strawberry).
Third letter = State of origin T (Tasmania), V (Victoria), Q (Queensland).
Fourth (number) = number specific to the isolate from that State.

Figure 3: *Trichoderma* TSQ7 antagonism of *B. cinerea*. This isolate has a mechanism strongly based on hyphal coiling and the finer *Trichoderma* hyphae can be seen to grow along and wrap around the larger *B. cinerea* hyphae.

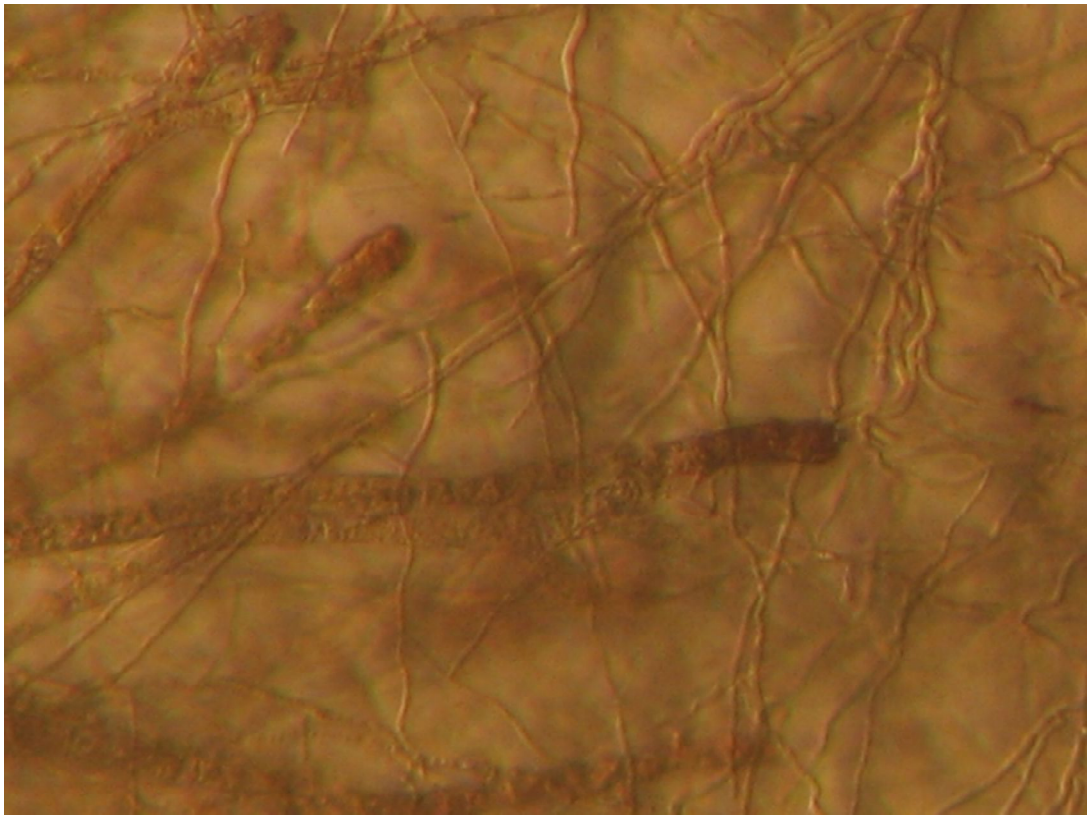

Figure 4 : *Gliocladium* GSV11 hyphae growing among the foremost hyphal tips of a *B. cinerea* colony. The larger *B. cinerea* hyphae can be seen to decay. This mode of inhibition suggests production of antibiotic compounds.

4.0. Screening Assays.

To test the ability of different potential biological control agents (BCAs) that were collected an assay was established on strawberry leaves. Strawberry leaves were wounded by dragging the upper leaf surface for 50mm along the surface of a sheet of sanding belt paper (Aluminium oxide GXK51 P80 130620) with the technicians index finger applying very light pressure. The wound site was marked by placing two spots 15mm either side of the wound zone using a white marker pen (Pentel 100WM). *Botrytis cinerea* spores were brushed directly from a sporulating strawberry fruit onto the wound area using a fine paint brush. The strawberry leaves were then individually placed in a 60mm deep, 110mm wide, 170mm long plastic trays lined with one sheet of tissue paper and 10 mls of sterile distilled water. Inoculum of each test BCA applied to the wound site as a 100µl drop containing 5 x 10^5 conidia which was applied to the centre of the wound and spread over the wound surface using a glass hockey stick which was sterilised between replicates. Each BCA treatment was replicated 5 times. The wounding only treatment (wounded but no spores of either *B. cinerea* or BCA added) and the untreated control (wounded with *B. cinerea* added but no BCA). The trays were sealed and incubated for 7 days in darkness at 22°C before assessment. The process is shown in Figures 5 & 6. An example of the end result is shown in Figure 7.

On assessment a 1cm^2 white paper frame was placed over the wound site and the following properties were recorded:

- The proportion of the wound area which had become necrotic. This was assessed on a 1 – 10 scale where 1 was completely green and had no visible signs of decay, and 10 was completely necrotic).
- The number of *Botrytis cinerea* conidiophores present in the 1cm^2 assessment area. This was recorded as a raw number.
- The presence of conidiophores of the test BCA within the 1cm^2 assessment area. This might be *Trichoderma*, *Gliocladium*, *Alternaria* or *Epicoccum* spores and was rated from 0 (none present) to 10 (covered in spores).

The results of these assays are shown in Table 3.

This was Metcalf Bio Control's first effort to develop an assay which would distinguish potential biological control agents. It is useful to measure the three properties of necrosis, conidiophore production of the pathogen and of the biological control agent because they provide different information about the way they work (or don't work). Results were often contradictory, largely because it was possible for organisms other than *B. cinerea* to invade the wound (most notably bacteria) which effected the necrosis value.

Leaves treated with 7 of the 34 fungi tested exhibited lower levels of infection than the untreated control (P=0.05) (Table 3). Leaves in 17 of the 34 fungi tested had lower numbers of *B. cinerea* conidiophores than the untreated controls. Conidiophore production by *B. cinerea* can be considered a reliable criteria for assessment of the biological control agents because it reflects *B. cinerea*'s successful infection (and the inhibition thereof by the biological control agent). Especially those isolates where no conidia of *B. cinerea* were produced are of interest for further screening.

There were no significant differences among treatments for sporulation of the biological control agent on the leaf wound (Table 3). It is encouraging that some of the biological control agents did sporulate at all on the leaf surface, as phyllosphere sporulation is not very commonly observed, certainly among *Trichoderma* spp. Sporulation is an indication that the biological control agent did establish in the wound and is therefore competent to grow on strawberry foliage. In the example of *Trichoderma* TSQ6

Trichoderma conidiophores were clearly visible among the inoculated *B. cinerea* conidia. There was no lesion on this specimen so TQS6 had scavenged nutrition for growth from the phyllosphere, likely including *B. cinerea* hyphae (Figure 8).

There are no clear standout biological control agents from the strawberry leaf antagonism assay, though it is encouraging to note that GSV13 and TSQ7 both were associated with reduced lesion size and reduced *B. cinerea* sporulation.

Table 3: The results of leaf infection assay 1 showing the area of necrosis on the 1cm^2 area of the leaf, the number of *B. cinerea* conidiophores and abundance of BCA conidiophores on the leaf

Treatment	**Necrosis of leaf (1-10)**	***B. cinerea* Conidiophores / 1cm^2**	**Biocontrol Agents Conidiophores**
TST1	3.80	0.20 *	0.00
TST2	6.00	2.20	0.00
TST3	4.60	0.00 *	0.00
TST6	2.60	0.20 *	0.60
TST7	5.80	0.60 *	0.00
TST8	2.00 *	1.00	0.00
GST4	3.80	0.00 *	0.00
GST9	1.60 *	2.20	0.20
GST10	3.80	1.40	0.20
GST11	2.40	2.00	0.00
GST12	2.80	2.00	0.00
TSV3	3.20	0.00 *	0.00
TSV4	6.00	0.40 *	0.20
TSV9	2.00 *	6.00	0.20
GSV5	4.60	0.00 *	0.00
GSV8	5.20	1.40	2.00
GSV11	1.60 *	1.20	0.00
GSV12	4.00	0.00 *	0.00
GSV13	1.60 *	0.20 *	0.00
GSV15	4.20	1.80	0.00
ASV6	5.40	0.00 *	0.00
ESV2	2.40	0.40 *	0.00
ESV7	2.20 *	2.80	0.00
ESV16	5.80	0.80	0.00
TSQ1	6.20	4.20	0.00
TSQ2	5.00	0.00 *	0.60
TSQ3	4.00	1.00	0.00
TSQ4	4.00	1.40	0.00
TSQ5	8.00	4.80	0.00
TSQ6	4.00	0.00 *	1.00
TSQ7	2.20 *	1.00 *	0.00
TSQ8	2.60	0.00 *	0.00
TSQ9	3.40	1.60	0.00
TSQ22239	4.20	0.00 *	1.00
Wounded only	2.64	0.00	0.00
Untreated : Wounded + inocul with *Botrytis*	5.88	3.48	0.00
P Value	0.010	6.07 x 10^{-13}	0.646
LSD0.05	3.62	1.88	
	1=healthy, 10=fully necrotic Values marked "*" have significantly lower necrosis than the untreated control.	Number of conidiophores in the 1cm^2 sample area. Values marked "*" have significantly lower sporulation than the untreated control.	0= no conidiophores 10= covered in conidiophores of No significant differences among means.

Figure 5: Wounding and inoculating leaves for the strawberry leaf antagonism assays.

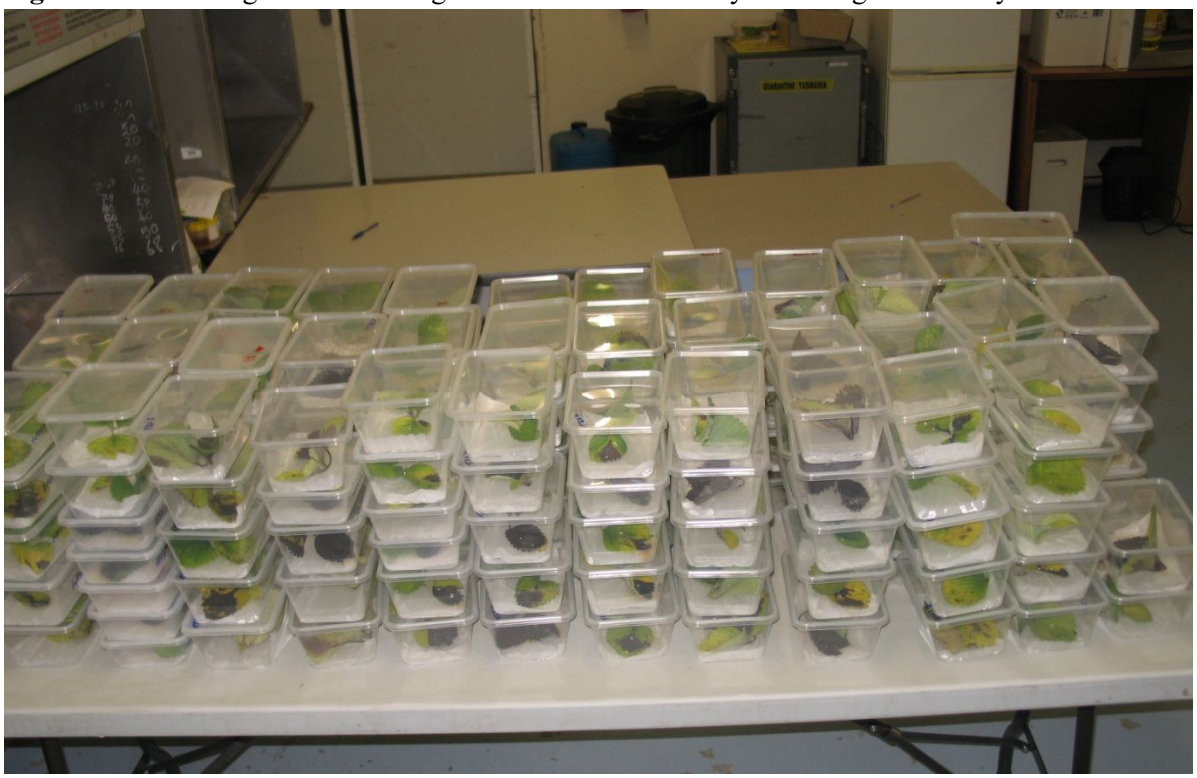

Figure 6: Strawberry leaves in trays from strawberry leaf antagonism assay 1.

Figure 7: Strawberry leaf in the antagonism assay. The wound site is marked between the two spots. *B. cinerea* conidiophores are beginning to appear from the necrotic area.

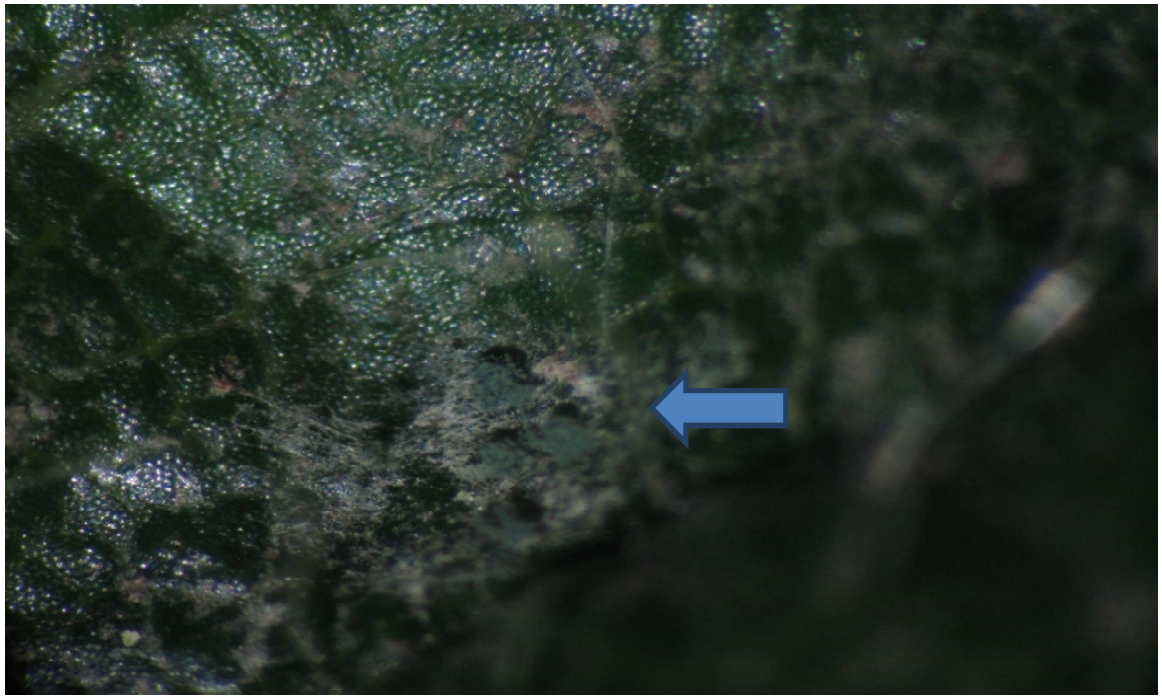

Figure 8: A wound site inoculated with *B. cinerea* and *Trichoderma* TSQ6. *B. cinerea* is visible as a brown/grey precipitate on the cuticle. TSQ6 has successfully germinated and grown to produce a clump of green conidiophores in the centre of the photograph (arrow).

Antagonism Assay 2:

A second replicate of the leaf antagonism assay was conducted to determine the capacity of each test biological control agent to inhibit the growth and sporulation of *Botrytis cinerea* in infected strawberry leaves. The procedure for the assay was as described for Antagonism Assay 1.

The results of leaf infection assay 2 are shown in Table 4. Whereas strong evidence of significant differences in comparison to untreated controls were obtained in assay 1, there are no significant differences between means in assay 2. The untreated control treatments (wounded, and wounded and inoculated) were conducted five times (each being of five replicates) and it was surprising that two of the five had lesions rated at zero, which influenced this result.

Table 4: The results of Antagonism Assay 2 showing the area of necrosis on the 1cm^2 area of the leaf, the number of *B. cinerea* conidiophores and abundance of BCA conidiophores on the leaf.

Treatment	**Necrosis of leaf (1-10)**	***B. cinerea* Conidiophores / 1cm^2**	**Biocontrol Agents Conidiophores**
TST1	3.0	0.0	0.0
TST2	1.4	0.0	0.0
TST3	3.2	0.0	0.0
TST6	3.4	0.2	0.0
TST7	1.8	0.2	0.6
TST8	4.0	0.0	0.0
GST4	2.6	0.0	0.0
GST9	4.0	0.0	0.0
GST10	2.4	0.0	0.0
GST11	3.0	0.0	0.0
GST12	2.0	0.0	0.0
TSV3	3.0	0.0	0.0
TSV4	2.0	0.0	0.0
TSV9	1.6	0.2	0.2
GSV5	3.6	0.2	0.0
GSV8	1.4	0.0	0.0
GSV11	2.2	0.2	0.0
GSV12	1.6	0.0	0.0
GSV13	2.2	0.0	0.0
GSV15	1.0	0.0	0.0
ASV6	2.0	0.0	0.0
ESV2	1.4	0.0	0.0
ESV7	1.4	0.0	0.0
ESV16	1.6	0.0	0.0
TSQ1	1.4	0.0	0.0
TSQ2	2.6	0.2	0.4
TSQ3	1.6	0.0	0.0
TSQ4	2.8	0.2	0.0
TSQ5	3.2	0.0	0.2
TSQ6	2.2	0.0	0.4
TSQ7	1.8	0.0	0.0
TSQ8	1.4	0.0	0.0
TSQ9	2.0	0.0	0.0
TSQ22239	1.0	0.0	0.0
Wounded only	1.6	0.0	0.0
Untreated : Wounded + inoculated with *Botrytis*	2.64	0.0	0.16
P Value	0.263	0.866	0.195
LSD0.05	-	-	-
	1=healthy, 10=fully necrotic	Number of conidiophores in the 1cm^2 sample area.	0= no conidiophores 10= covered in conidiophores.

It was not possible to include every biological control agent which was determined to have some positive attributes by the three assays, in the field experiments. To assist in selecting the best candidates , a decision support table was prepared (Table 5) which indicated simply using "+" or "-" whether the biological control agent performed well against the overall criteria of the assay. All four agents which were assessed positively in each assay were listed for further field experimentation, together with a selection of those that scored well in two out of three assays. In most cases the decision to take one of these and not another was based simply on whether the isolate exhibited abundant sporulation, which is an important attribute for manufacture. There was also at least one isolate from the three different states (TAS, VIC & QLD) included.

Table 5: Decision support table for selecting biological control agents for field experiments. Positive or negative performance are indicated by "+" or "-".

Treatment	Agar assay	Leaf assay 1	Leaf assay 2	Include in field trial Yes/No
TST1	+	+		
TST2	+		+	
TST3	+	+		
TST6	+	+		Yes
TST7	+	+	+	Yes
TST8	+	+		
GST4	+	+		
GST9		+		
GST10	+	+		Yes
GST11				
GST12				
TSV3	+	+		
TSV4		+	+	Yes
TSV9		+	+	
GSV5	+	+		
GSV8	+		+	
GSV11	+	+		
GSV12		+	+	
GSV13			+	
GSV15			+	
ASV6		+		
ESV2		+	+	
ESV7		+	+	
ESV16			+	
TSQ1	+		+	
TSQ2		+	+	Yes
TSQ3			+	
TSQ4	+			
TSQ5	+			
TSQ6	+	+		Yes
TSQ7	+	+	+	Yes
TSQ8	+	+	+	Yes
TSQ9				
TSQ22239	+	+	+	Yes

5.0. Queensland field experiment 2014.

A field experiment was conducted at Strawberry Fields, Palmview, Queensland, a business which combines "pick your own" with conventional strawberry production. The variety Camerosa was used.

The objective was to test biological control agents from Qld, Vic and Tasmania which have been identified as having potential for biological control of *B. cinerea* in strawberries in culture and leaf assays.

A prototype spore powder was produced for each isolate in the study, to be used in the field trial experiment. The concentration of propagules in the spore powder was tested by assessing the Haemocytometer count of 0.01g in 10ml water solution. The results are shown in Table 6. Most of the prototype powders were in the 10^9 Colony Forming Units range. This is lower than would be preferred in a final formulation, but experience has shown that production processes can be fine-tuned to improve CFU yield.

The field experiment was a randomised complete block design with 10 replicates of each treatment and approximately 20 plants in each replicate (3 linear metres of bed). Biological control agents applied at 200g/Ha using a Solo 475 knapsack sprayer delivering 14.7mls per second (117.6mls containing 0.03g of biological control agent prototype powder to each plot). The spray was applied only to foliage and not the bare ground between rows.

The trial was marked out and the first treatment applied on 26/8/14 and was sprayed on a weekly basis until 15/10/14. The trial site is shown in Figure 9. The variety *Fortuna* was used.

Treatment list for the 2014 Queensland field experiment.

(A) Untreated
(B) Untreated 2
(C) Untreated (sprayed with water only)
(D) TSQ2
(E) TSQ6
(F) TSQ7
(G) TSQ8
(H) TSQ22239
(I) TSV4
(J) GST10
(K) TST6
(L) TST7
(M) Td81b

Botrytis cinerea infects strawberry flowers and most effect of control measures is not seen until those flowers grow into fruit. To assess the duration required for maturation, flowers were tagged with the date of bloom and maturity monitored as in Figure 10. This information was used to schedule assessments, so that assessments were conducted at a time when the first fruit which had been sprayed as flowers, and had been sprayed weekly during development.

The ability of BCA's tested to prevent latent infections in flowers was tested by sampling five flowers from each plot in the experiment which were incubated on RASP medium (Appendix 1). *Botrytis* spp. is one of few other fungi which can grow on this medium so incubation of specimens provides an indication of the distribution of both fungi.

The flower samples were surface sterilised in 3% NaOCl for 1 minute before being washed in two changes of sterile distilled water for one minute. Samples were aseptically placed on RASP medium (the five from a replicate on a single plate) and incubated for seven days before inspection. Samples frequently sporulated and produced *B. cinerea* sclerotia (Figure 11). The difference between effective treatments and untreated controls was immediately obvious, with untreated flowers covered in grey clumps of *B. cinerea* conidiophores, whilst effective treatments were covered in green *Trichoderma* conidiophores (Figure 12). The results are shown in Table 7. In the untreated controls 97% of flowers contained latent *B. cinerea* inoculum. This indicates the extreme danger of infection at the site, that almost every developing fruit carries *Botrytis* and will either decay prior to picking (in conducive weather) or in storage. Nine of the agents provided significant reductions in latent *B. cinerea* infection, the best were TSQ22239 and TSV4 which reduced latent infection to 12 and 6% respectively. *Trichoderma* spp. were found to have colonised internal regions of the strawberry flower in 4% of fruit in untreated controls, some of this would have arrived by spray contamination from nearby treatments. There was internal *Trichoderma* colonisation (which means at least one floret of the many in a flower) in 100% of flowers treated with six of the BCA's. This indicates potential for internal systemic protection and eradication of *B. cinerea* infections, which is a useful attribute. *B. cinerea* infection and *Trichoderma* colonisation were inversely related. The suppression of these latent infections is a good indicator of BCA efficacy because latent infection is a major determinant of final disease incidence, even though fruit with latent infections may never express infection in dry weather.

The incidence of disease in the field trial experiment was recorded five weeks after establishment by picking the fruit in each plot. *Botrytis cinerea* infects strawberry flowers and most effect of control measures is not seen until those flowers grow into fruit. To assess the duration required flowers were tagged with the date of bloom and maturity monitored as in Figure 10

The fruit of irregular shape and size and those with disease were separated and the marketable fruit were weighed to calculate yield. The number of marketable fruit were counted, the number of *Botrytis* infected fruit were counted and the number of reject fruit and the number of fruit infected by other diseases.

In the Palm View experiment, *Rhizopus* spp. was the main cause of fruit decays other than *Botrytis*. The disease percentage is perhaps over expressed, as a scheduled commercial pick was delayed for three days prior to the experimental assessment. The results of the field trial experiment are shown in Table 8. The marketable yield in the untreated controls averaged at 0.4kg per plot. There was no significant difference among the treatments. The percentage of *Botrytis* infected fruit in the untreated controls averaged at 15.32% The percentages of infection were quite variable between replicates and accordingly, no significant differences in infection were recorded between treatments. It is pleasing to note though that the untreated controls are at the more severely infected end of the result spectrum and that TSQ6, TSQ7, TST6 & TST7 all have less than half the level of infection recorded in the untreated control. TSQ7 at 5.28% infection has 65.6% less infection than the untreated control. TSQ7, TST6 & TST7 all provided significant reductions in *Botrytis* latent infection of fruit (Table 8) so the results do support each other somewhat. The ANOVA on the other diseases (which were primarily *Rhizopus*) indicated significant differences among means, but no values are significant according to LSD(0.05).

A second assessment was conducted at the site two weeks after the first, but the temperature and humidity had altered in this time such that negligible levels of *Botrytis* infection were detected, even after incubating picked fruit at 20°C for 4 days.

Table 6: CFU counts of prototype spore powders used in the 2014 biological control field experiments at strawberry fields.

Biological Control Agent	CFU x 10^9
(D) TSQ2	2.85
(E)TSQ6	1.05
(F)TSQ7	2.35
(G) TSQ8	2.8
(H)22239	1.85
(I)TSV4	2.5
(J)GST10	2.35
(K)TST6	1.05
(L)TST7	2.1
(M)Td81b	2.8
P Value	0.00359
LSD0.05	1.03

Figure 9: Dean Metcalf assessing the 2014 trial at strawberry fields.

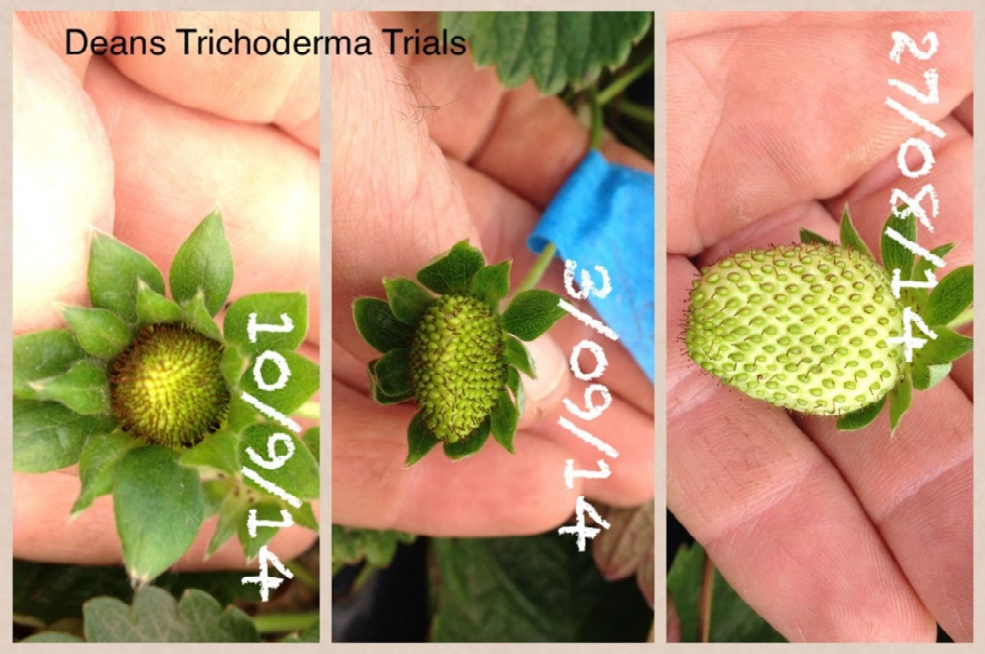

Figure 10: Picture taken on 17/9/14 of developing fruit which flowered on dates as labelled.

Figure11: Strawberry flower infected by *Botrytis cinerea* exhibiting profuse sporulation and three sclerotia in the centre of the picture.

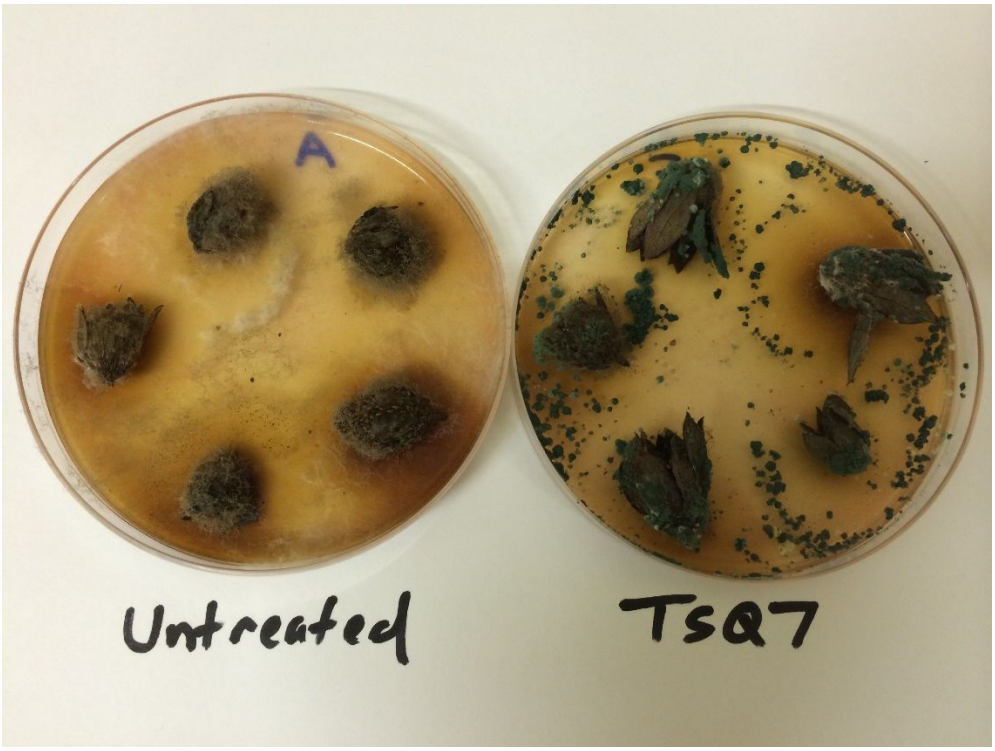

Figure 12: Strawberry flowers incubated on RASP agar. The flowers marked "Untreated" are infected by *B. cinerea*. The flowers marked "TSQ7" are covered in *Trichoderma* sporulation.

Table 7: Percentage of strawberry flowers which were internally colonised by *B. cinerea* and *Trichoderma* spp. as assessed by incubation on RASP medium for seven days.

	% *Botrytis*	**% *Trichoderma***
(A) Untreated	94	4
(B) Untreated2	100	4
Ave Untreated	**97**	**4**
(C) Water only	92	6
(D) TSQ2	60 *	80 *
(E)TSQ6	84	58 *
(F)TSQ7	36 *	100 *
(G) TSQ8	44 *	100 *
(H)22239	12 *	100 *
(I)TSV4	6 *	100 *
(J)GST10	32 *	88 *
(K)TST6	68 *	80 *
(L)TST7	60 *	100 *
(M)Td81	54 *	100 *
P Value	3.08×10^{-23}	3.76×10^{-36}
LSD0.05	19.19	24.80
	Values followed by "*" have significantly lower *Botrytis* flower infection than the untreated control.	Values followed by "*" have significantly greater BCA internal flower colonisation than the untreated control.

Table 8: Results of the 2014 biological control field experiment at Palmview showing the yield per plot, average number of infected fruit, percentage of *Botrytis* infection and percentage of other rots.

	Marketable Yield (kg / plot)	**Average number of *Botrytis* infected fruit / plot.**	**% *Botrytis* infected.***	**% other rots. ****
(A) Unt 1	0.397	6.2	15.13	18.15
(B) Unt 2	0.404	7.1	15.52	24.84
Ave Untreated	**0.400**	**6.65**	**15.32**	**21.50**
(C)Water only	0.424	4.9	11.43	18.23
(D) TSQ2	0.328	7.0	16.81	29.08
(E)TSQ6	0.374	2.9	6.61	25.04
(F)TSQ7	0.350	2.5	5.28	25.37
(G) TSQ8	0.308	3.5	10.33	29.66
(H)22239	0.386	3.9	9.82	26.55
(I)TSV4	0.514	5	10.86	17.95
(J)GST10	0.392	3.7	8.30	16.63
(K)TST6	0.320	2.8	5.72	20.61
(L)TST7	0.300	2.4	5.59	24.24
(M)Td81	0.278	3.4	9.56	29.12
P Value	0.142	0.269	0.559	0.047
LSD0.05	-	-	-	9.69

*- average is calculated as *Botrytis* rots / (total marketable fruit + reject fruit + other rots) *100

**- other rots were predominantly *Rhizopus* spp.

6.0. Screening isolates in Tasmanian conditions.

Molesworth field experiment 2014/5.

A field experiment was conducted at Dean Metcalf's farm at Molesworth, Tasmania, a business which combines "pick your own" with conventional strawberry production. The variety Red Gauntlet was used.

The objective was to test biological control agents from Qld, Vic and Tasmania which have been identified as having potential for biological control of *B. cinerea* in strawberries in culture and leaf assays.

The field experiment was a randomised complete block design with 10 replicates of each treatment and approximately 20 plants in each replicate (3 linear metres of bed). Biological control agents applied at 200g/Ha using a Solo 475 knapsack sprayer delivering 14.7mls per second (117.6mls containing 0.03g of biological control agent prototype powder to each plot). The spray was applied only to foliage and not the bare ground between rows.

The trial was marked out and the first treatment applied on 5/11/14 and was sprayed on a weekly basis until 13/2/15. The trial site is shown in Figure 1. Assessments were performed on 31/11/14, 4/12/14, 16/12/14, 20/12/14, 13/1/14, 24/1/15, 6/2/15 & 13/2/15. The number of mature fruit per plot were counted and weighed and the number of *B. cinerea* infected fruit were counted. The results for the eight assessments were pooled for analysis.

Treatment list for the 2014/5 field experiment in Tasmania.

(A) Untreated
(B) Untreated 2
(C) Untreated (sprayed with water only)
(D) TSQ2
(E) TSQ6
(F) TSQ7
(G) TSQ8
(H) TSQ22239
(I) TSV4
(J) GST10
(K) TST6
(L) TST7
(M) Td81b

The ability of Biological Control Agents tested to prevent latent infections in flowers was tested by sampling 5 flowers from each plot in the experiment which were incubated on RASP medium (Appendix 1). This is a selective medium for isolation of *Trichoderma* spp. *Botrytis* spp. is one of few other fungi which can grow on this medium so incubation of specimens provides an indication of the distribution of both fungi.

The flower samples were surface sterilised in 3% NaOCl for 1 minute before being washed in two changes of sterile distilled water for one minute. Samples were aseptically placed on RASP medium (Appendix 1), with the five from a replicate on a single plate) and incubated for seven days before inspection. The results (Table 9) show significant and sometimes total reductions in *B. cinerea* latent

infection of flowers, and significant increases in the colonisation of internal flower parts by *Trichoderma* or *Gliocladium.*

The results of the eight assessments of disease incidence were added together for analysis. The results are shown in Table 10. There was 13.34% infection among the three untreated control treatments. The lowest disease incidence in the experiment was among plants treated with TSQ6 (originally collected from Queensland) which had 2.77% infection, which is 79.4% less than the untreated controls.

Figure 13: Application of treatments to the trial site at Molesworth.

Table 9: Percentage of strawberry flowers which were internally colonised by *B. cinerea* and *Trichoderma* spp. as assessed by incubation on RASP medium for seven days. For GST10 the isolation figure shown is *Gliocladium.*

	% *Botrytis*	**% *Trichoderma***
(A) Untreated	23.99	16.00
(B) Untreated2	16.66	13.00
(C) Water only	13.00	19.00
Average of untreated	**17.88**	**16.00**
(D) TSQ2	3.00 *	40.00 *
(E)TSQ6	0.00 *	32.50 *
(F)TSQ7	0.00 *	47.14 *
(G) TSQ8	0.00 *	50.00 *
(H)22239	0.00 *	50.00 *
(I)TSV4	0.00 *	50.00 *
(J)GST10	0.00 *	46.00 *
(K)TST6	0.00 *	47.50 *
(L)TST7	0.00 *	50.00 *
(M)Td81	15.00	47.50 *
P Value	1.3×10^{-4}	7.4 x 10-38
LSD0.05	11.66	4.86
	Values followed by "*" have significantly lower *Botrytis* flower infection than the untreated control.	Values followed by "*" have significantly greater BCA internal flower colonisation than the untreated control.

Table 10: The percentage of infection among strawberry fruit from the field trial at Molesworth.

Treatment.	**Percentage fruit decay.**
(A) Untreated 1	11.37
(B) Untreated 2	12.53
(C) Water only	16.13
Average of Untreated	**13.34**
(D) TSQ2	8.18
(E)TSQ6	2.77
(F)TSQ7	6.18
(G) TSQ8	5.44
(H)22239	6.81
(I)TSV4	9.18
(J)GST10	10.79
(K)TST6	7.36
(L)TST7	5.20
(M)Td81	7.75
P Value	0.16

7.0. Testing best candidates in Queensland in 2015.

The second Queensland field trial experiment was established at Strawberry Fields (Palmview) on 6th August 2015. VARIETY The objective was to test the four best biological control agents which were identified as having potential for biological control of *B. cinerea* in strawberries. These have been collected from strawberry foliage in Qld, Vic and Tas and tested in culture for ability to kill *B. cinerea* mycelium.

The experiment was a randomised complete block design with 10 replicates of each treatment (Table 1) and approximately 20 plants in each replicate (5m x 1.6m bed wide). Biological control agents were applied at 100g/Ha using a knapsack sprayer.

Treatment list for the 2015 Queensland field experiment was as follows:

(A) Untreated
(B) Untreated 2
(C) *Trichoderma* TST6
(D) *Trichoderma* TST7
(E) *Trichoderma* TSQ6
(F) *Trichoderma* TSQ7
(G) Blend of TST6, TST7, TSQ6, TSQ7 (in equal portions adding up to 100g/Ha).

B. cinerea infects flowers to create latent infections, and colonisation of flower receptacles to prevent infection by *B. cinerea* is a key performance criteria in a biological control agent. Flowers were sampled to assess colonisation by *Trichoderma* and infection by *B. cinerea*, using the same procedure outlined in MS103. Ten flowers per replicate were surface sterilised and incubated on RASP agar (Appendix 1). The results are shown in Table 2. It can be seen that 6% of flowers in untreated controls were colonised by *Trichoderma*. Much of this is likely to have spread from nearby treated plots. In all of the *Trichoderma* treatments, upwards of 70% of fruit were internally colonised by *Trichoderma* In the same fruit samples, the percentage of fruit colonised by *B. cinerea* in the untreated controls was 86.5%, emphasising that the site used for the experiments was one of very high disease pressure if the humidity or leaf wetness should rise. The *Trichoderma* treated fruit all had a significantly lower number of latent *B. cinerea* infections than the untreated controls.

The field trial was assessed twice, on 10/9/15 and 24/9/15. The total number of fruit in each plot were harvested, the number infected were separated into two categories, *B. cinerea* and other rots. The number of "other rots" was quite small and results are not presented. The healthy fruit were weighed to determine the yield. Table 12 shows that the percentage of infected fruit in the untreated controls averaged at 30.41%. The biological control agents tested (which are the best from 2014 experiments) all reduced infection by a proportion of one half to two thirds, and all provided significant reductions in disease incidence. The best reduction was provided by *Trichoderma* TSQ6, which reduced infection to 10.35% (which is a reduction of 66% relative to the untreated control). There were no significant differences among the yields in the trial (Table 12).

The site for the trial was shaded more heavily on the western side than the eastern side by surrounding forest trees and the shaded area seemed to have greater disease pressure than the unshaded (Table 11). There was also a row where a dripper line problem appeared late in the trial and the affected plots seemed to have lower disease incidence. This effect was most noted at the final disease assessment. Some consideration of these forces may be possible in a later analysis.

Table 11: Layout of the second Queensland field trial at Palm View in 2015. The eastern (left) side of the trial was shaded in the afternoons which altered the disease expression in part of the site.

	D58					
G69	B57	B47	E37	B70		
A68	E56	G46	A36	A27	A18	D9
F67	F55	C45	A35	F26	G17	B8
C65	C54	A44	F34	C25	E16	G7
D64	D53	F43	E33	G24	C15	C6
A63	A52	D42	C32	B23	A14	D5
C62	G51	B41	B31	D22	C13	A4
E61	B50	G40	G30	B21	E12	F3
G60	E49	F39	D29	D20	G11	B2
F59	D48	C38	E28	F19	F10	E1

Table 12: Results of the second Queensland field trial at Palm View. The percentage of flowers colonised by *Trichoderma*, the percentage of flowers infected by *B. cinerea*, the percentage of fruit infected by *B. cinerea* at harvest and the yield in kg/5m plot. The disease incidence and yield are pooled values from two assessment dates.

Treatment	**Percentage of *Trichoderma* in flowers**	**Percentage of *B. cinerea* in flowers**	**% Disease incidence**	**Yield (kg/plot).**
A. Untreated1	10	86	30.33	2938.6
B. Untreated 2	2	87	30.50	3090.0
Average of Untreated Controls	**6**	**86.5**	**30.41**	**3014.3**
Trichoderma TST6	97	23 *	13.67 *	2629.5
Trichoderma TST7	100	31 *	13.13 *	2896.0
Trichoderma TSQ6	70	68 *	10.35 *	2722.0
Trichoderma TSQ7	100	28 *	16.47 *	3069.5
Blend of TST6, TST7, TSQ6, TSQ7.	100	19 *	13.78 *	2911.0
P Value	1.2×10^{-35}	4.9×10^{-15}	8.6×10^{-4}	0.65
LSD0.05	10.03	17.6	11.25	-

Figure 14: The site for the second Queensland field trial experiment at Palm View.

8.0. Testing the best candidates in Tasmania

The trial to test the best candidates in Tasmania was conducted on Metcalf's farm using the variety Red Gauntlet and used the same treatment list as the previous trial in Queensland except that Td81b (successful as a biological control of Botrytis in grapes) was added to the treatment list. The trial was sprayed with 100g/Ha of each biological control agent on a weekly basis beginning on XXXX until one week before the final sampling on 23/3/16. Disease was assessed on six dates on a weekly basis leading up to 23/3/16. The trial site had a few patches of *Fusarium* root rot, and the plots each of 5 metres were placed to avoid the infected area. However, some plots in the trial did suffer stunting to *Fusarium*. There were ten replicates of each treatment.

Four weeks after the sprays began, samples were taken (10 per plot) to assess the colonisation by *Trichoderma* spp. and of *B. cinerea* in developing fruit. The samples were surface sterilised as in previous chapters and incubated on RASP medium (Appendix 1) for 7 days before assessment of colonies. The results show that Trichoderma spp. colonised 15% of fruit in untreated controls, where in the biological control treatments the colonisation was significantly higher at between 40 – 50%. A higher spray intensity or frequency would be needed to achieve higher colonisation. The presence of *Trichoderma* spp. in untreated controls is far higher than it would be in most natural circumstances and has spread primarily by bees which had visited treated flowers in nearby plots. There were latent *B. cinerea* infections in 8.5% of fruit and this was significantly reduced to 2% or lower in each of the biological control treatments.

On each harvest date the total number of ripe fruit were counted and the number infected by *B. cinerea* or any other disease (clumped into a category of "other rots"). The 2015/6 season was the driest on record in Tasmania, so the weather was not conducive to spread of *B. cinerea*. Although there were 8.5% of fruit with latent *B. cinerea* infections, the level of infection in the untreated control fruit averaged at 0.485% With such a low percentage of infection in the untreated control there were no significant reductions in percentage infection and no significant differences in yield. Some of the actual variances in yield can be attributed to Fusarium stunt to plants and some adjustment could be made to data based on the number of healthy plants in each plot at the end of the trial. However, there is little to be gained when the dry weather lead to such low disease incidence.

Table 13: Results of the second Tasmanian field trial at Molesworth. The percentage of flowers colonised by *Trichoderma*, the percentage of flowers infected by *B. cinerea*, the percentage of fruit infected by *B. cinerea* at harvest and the yield in kg/5m plot. The disease incidence and yield are pooled values from six assessment dates. The season was very dry leading to low infectivity of *B. cinerea*.

	***Trichoderma* in flower samples**	***Botrytis* in flower samples**	**% Infection**	**Yield per plot (grams)**
A) Untreated	16	9	0.41	834.220
B) Untreated 2	14	8	0.13	620.900
Average of untreated	**15**	**8.5**	**0.485**	**727.560**
C) TST6	47	0	0.00	662.080
D) TST7	41	1	0.64	807.680
E) TSQ6	47	1	0.06	502.180
F TSQ7	44	2	0.39	499.900
G Blend	37	1	0.05	438.130
H Td81b	44	2	0.63	601.240
P Value	2.8×10^{-6}	2.2×10^{-4}		
LSD0.05	15.45	4.52		

9.0. Testing the best candidates in Victoria.

The field trial in Victoria to screen the best candidate biological control agents was conducted on the farm of Mr Sam Violi at Coldstream. The variety Albion was used. *B. cinerea* generally manifests in the autumn crop in Victoria so the trial was established on 9/2/16 and sprayed with 100g/Ha of each biological control agent on a weekly basis.

10 samples flowers were taken from each plot, surface sterilised and incubated on RASP medium as described in previous experiments. It is interesting that in the untreated controls the flowers had not been colonised by *Trichoderma* at all (Table 14), where this had occurred in all of the four preceding experiments. There were also no colonies of TSQ6 within flowers. There may be an interaction of *Trichoderma* races with strawberry varieties, or even climates leading to these observations. It is also worthy of note that the number of fruit with latent *B. cinerea* infections was not reduced in comparison to the untreated control, and this is the only trial where this did not occur,

It was intended to begin assessments as soon as *B. cinerea* was seen in the trial, but the weather remained warm and dry and two disease assessments conducted on 3/4/16 and 18/4/16. The assessment procedure is shown in Figure 15 where the fruit from each plot are picked into trays with the B. cinerea infected fruit and "other disease" fruit separated into individual containers. The fruit were then counted and weighed (Figure 16). Following the second assessment the crop was terminated by the grower so further assessments were not possible. The two assessments are added together and the results shown in Table 14 where the percentage of infection is 2.36% in the untreated controls. Although the disease incidence is low, significant reductions in disease incidence were provided by some of the biological control treatments, with the blend of four agents reducing infection to 0.97%

Table14: Results of the second Tasmanian field trial at Molesworth. The percentage of flowers colonised by *Trichoderma*, the percentage of flowers infected by *B. cinerea*, the percentage of fruit infected by *B. cinerea* at harvest and the yield in kg/5m plot. The disease incidence and yield are pooled values from two assessment dates.

	% flowers colonised by *Botrytis*	**% flowers colonised by *Trichoderma***	**% Infection**	**Yield (grams/plot)**
Untreated 1	44	0	2.24	1352.1
Untreated 2	38	0	2.48	1283.5
Average of Untreated	**41**	**0**	**2.36**	**1317.8**
C) TST6	41	20 *	1.91	1183.0
D) TST7	23	37 *	1.00 *	1227.8
E) TSQ6	47	0	1.64	1116.2
F) TSQ7	43	14 *	1.17 *	1337.8
G) Blend	33	11 *	0.97*	1005.4
H) Td81b	45	1	1.10 *	1351.0
P Value	0.065	1.4×10^{-5}	0.048	0.288
LSD0.05		1.52	1.13	

Figure 15: Harvesting fruit at the Coldstream (Vic) field trail site. The infected fruit are separated into containers which can be seen sitting in each plot.

Figure 16: Strawberry IDO, Jason Hingston participating in the trial assessment at Coldstream. The healthy fruit in each tray are counted and weighed.

10.0 Sclerotial parasitism experiments.

To assess the effect of each *Trichoderma* isolate in the experiment on the survival of *B. cinerea* sclerotia in strawberries, infected strawberry bulbs were collected from unsprayed fruit and allowed to decay in plastic trays in a farm shed until sclerotia were formed. Sclerotia were carefully picked from the remnant fruit in a manner to avoid abrasion of the rind and groups of 10 sclerotia were sealed in 50 x 50 mm nylon mesh bags (Figure 17). The bags were buried in 10 x 20 x 7cm plastic trays containing 5cm of soil amended with 0.1g of biological control agent inoculum as used in the field trial experiment. The soil was from the strawberry field on Metcalf's farm and 50% of field moisture capacity. Each treatment was replicated 10 times (plus untreated control x 20 replicates) and the trays were sealed and incubated at 10°C. An additional two replicates of each treatment were performed for destructive assessment on a monthly basis, where a replicate was removed from the soil to determine whether the sclerotia had begun to decay, at which time it would be appropriate to destructively assess the entire experiment. The trays were sealed and incubated in a randomised complete block design. The experiment was established in the third week of April 2015.

In the third week of May 2015 a replicate of bags was recovered from the soil and assessed. The process of sclerotial decay had not progressed enough at that time for full assessment of the experiment, so the sclerotia continue to be incubated, the same continued through June 2015 but decays were noted in July 2015. Viable sclerotia were counted and the results are presented in Table 15. The results show that over 90% of *B. cinerea* sclerotia lost viability by the third month of storage in soil. There were no significant differences among treatments and where soil was inoculated with different biological control agents.

The sclerotia of *B. cinerea* are generally known to survive long enough to produce spores at flowering time in the season following their formation, so the sclerotia decayed faster than might have been expected. The soil used for the experiment was from a field which had been managed with soil health in mind, so it is possible that the rate of decay in this soil was greater than it might have been in a depleted soil.

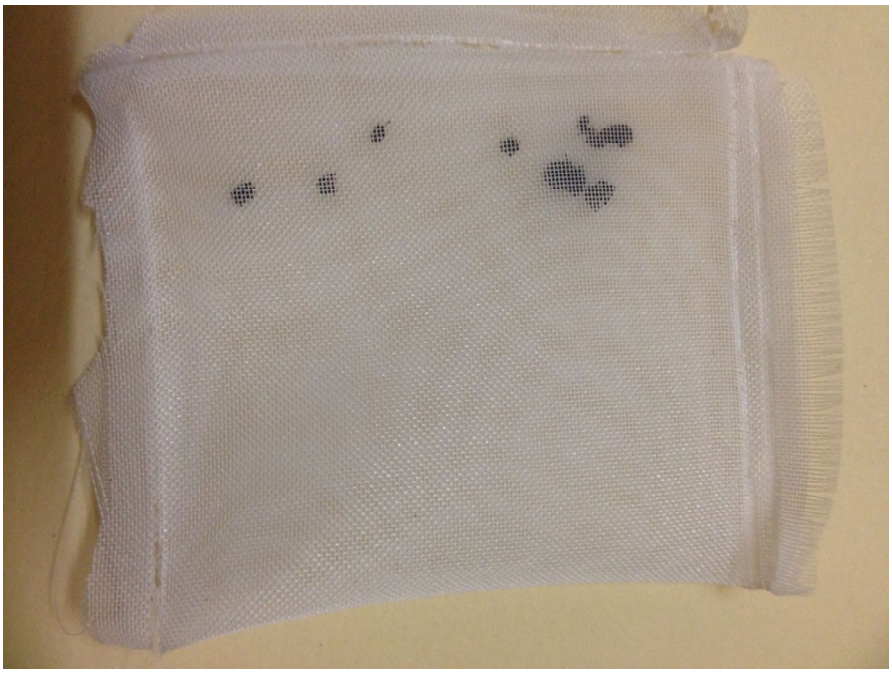

Figure 17: *Botrytis cinerea* sclerotia sealed in a nylon mesh bag for the sclerotial parasitism experiments.

Table 15: The number of *B. cinerea* sclerotia which were found to be viable following three months storage in soil at 10°C, with and without biological control inoculants.

Treatment	**Surviving sclerotia / 10**
Untreated 1	1.1
Untreated 2	1.1
Untreated 3	0.6
Average of untreated	**0.8**
TSQ2	1.1
TSQ6	0.4
TSQ7	0.4
TSQ8	0.6
TST6	1.2
TSV4	0.8
GST10	0.9
22239	1.1
Td81b	0.7
P Value	0.66

11.0. Rate of application

The rate of application experiment was conducted on Metcalf's farm at Molesworth in Tasmania using the variety Red Gauntlet The trial was sprayed with 100g/Ha of each biological control agent on a weekly basis beginning on 10/1/16 and on a weekly basis thereafter. There were ten replicates of each treatment which were sprayed by knapsack at 0, 100g. 200g or 500g of the Trichoderma blend spore powder containing TSQ6, TSQ7, TST6 and TST7.

Four weeks after the sprays began, samples were taken (50 per plot) to assess the colonisation by *Trichoderma* spp. and of *B. cinerea* in developing fruit. The samples were surface sterilised as in previous chapters and incubated on RASP medium (Appendix 1) for 7 days before assessment of colonies.

The results show that there was a low level of background *B. cinerea* inoculum relative to the trials in Qld and Victoria. There were 4.2/50 flowers with latent infection in the untreated control. The 100g rate of application reduced this by 80% and the 500g rate by 97.8% 8.4% of flowers in the untreated control were colonised by *Trichoderma,* which is likely a result of bee activity. There was a linear increase in colonisation of flowers, with the three rates of application. The 100g rate allowed Trichoderma spp. to colonise 45.6% of flowers which is lower than might be desired but should have an impact on a moderate disease problem. The 200g rate allowed 83% colonisation of flowers which is probably the maximum economically possible. The 500g rate allowed almost complete flower colonisation.

Table 16: The average number from 50 flower samples incubated on RASP medium which were either infected by *B. cinerea* or colonised by *Trichoderma* spp after being sprayed with 0, 100, 200 or 500g/Ha of the *Trichoderma* blend.

	***Botrytis* in fruit / 50**	***Trichoderma* in fruit / 50**
Untreated	4.20	4.20
100g/Ha	1.00	22.80
200g/Ha	0.45	41.50
500g/Ha	0.09	48.40
P Value	8.4×10^{15}	3.4×10^{29}
LSD0.05	0.68	5.76

12.0 Compatibility with chemical practice.

The objective of this experiment was to determine whether common chemical practices in strawberry production would inhibit *Trichoderma* spp. From a search of registered fungicides the main concerns would be chemicals used in powdery mildew control. These are often sprayed on a weekly basis. Bacterial rots are the next largest concern. The objective was not to consider every possible chemical option, but to determine what powdery mildew chemicals could be used at the same time as *Trichoderma* formulations with least injury to the establishment of the Trichoderma population. Experience in the grape industry has shown that chemicals can be rotated to use the non injurious chemicals at the time *Trichoderma* needs to grow, and then it is possible to return to the harsher chemicals. The chemical list inclu50ded sulphur, potassium bicarbonate, myclobutanil, and trifloxystrobin for powdery mildew and copper oxychloride and copper hydroxide for bacterial infections.

The experiment was conducted using the variety Red Gauntlet on Metcalf's farm at Molesworth in Tasmania. A spray of the TSQ6, TSQ7, TST6, TST7 blend was used in the experiment and was applied at 100g/Ha on 10/11/16. The selected chemicals were applied two days later in 500L of water / Ha. Each treatment was replicated ten times, a single replicate being 10m of bed. A further seven days after the chemical application, fifty samples were taken from each plot and transferred to the laboratory for testing. The samples were carefully selected to be one week from flowering, so that they would be flowers which were open at the time the *Trichoderma* blend was applied.

The samples were surface sterilised in 1% NaOCl for 3 minutes, washed in two changes of sterile distilled water for one minute each and using sterile technique transferred to RASP medium for incubation. After 10 days incubation colonies of *B. cinerea* or *Trichoderma* spp were identified.

The results (Table 17) show that 48.5% of flowers in the *Trichoderma* only treated controls were internally colonised by *Trichoderma* spp. Among the chemical treated flowers most colonisation percentages are slightly lower, but the only treatment with a statistically significant reduction in colonisation is the myclobutanil treatment which reduced colonisation by around 40%

There were no significant differences in the *B. cinerea* infection of flowers. This interaction was potentially complex because some of the chemicals might be inhibiting B. cinerea in addition to the protection provided by *Trichoderma.* Inhibition of B. cinerea by trifloxystrobin might have been anticipated.

Table 17: The colonisation of strawberry flowers by *Trichoderma* spp or infection by *B. cinerea* after the plants were treated with the *Trichoderma* blend and two days later with different listed chemicals.

	Composition	**Rate / 100L**	**% Colonised by *Trichoderma* spp**	**% Infected by *B. cinerea***
Untreated 1			50.40	2.6
Untreated 2			46.60	2.8
			48.50	**2.7**
Sulphur	800g/L	300g/100L	39.20	1.8
Copper hydroxide	500g/kg	105g/100L	44.00	2.6
Copper oxychloride	500g/kg	150g/100L	43.80	2.8
Myclobutanil	400g/kg	24g/100L	29.20 *	2.8
Potassium bicarbonate	400g/kg	400g/100L	38.40	2.0
Trifloxystrobin	25g/kg	25g/100L	32.60	2.6
P Value			9.5×10^9	0.78
LSD0.05			20.8	

13.0. General Discussion.

Experience in biological control has shown that the selection of a biological control agent requires a micro organism that it adapted to growth and survival on the host plant and in the climate where the crop is grown, and that microbe needs to be capable of inhibiting the growth of the pathogen. For this reason a variety of micro organisms were collected from different environments and naturally colonising strawberry leaves were tested for the ability to cause mycelial lysis in culture. Quite a large proportion of the flower samples collected contained latent *B. cinerea* inoculum, demonstrating the widespread latent nature of the disease. More than half of the collected isolates were able to cause lysis of *B. cinerea* mycelium in culture, which was promising, but also set a high standard for selecting only the best. *B. cinerea* is a captive target in these assays, and assays on plant tissue are a harder test.

An interesting array of mycelial interactions were seen. In some cases physical coiling was noted, in some cases distortion of growth was seen suggesting antibiotic production, and in some cases *B. cinerea* hyphae dissolved in the pathway of antagonist hyphae suggesting secretion of extracellular chitinases.

The microbes (which were mainly *Trichoderma* or *Gliocladium* spp) were tested for ability to restrict lesions on planta and inhibit sporulation. Using this method the microbes capable of invading *B. cinerea* lesions and attacking the disease were selected. Using the results of multiple leaf assays 34 candidates were reduced to nine best candidates for the first round of field trials (Table 5).

Before going in to field trials it was necessary to verify that each candidate could be mass cultivated. Prototype spore powders containing around 10^9 CFU per gram were prepared.

It is useful to know not only the effects of each biological control agent on disease incidence, but also to know how successful the biological control agent is in colonising the " infection court " (site of infection). A semi selective medium for *Trichoderma* spp. named RASP (Metcalf *et al.*, 2003) was used to monitor *Trichoderma* colonisation. By chance, *B. cinerea* is one of very few other fungi that can be detected on this medium. *B. cinerea* does not so much grow on the medium as sporulate in tissue segments placed in the medium. The ability to detect both *Trichoderma* spp. and *B. cinerea* on a single sample was a very valuable tool for monitoring population dynamics and was used throughout the series of field trials.

In the first field trial at Palm View Qld, nine isolates and *Trichoderma harzianum* Td81b were tested. It was necessary to monitor the progress of fruit development to determine the duration before the first treated fruit would ripen. It was found that the ripening duration was 4-5 weeks. This is important for both trial work and commercial use. It is not possible to judge the full effect of the biological control agent without applying for a month, so that the effect on latent infection within ripening fruit can be seen. The strawberry flower is infact a cluster of dozens of ovules (as marked by "achenes" or seeds on the ripe fruit). For a flower sample to show *B. cinerea* sporulation, only one of these would need to contain a latent infection. Similarly, if *Trichoderma* is found to be established within surface sterilised fruit, it need only have colonised a single ovary of the dozens present. However, the test does provide a generalistic indication of population dynamics

In the first Queensland experiment the effect on latent infection of applying biological control agents was pronounced as can be seen in Figure 12 and Table 7, where 97% of fruit contain latent infection in the untreated controls and this is reduced, often by more than half in treated fruit where *Trichoderma* is detected is as much a 100% of surface sterilised fruit. There were 15.32% infected fruit in untreated controls and four treatments had one third as many infected fruit (not statistically significant, but something to work with).

The first trial in Tasmania tested ten isolates in a similar field experiment. Highly significant reductions in latent infection were recorded. Though the rate of application was the same as the Queensland experiment the percentage of fruit in which *Trichoderma* colonised the ovary (as measured by incubation on RASP medium), was about half of the Qld experiment. The variety and climate are different. The level of infection in the untreated controls was 13.34% The lowest percentage of infection in the trial was TSQ6 at 2.77%

In the second Queensland experiment the treatment list was reduced to the best candidates. Based on the two previous field experiments and the screening assays the isolates selected were *Trichoderma* TSQ6, TSQ7, TST6 and TST7. The latent flower infection in untreated controls was 86.5% which provided a robust disease challenge, but Trichoderma colonisation significantly reduced this latent infection and all biological control agents provided statistically significant reductions in disease. Isolate TSQ6 provided the best reduction from 30.41% to 10.35%. The *Trichoderma* blend provided reduction to13.78%

The second field trial using the best candidates in Tasmania was inconclusive due to a hot and dry season and no infection, despite latent infections in 8.5% of fruit. Samples on RASP medium suggested that latent infection was reduced to 0 -2% by *Trichoderma* treatments.

The field trial at Coldstream in Victoria tested the best candidates. Flower colonisation by *Trichoderma* was lower among these treatments than in other experiments and no significant reductions in latent infection were demonstrated. Disease incidence was low at 2.36%, but there were statistically significant reductions in infection provided by TST7 (to 1.0%), TSQ7 (to 1.17%) and the *Trichoderma* blend (to 0.97%). *Trichoderma* spp. seemed to behave a little differently in colonisation in the Victorian experiment and it may raise the interaction of *Trichoderma* races with strawberry varieties.

Sclerotia are generally regarded as an important source of inoculum for *B. cinerea*, as they are known to over winter and produce conidia in spring. In our experiment in parasitism of sclerotia there was no difference in survival of sclerotia between untreated control and *Trichoderma* treated sclerotia. The soil used in the experiment seems to have contained the microbes necessary for sclerotial parasitism without amendment of additional biological control agents. This may be generally the pathway of decay for buried *B. cinerea* sclerotia, and it would be worthwhile to know whether the same result would be obtained if the sclerotia were incubated in the open air.

The rate of application experiment showed that the 100g rate of spore powder application was enough to reduce the level of latent infection in fruit by around 80% and 200g reduced this to 90%. Application of 500g reduced latent infection to around 2% The 100g rate seems to be the optimum from a cost:benefit perspective.

The studies of chemical compatibility showed that there was little if any inhibition of *Trichoderma* from being oversprayed with sulphur, copper, or potassium bicarbonate. One third inhibition from application of myclobutanil or trifloxystrobin. These sprays give the grower an option of protection from powdery mildew if needed during the time *Trichoderma* is growing in the flowers.

Overall, the study has successfully identified microbes that can be used to colonise strawberry flowers and inhibit *B. cinerea*. A prototype spore formulation has been prepared which is ready for commercial trials. Results suggest the formulation will reduce losses to *B. cinerea* by around 65% if used at 100g/Ha on a weekly basis with only compatible chemicals. Growers would need to trial the formulation for a full month to assess the effect on the disease.

13.0. Citation list.

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Appendix 1: Media

RASP Agar

Developed by Dean Metcalf for semi selective isolation of *Trichoderma* spp.

RASP selective agar (Metcalf *et al*., 2004) consisted of: 2.0 g $(NH_4)_2SO_4$; 4.0 g KH_2PO_4; 6.0 g Na_2HPO_4; 0.2 g $FeSO_4 \cdot 7H_2O$; 1 mg $CaCl_2$; 10 µg H_3BO_3; 10 µg $MnSO_4$; 70 µg $ZnSO_4$; distilled water 1 L; 20 g Agar; 5 g cellulose powder (Sigma Australia); adjusted to pH 4.0 before autoclaving. After the medium cooled to 70 °C, 10 ml of a sterile distilled water stock solution containing fungicides and antibiotics was added to provide the following concentrations per litre: 0.05 g streptomycin sulphate; 0.016 g rose bengal; 0.004 g Allisan (750 g/litre dichloran, Ag-Chem Pty Ltd, Australia); 3.2 ml Previcur (600 g/litre propamocarb, Schering Pty Ltd, Germany).

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Pectin Agar

Pectin Agar is a general agar developed by Dean Metcalf for use in plant disease diagnostics.

Pectin Agar is made from $NH_4H_2PO_4$;0.9g, $(NH_4)2HPO_4$; 2.0g , $MgSO47H_2O$; 0.1g, KCl ;0.5 g, Citrus pectin; 10g, Agar; 15g, per litre of distilled water, buffered to pH 4.0 using HCl.