Final Report

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Biological control of serrated tussock

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Abstract

Two major serrated tussock die back events have recently occurred in Australia during 2009 and now 2011. The pathogen, *Fusarium oxysporum* was identified infecting serrated tussock plants at both locations and was implicated in the die back. *Fusarium oxysporum* varieties can be quite pathogenic and have been used as classical biological control agents. This project has investigated this possibility. A detailed host specificity trial tested the impact of the *F. oxysporum* on 10 serrated tussock provenances across Australia and 5 agricultural and 5 indigenous grass species. The *F. oxysporum* did not have any impact on host plant survival suggesting that it may have been a saprophytic variety rather than pathogenic or that other serrated tussock grazers are required to enable entry of the pathogen. A follow up trial using tiller pieces from sick and dying serrated tussock from Bathurst Lake in NSW, did produce significant impacts on serrated tussock survival. The identified pathogens in these plants were *Fusarium oxysporum* and *Epicoccum* sp. While a later visit to the NSW affected property identified many serrated tussock plants showing white mycelium growth from their crowns and lower shoots produced by *Rhizoctonia* sp. and significant root damage by the nematodes *Paratrichodorus* sp. and *Rotylenchus* sp. This evidence suggests there could be a possible relationship between the nematode damage and soil pathogens infection requiring further investigation. Such a project could provide meat producers with a biological solution to managing this serious Weed of National Significance.

Executive Summary

A beef farmer in NSW and a sheep farmer in Tasmania have each experienced dieback of serrated tussock populations without herbicide application. A detailed host specificity trial using a pathogen identified from these infestations failed to have a significant impact on serrated tussock survival. A follow-up pot trial using leaf pieces from field collected infected plants did result in significant serrated tussock die-back. Identification of high populations of root feeding nematodes at the NSW site suggests a possible relationship between root damage and soil pathogen effects requiring further investigation as a potential biological control solution for serrated tussock.

Contents

Background

Serrated tussock (*Nassella trichotoma* Trin. & Rupr Barkworth) is a declared Weed of National Significance (Thorp and Lynch, 2000) that has been estimated to conservatively cost Victoria $5 million per year (Nicholson *et al.* 1997) and New South Wales $40.3 million per year (Jones and Vere 1998). It has been described as causing a greater reduction in pasture carrying capacity than any other weed in Australia with heavily infested paddocks in NSW carrying only 0.5 dry sheep equivalent (d.s.e.) per hectare compared to 7 to 15 d.s.e. on improved pasture without the weed (Parsons and Cuthbertson 1992). It is also a significant environmental weed, threatening endangered native grasslands (McLaren *et al.* 1998). Serrated tussock is one of the most serious weeds affecting the meat and livestock industry in Australia (McLaren *et al.* 2002). The potential distribution of serrated tussock in Australia has been estimated at 32 million ha with substantial areas of New South Wales (NSW), Victoria and Tasmania at risk of invasion (McLaren *et al.* 1998). The recent identification of herbicide resistance to the most popular herbicide (flupropanate) for serrated tussock control highlights the need to identify new control tools for the debilitating noxious weed (McLaren *et al.* 2010).

A project initiated in 1999 through a consortium of research funders including MLA, Shires and Cities (Victoria and NSW), State Departments (Victoria and NSW) and the Federal Government (DAFF) investigated classical biological control of serrated tussock from 1999-2005. Detailed surveys and research studies in Argentina (the country of origin of serrated tussock) identified three potential classical biological control pathogens for serrated tussock. Unfortunately, these biological control candidates were either not host specific (*Puccinia nassellae*), not sufficiently pathogenic to Australian accessions of the weed (*P. nassellae*, *Tranzscheliella* spp.) or their biology and life cycle could not be fully determined (*P. nassellae, Tranzscheliella* spp., *Corticiaceae* sp.), precluding further work (Anderson *et al*. 2006)

Figure 1. *Patches of serrated tussock dead for no apparent reason at Droughty point near Hobart in Tasmania (no herbicide had been applied).*

During August 2009, large patches of dense serrated tussock near Hobart in Tasmania were observed dying back for no apparent reason (Figure 1). No herbicides had been applied to the regions in question. DPI Frankston were alerted and after discussions with the Tasmanian Weeds Officer, samples of the affected serrated tussock were sent to DPI's pathologist, Dr James Cunnington, who identified *Fusarium oxysporum* and several other pathogens in the samples.

Initially *Fusarium oxysporum* appeared to be the most likely candidate causing the serrated tussock dieback.

Fusarium oxysporum is a ubiquitous soil pathogen found all around the world containing many non pathogenic strains (Nelson *et al.* 1983). *Fusarium oxysporum* causes fusarium wilt in wide range of plant species including carnations, lettuce, tomatoes, sweet potatoes, cotton, radish, wheat, celery, bananas, peas, flax, *Eucalyptus*, strawberries, carrots, beans, asparagus, chickpeas among others (Gordon and Martin 1997, Hubbard and Gerik 1993, Katan 1971, Rowe 1980). It can be an extremely damaging pathogen and there are lots of biocontrol projects to contain *Fusarium oxysporum* outbreaks on beneficial species (Fravel *et al.* 2003). However, some strains can be quite host specific and it has been previously used for biological control of numerous weed species including leafy spurge (*Euphorbia elula*) (Caesar *et al.* 1999), sicklepods (*Senna obtusifolia*) (Boyette *et al.* 1993), *Striga hermaonthica* (Ciotola *et al.* 1994), broomrape species (*Orobanchie* spp.) (Alla *et al.* 2008), parthenium ragweed (*Parthenium hyserophus*) (Pandey *et al.* 1992) and other species.

Figure 2. *Farmer Hans Kazmaeir inspects dead serrated tussock that was dying for no apparent reason. It had not been treated with herbicide on his property near Bathurst Lake in NSW.*

Previously *Fusarium oxysporum* has been extracted from serrated tussock buried seed banks as part of a PhD project investigating serrated tussock biotypes across Australia (Casonato 2003). Seona Casonato conducted preliminary virulence and host specificity trials with a strain of *Fusarium oxysporum*. Her results showed that this particular *F. oxysporum* strain infected serrated tussock and killed serrated tussock seedlings but was not particularly virulent or host specific on mature serrated tussock plants.

During February 2011, a second serrated tussock die-back event was brought to our attention occurring on a farmers property near Bathurst Lake in NSW. The beef Farmer, Hans Kazmaeir had areas of his property where no herbicide had been applied yet serrated tussock was selectively dying amongst other unaffected grasses (Figure 2). This site was subsequently visited and diseased serrated tussock samples sent to DPI Knoxfield for pathogen assessment. *Fusarium oxysporum* was again identified as a potential candidate for causing this damage.

This current project investigated the potential of the isolated *Fusarium oxysporum* pathogen from Tasmania and NSW affected serrated tussock as a potential biocontrol agent for serrated tussock.

Project objectives

By March 2012, determine the virulence and selectiveness of a pathogen, *Fusarium oxysporum* observed killing large areas of serrated tussock in Tasmania and NSW.

Success in achieving milestone

Milestone completed. The honours project trial did not indicate that *Fusarium oxysporum* was the definitive causal agent of the observed serrated tussock dieback but may have been a saprophytic pathogen present as a result of the causal agent which has yet to be determined. A follow-up trial was conducted to attempt to identify this pathogen or causal agent.

Methodology

An honours student, Richard Cowan was identified and appointed to undertake research for MLA on the "Biological Control of Serrated Tussock" project at RMIT University during March 2011. Richard was supervised by Professor Ann Lawrie and Dr David McLaren. Richard thesis reporting on site surveys, pathogen assessments, DNA analysis of samples and host specificity testing of Tasmanian and NSW pathogen samples is attached (pages 15-83).

The glasshouse host specificity experiments undertaken by Richard Cowan did not indicate any significant pathogenicity caused by *Fusarium oxysporum.* Ongoing discussions with the beef farmer, Hans Kazmaeir at Bathurst Lake suggested that the serrated tussock populations were continuing to be selectively impacted by an apparent pathogen. As a consequence repeat collection of affected plants was undertaken and the affected plants used to see whether the apparent diseased plants could affect untreated plants in a glasshouse experiment.

Serrated tussock plants from Bathurst Lake showing symptoms of pathogen attack were collected and dispatched to DPI Frankston during December 2011 (Figure 3). The collected plants still had soil attached to their roots. The plants were broken up into tillers, their leaves trimmed (to help survival of transplanted tillers) and were planted with their soil into pots containing mature previously untreated control serrated tussock plants from the 10 serrated tussock accessions used in glass house experiments 1 and 2 (control plants from Experiments 1 and 2) (Figure 4).

Figure 3. *Tillers of serrated tussock collected from Bathurst Lake showing white fungal damage being prepared for transplant into untreated control serrated tussock plants.*

Treated plants were compared to untreated control plants from the Rowsley valley and St Albans accessions kept under equivalent conditions in a separate glasshouse. Control plants also had serrated tussock tiller pieces planted from untreated serrated tussock plants. Serrated tussock plants were periodically assessed for damage using a 0-9 scale (0=healthy, 9=dead). The treatment protocol is shown in Table 1. A serrated tussock plant from each of the ten treated provenances was allocated to a bench and arranged randomly. Three benches provided the block replication.

Due to serrated tussock availability, untreated controls were restricted to the Rowsley valley and St. Albans provenances. Two plants of each of the two untreated control plants were allocated to bench and arranged randomly. Three benches provided the block replication. Plants within block were re-arranged at random each fortnight. Treated and control serrated tussock plants were maintained in separate glasshouses. Glasshouses were maintained at a constant 20-25^{0}C and 60-80% humidity.

Figure 4. *Previously healthy serrated tussock plants (controls from previous trials) treated with trimmed serrated tussock tiller pieces (foreground) from sick and dying serrated tussock plants collected from Bathurst Lake.*

Table 1 Glasshouse Protocol for Glasshouse Experiment 3

Serrated tussock Provenances	Replications	Treatments	Glasshouse
Bulburn Rd , Werribee	3	NSW Tiller transplant	2
Rocklands	3	NSW Tiller transplant	2
Bungleeltap Rd, Mt. Wallace	3	NSW Tiller transplant	2
Missen, Rowsley Valley (Flupropanate resistant)	3	NSW Tiller transplant	2
St Albans (Flupropanate susceptible)	3	NSW Tiller transplant	2
Rokeby	3	NSW Tiller transplant	2
Armidale	3	NSW Tiller transplant	2
Goulburn	3	NSW Tiller transplant	2
Abercombie	3	NSW Tiller transplant	2
Bathurst Lake	3	NSW Tiller transplant	2
Rowsley Valley (Flupropanate resistant)	6	Untreated control	3
St Albans (Flupropanate susceptible)	6	Untreated control	3

Figure 5 . Treated serrated tussock plants began showing symptoms of tillers turning bright red before senescing.

Results

A two way analysis of variance assessed the affect of the NSW tiller treatment additions versus the untreated control plants (Table 2). This showed significant impacts on treated compared to untreated plants and also significant differences within serrated tussock provenances in the treated plants. Treated plants began showing symptoms such as bright reddening of tillers (Figure 5) followed by black spotting within the tiller. Control plants remained green.

Table 2. Two way analysis of variance – Serrated tussock Treatments vs Provenances

Source of Variation	d.f.,	s.s.,	m.s.,	v.r.,	F pr.
Treatments	1	57.235	57.235	30.77	<.001
Provenances	11	90.633	8.239	4.43	<.001
Residual	35	65.111	1.86		
Total	47	212.979			

Results showing the average damage score and standard errors for the different serrated tussock provenances treated are shown in Figure 6. The Victorian serrated tussock from the provenance of Little River had the greatest damage score while the NSW provenance from Abercombie, Armidale and Bathurst Lake were the least damaged compared to the untreated controls. A plant showing disease symptoms was sent to DPI Knoxfield for pathogen assessment by the Plant Health Services Section. The roots and crown samples were positive to *Fusarium oxysporum* while the shoots were attacked by an *Epicoccum* sp.

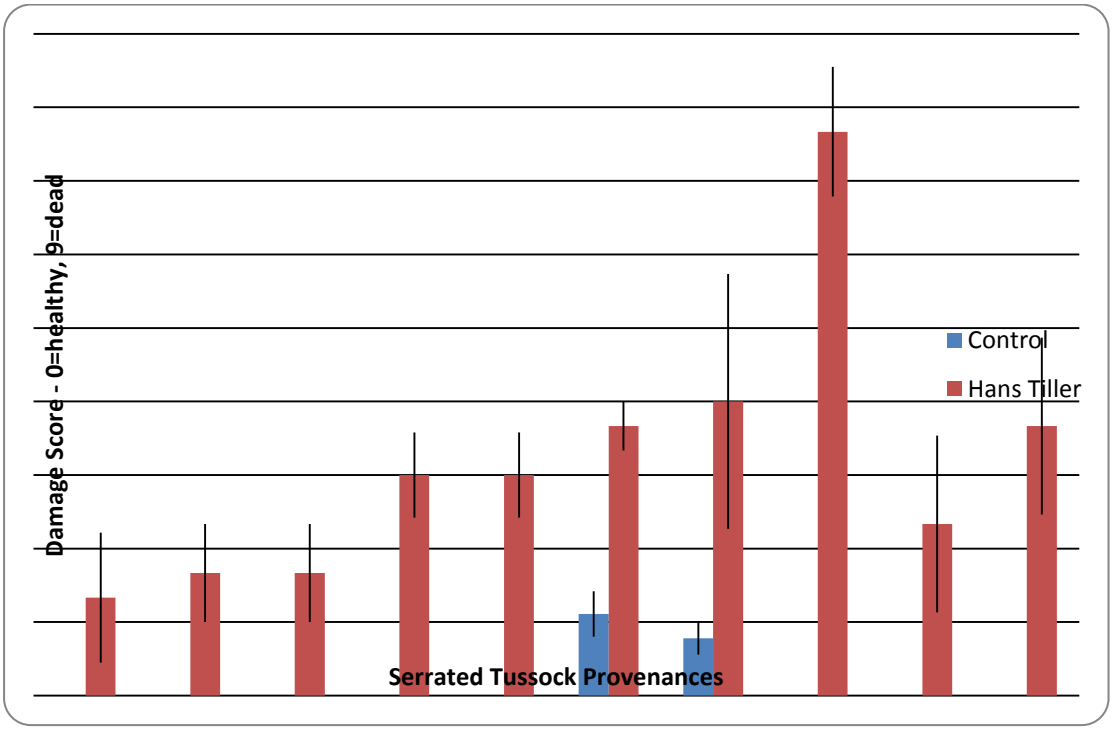

Figure 6. Effect of pathogen tiller transplants on serrated tussock damage scores 51 days after tiller transplants.

This result prompted a follow-up visit to the NSW property with Dr Jacky Edwards who is the Science Leader of the Victorian Department of Primary Industries Plant Pathology team on March 14th 2012. White fungal masses seen observed commonly on serrated tussock crown bases (Figure 7.) that were confirmed as *Rhizoctonia* sp. that are known pathogens of crops such as wheat, tomatoes and cucumbers among many more. It has also been reported that pigweed (*Amaranthus palmeri*) is susceptible to *Rhizoctonia solani* (Boosalis and Scharen 1960).

Plant and root samples were again assessed by the Plant Crop Health Diagnostics section at the Victorian Department of Primary Industries for pathogen and nematode attack. The roots and crowns were again infected by *Fusarium oxysporm* while a nematode assessment identified 1,613 stubby-root nematode (*Paratrichodorus* sp.) and 93 spiral (*Rotylenchus* sp.) in 200 ml of soil surrounding the serrated tussock roots. Stubby-root nematode feed externally on plant roots and a generalist feeders attacking a range of grass species. No nematodes were identified inside the roots or shoots of the serrated tussock plants sampled. The density of stubby and spiral nematodes were considered very high and likely to be responsible and/or be contributing to the observed serrated tussock symptoms at the Bathurst Lake site.

Discussion

Serrated tussock plants dying for no apparent reason have been collected from Droughty Point in Tasmania and Lake Bathurst in NSW. A range of possible pathogens including *Fusarium oxysporum, Alternaria* sp., *Rhizoctonia* sp., *Mucor* sp., *Phytophthora cryptogea* and the nematodes, *Paratrichodorus* sp. and *Rotylenchus* sp. were identified as possible causal agents. *Fusarium oxysporum* and *Phytophthora cryptogea* were positively identified using PCR techniques. *Fusarium oxysporum* appeared the most likely candidate for causing selective die-back of

serrated tussock populations and was targeted for virulence and host specificity testing.

Figure 7. White fungal mycelium of *Rhizoctonia* sp. on the crown and tiller bases of serrated tussock at Lake Bathurst, NSW.

The laboratory host specificity testing of *F. oxysporum* as a potential biological control agent was conducted by Richard Cowan from RMIT University under the supervision of David McLaren (DPI) and Professor Ann Lawrie (RMIT University) as an honours project. The *Fusarium oxysporum* collected from Tasmania and NSW were tested in separate host specificity trials but did not result in any significant die-back of serrated tussock accessions or of native and agricultural grass species tested during the 66 days of this trial (see Richard's Honours thesis below).

Unintended introductions of pathogens with significant weed biological control impacts are not uncommon in Australia. Some examples include:

- Tutsan rust (*Melampsora hypericorum*) that has successfully controlled tutsan, *Hypericum androsaemum* in the Otways and Gippsland in Victoria (McLaren *et al.* 1998)
- Blackberry rust (*Phragmidium violaceum*) that was found affecting blackberry, *Rubus fruiticosis* in Gippsland before deliberate releases in the classical biological control program could be made (Bruzzese and Field 1984)
- Weedy *Sporobolus* fungi (*Nigrospora oryzae*), has been identified affecting widespread infestations of giant Parramatta grass, *Sporobolus fertilis* near Grafton in NSW (Ramasamy *et al.* 2007).

Ongoing contact with the property owner at Bathurst Lake near Tarago in NSW suggested that a natural (not herbicide) die-back of serrated tussock populations was still taking place implying that a natural biological control (pathogen, nematode or insects) agent was still affecting its survival. A follow-up experiment investigating whether infected Bathurst Lake NSW serrated tussock tillers planted in with untreated serrated tussock plants could transfer the "disease" showed significant impacts on serrated tussock damage scores compared to the untreated controls (Figure 5). A diagnostic pathogen assessment of one of the infected serrated

tussock plants from this experiment revealed significant *Fusarium oxysporum* from the roots and crown. Black pathogen lesions were also identified on the leaves caused by *Epicoccum* sp. *Epicoccum* sp. are mostly recorded as saprophytes but are also known to be parasitic on apple and millet (Kortekamp 1997).

This experiment also showed considerable variation in damage scores between serrated tussock provenances suggesting that there may be some genetic variation between serrated tussock populations in Australia. Seona Casonato assessed the taxonomy and variation of 36 serrated tussock provenances of serrated tussock across Australia (Casonato 2003). She determined that taxonomically and molecularly they were all serrated tussock but populations behaved differently in relation to seed germination, growth and flowering. This trial also indicates some variation in serrated tussock provenance susceptibility to possible pathogen/nematode attack.

After several site visits to the Bathurst Lake site in NSW and collecting serrated tussock plants showing die-back symptoms we now know there is a guild of intertrophic organisms associated with these plants. We have a combination of several pathogenic fungi (*Fusarium oxysporum, Rhizoctonia* sp., *Epicoccum* sp) plus two root damaging nematodes attacking the serrated tussock plants. The actions of a grazer and a pathogen have often been linked to spectacularly successful biological control programs. The success of the Prickly pear cactus (*Opuntia stricta)* biological control program in Australia was due largely to the combined actions of cactus cladode eating *Cactobalstis cactorum* larvae and an assortment of fungi including *Fusarium* spp. (Dodd 1940). Caesar (2011) describes the observations of Dodd to suggest that the prickly pear cactus was weakened by the attack of many *C. cactorum* larvae that also provided entry points and opportunities for pathogens and bacteria to complete the work of rotting the plant to its eradication. Another good example of this is in biological control of leafy spurge, *Euphorbia esulalvirgata,* using the flea beetles, *Aphthona* spp. in the USA (Caesar 2003). It was found that soil pathogens such as *Fusarium oxysporum, Rhizoctonia* sp. and *Pythium* spp. are associated with the flea beetle damage to leafy spurge and laboratory experiments showed that plant mortality was linked to a combined attack compared to either the flea beetle or the pathogens by themselves.

The recent discovery of nematode damage to the serrated tussock roots may provide an important clue to the observed serrated tussock mortality happening at the Bathurst Lake property in NSW. We hypothesise that the damage caused by the nematodes may be enabling soil borne pathogens such as *Fusarium oxysporum* and *Rhizoctonia* sp. to gain access to serrated tussock roots causing the observed die-back. The land owners observations were that the die-back was also affecting a species of native grass (*Austrostipa scabra* we think) while other native grasses were unaffected (Figure 8.). The nematodes, *Paratrichodorus* sp. and *Rotylenchus* are reportedly generalists but other exotic beneficial grasses on the property did not appear to be affected. The lack of response in the glasshouse trials undertaken by Richard Cowan using *Fusarium oxysporum* as a sole biological control agent may have been because the serrated tussock plants did not have the nematode creating access points for entry of the pathogen. In this last trial, we applied serrated tussock tillers with their soil attached (presumably containing nematodes) into healthy serrated tussock plants.

Additional investigation into the relationships of serrated tussock to the nematodes - *Paratrichodorus* sp. and *Rotylenchus* sp. and the pathogens *Fusarium oxysporum* and *Rhizoctonia* sp. could potentially provide some important information that could

help land managers selectively control this debilitating weed. We would recommend conducting similar studies to Caesar (2003) who investigated the relationships of the flea beetle *Aphthona* spp and the soil pathogens *Fusarium oxysporum* and *Rhizoctonia* sp in the successful biological control of leafy spurge in North America.

Figure 8. An affected serrated tussock plant (left) next to an unaffected native grass species. Note other grasses in foreground are also unaffected.

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BIOTECHNOLOGY & ENVIRONMENTAL BIOLOGY
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An Investigation Into a Potential Biological Control of Serrated Tussock

Honours Thesis

Richard Cowan

October-2011

Two major serrated tussock die back events occurred in Australia during 2009 and now 2011. The pathogen, *Fusarium oxysporum* has been identified infecting serrated tussock plants at both locations and is implicated in the die back. *Fusarium oxysporum* varieties can be quite pathogenic and have been used as classical biological control agents in the past. This project investigates the strain isolated from these locations for their virulence against serrated tussock and host specificity to important agricultural and environmental species.

I declare this thesis is my own work and has not been reproduced from any previously submitted materials for any other Diploma, Degree or any other award and that all references used have been fully acknowledged in this thesis.

Richard Leigh Cowan
31-October-2011

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Chapter 1: Background

1.1. Introduction

Weeds cause significant problems in agriculture throughout the world, reducing yield and quality of crops by competing for the water, nutrients, and sunlight essential for crop growth. However, despite massive efforts, weeds continue to cause significant losses. These losses result from the selection and emergence of species that are not controlled by currently available herbicides, the inability of a herbicide to selectively control certain weedy species without injury to crop species, and hence the development of herbicide resistant strains. Plant pathogens have been suggested as one of several possible means of controlling the weeds that remain problematic in otherwise successful weed control programs in intensive agriculture or even as an alternative to chemical herbicides (Te Beest, et al., 1992). Serrated tussock is one of Australia's worst weeds due to its invasiveness, potential for spread and its social, economic and environmental impacts (Thorp and Lynch, 2000). It probably accounts for a greater reduction in pasture carrying capacity than any other weed in Australia (Parsons and Cuthbertson, 1992). For example, pastures that normally carry 7–15 dry sheep equivalent (DSE) per hectare can be reduced to a carrying capacity of 0.5 DSE per hectare under heavy infestation (Campbell and Vere, 1995). A survey undertaken in 2006 confirmed the massive impacts this weed is having on Australian agriculture with average annual management costs ranging from $15,000 to $20,000 per year per respondent (McLaren, et al., 2006). With serrated tussock already occupying over 870,000 ha in NSW alone, with the potential to spread to 32 million ha, Australia-wide (McLaren, et al., 2004), it is understandable that serrated tussock has been designated a Weed of National Significance (Thorp and Lynch, 2000).

Unfortunately, options for controlling serrated tussock are very limited. Current best practice as recommended by (Osmond, et al., 2008) involves a strategy known as Integrated Weed Management, which combines several techniques including physical, chemical, biological and social control practices. These techniques tend to be costly, complex, and not fully understood by landowners. At present, flupropanate is the most effective and widely used herbicide to combat Serrated Tussock (Campbell and Vere, 1995). However, the weed has recently been showing an alarming trend of developing a resistance to this herbicide. (McLaren, et al., 2010).

Detailed regional surveys to assess the extent of serrated tussock resistance to flupropanate have been conducted in Armidale in NSW, Diggers Rest and the Rowsley Valley, both in Victoria (McLaren, et al., 2010). The data show that the spread of flupropanate resistant serrated tussock is spreading and this has far reaching implications including expounding the already extensive herbicide usage, and the other costs associated with weed management for farmers. Consequently, identifying new methods for controlling this noxious weed is of paramount importance.

Fungal plant pathogens are now being used to control weeds throughout the world by the introduction of specific isolates of exotic pathogens or by the commercialisation of endemic pathogens. The evidence is clear: fungal plant pathogens can effectively control weeds (Te Beest, et al., 1992). During a PhD in 2003, a fungus called *Fusarium oxysporum* was identified as having potentially pathogenic effects on serrated tussock (Casonato, 2003). The pathogen was tested but unfortunately, that variant of *F. oxysporum* was not host specific, causing more damage to beneficial species than to serrated tussock. However, in 2009 large patches of Serrated Tussock in Tasmania were observed to be dying back for no apparent reason. After an investigation by the local Weeds Officer, samples of the affected weed were sent to the Victorian Department of Primary Industries (DPI) where (amongst other pathogens) *F. oxysporum* was isolated. At this stage a new variant of *F. oxysporum* was possibly the pathogen causing the serrated tussock dieback.

This project was an investigation into the observed naturally occurring serrated tussock dieback phenomenon, apparently associated with this strain of *F. oxysporum a*nd the possibility that this, or other, pathogen(s) could be used to control serrated tussock infestations in other parts of Australia. Developing a classical biological control method is a potential long-term, low cost solution that could significantly reduce the impacts of this weed.

Figure 1 Indigestible serrated tussock invading grazing land, reducing the Carrying capacity. (Photo: Ryan Melville)

1.2. Literature review

1.2.1. Serrated tussock

Serrated tussock is a perennial tussock forming grass that is one of the worst weeds that has ever entered Australia. It is classified as both an environmental weed and a pasture weed, and probably causes a greater reduction in stock carrying capacity than any other weed in Australia (Parsons and Cuthbertson, 1992). Infestations mostly occur on pastureland, where it causes significant loses in productivity but they're also increasingly prevalent in natural environments such as National Parks and water catchment areas, where the noxious weed is threatening native grassland and adversely affecting native ecology and biodiversity (Casonato, 2003).

1.2.1.1. Preferred habitat

Serrated tussock is a highly resilient weed that infests a wide range of habitats. It quickly invades bare ground and disturbed areas such as roadsides or overgrazed pastures (Osmond, et al., 2008). In its native country of Argentina serrated tussock is a minor weed invading cleared woodland, ploughed fields and neglected areas. (Parsons and Cuthbertson, 1992). Unfortunately in Australia, serrated tussock is far more prolific, taking to dry coastal vegetation, grassland, grassy woodlands, dry sclerophyll forests and rock outcrop vegetation (Carr, et al., 1992). Serrated tussock is not limited by soil type or fertility and is often found growing in soil where there is little nutrition and low water availability. It grows on soils derived from slate, shale,

limestone, ironstone, granite, basalt, sandstone, and mudstone (Campbell and Vere, 1995). Serrated tussock grows in acidic soils but less so in soils which are affected by salinity or in damp or swampy ground, preferring well drained areas. It also does not grow in shaded areas, such as under a dense canopy of trees (Osmond, et al., 2008).

1.2.1.2. Agricultural Impacts

Serrated tussock and other exotic stipoid grasses are one of the most significant threats facing grazing industries and indigenous grasslands in Australia (McLaren, et al., 1998). In 2010 a survey of 5000 land managers across Australia with land infested with serrated tussock showed that the weed is costing each property between $15,000 and $20,000 annually in control and lost production costs (McLaren, et al., 2010). The lost productivity is a result of the fact that, unlike similar grasses, serrated tussock is virtually indigestible, and livestock will only attempt to eat it as a last resort. If forced to graze it, stock lose weight and may die (Campbell and Vere, 1995). The low palatability of the grass is primarily due to the high sclerenchyma content in each leaf and dead leaves persist on the tussocks (Campbell and Vere, 1995), and so a pasture infested with serrated tussock has a significant drop in its livestock carrying capacity. This reduction is best illustrated using the dry sheep equivalent (DSE), which is a standard unit that can be used to assess the carrying capacity and potential productivity of an area of grazing land. One unit of DSE is the amount of feed required by a two year old, 45 kg Merino sheep to maintain its weight (Osmond, et al., 2008). Heavily infested paddocks in NSW carry only 0.5 DSE per hectare compared to 7 to 15 DSE on pasture without the weed (Parsons and Cuthbertson, 1992). The palatability can be increased with the addition of supplements but the nutritional value of the feed is still poor and it cannot be grazed economically (Campbell and Vere, 1995).

1.2.1.3. Environmental Impacts

Native grasslands are one of Australia's most threatened ecosystems Less than one per cent of their original extent remains (Ross, 1999) and these are in various stages of degradation throughout south-east Australia. In Victoria, serrated tussock is either actively invading or has the potential to invade some of the most endangered native grassland remnants (Carr, et al., 1992) and its presence is a serious threat to the native flora and fauna. Being very similar in appearance to many native grasses, it is

able to go unnoticed in grasslands for many years, and by the time it is recognised, the native grasses have been replaced by significant infestations of serrated tussock, reducing the biodiversity of the area. Serrated tussock also increases the fire risk in peri urban areas due to large build-ups of very dry combustible biomass, once the seed heads ignite they can then detach and travel long distances, spreading fires across large areas. The presence of vast infestations of serrated tussock has reportedly extended the fire season by 2 months in the Geelong region of Victoria (McLaren, et al., 2004).

1.2.1.4. History & Distribution

Serrated tussock is native to South America, and is spread over large areas of the Argentinian pampas grasslands which is likely to be the plant's origin (Taylor, 1987). It has since been introduced to several other countries, with small infestations occurring in England, France, Italy, Scotland and the United States (Campbell, 1982). However, in Australia, New Zealand and South Africa, Serrated tussock has invaded to such an extent that it has become naturalised (Wells, 1978). In Australia, the main areas of infestation lie in latitudes 33 - 38°S, and it currently occupies about 1,000,000 ha in the southern and central tablelands of New South Wales and central southern Victoria (McLaren, et al., 1998). It was probably first introduced into Australia in the early 1900s but was not officially recorded until 1935 after it was discovered at Yass (located 55 km from Canberra) (Campbell and Vere, 1995). It is believed to have originated from seed contained within fodder imported during a drought (Campbell and Vere, 1995, McLaren, et al., 1998). In Victoria, serrated tussock was first recorded in 1954 at Broadmeadows and spread rapidly so that by 1995 it was occupying over 130,000 ha of land (McLaren, et al., 1998). Due to continued hard work by landowners, the estimated coverage has been reduced to around 82,000 hectares (Seager, et al., 2011).

Control measures have been costly and time-consuming, involving strict quarantine on the movement of vehicles, fodder and stock (Seager, et al., 2011). Serrated tussock can also found in Tasmania, with about 1500 hectares being infested in 1998. Fortunately for Tasmania, it has been possible to control the spread of serrated tussock and there is the possibility of eradication (Casonato, 2003), but on mainland Australia the spread of serrated tussock is too great and there is no possibility of total eradication (McLaren, et al., 2004). For all of Australia, the potential distribution of errated tussock has been estimated at 32 million hectares

(McLaren, et al., 1998). Serrated tussock is currently proclaimed a noxious weed in the Australian Capital Territory, New South Wales, Victoria, South Australia and Tasmania, which means that landholders have a legal responsibility to prevent the plant's growth and spread from their property and a lack of compliance can result in prosecution (Miller and Wilsher, 1999).

1.2.1.5. Identification

Serrated tussock is the somewhat misleading name for *Nassella trichotoma*. It is misleading because many other native tussock grasses exhibit similar serrations on their leaves (Campbell, 1982) and this can lead to confusion in identification. It can be difficult for those unfamiliar with serrated tussock to identify it and it is often misidentification that has enabled it to spread so greatly. Indeed, identification of all grasses can be difficult, especially without a seed head present. Serrated tussock forms a very dense tussock, up to 50 cm high and 60 cm across, composed of numerous fine leaves. Flowering stems emerge in spring and grow to a length of 95 cm, roughly twice as long as the leaves. They are initially erect but then droop at maturity. Serrated tussock leaves have small spines or serrations that can be felt when drawn between the fingers from the tip to the base (Campbell, 1977). Some other tussocks have similar serrations and the degree of serration depends often on the environmental conditions. It is only with 8-10 x magnification that the differences in serration between different grasses can be accurately determined (Campbell, 1977). However, when rolled between the index finger and thumb, the leaves are smooth like a needle. This distinguishes them from some similar native grass species, which will feel like they have flat edges (Faithfull, et al., 2004). The best identification feature is the white, hairless ligule (Campbell, 1977). The ligule is found at the junction of the leaf blade and leaf sheath, and is about 1 mm long, rounded, membranous and glabrous. Other tussocks either have a hairy ligule or no ligule at all (Campbell and Vere, 1995). Although this is a relatively easy method for identification, many people find the easiest way to identify serrated tussock is when the plants flower as it possesses distinctive purple glumes (Campbell, 1977).

1.2.2. Control Methods

Unfortunately, options for controlling serrated tussock are very limited. Current best practice as recommended by (Osmond, et al., 2008) involves a mixed strategy known as Integrated Weed Management, which combines several techniques

including physical/mechanical, chemical, biological and cultural or social control practices. These techniques are more often than not costly, complex, and not fully understood by landowners. Presently, flupropanate is the most effective and widely used herbicide to combat serrated tussock (Campbell and Vere, 1995), however the weed has recently been showing an alarming trend of developing resistance to flupropanate (McLaren, et al., 2010). This growing resistance spells the need to identify new tools for controlling this noxious weed.

1.2.2.1. Non-chemical control

The simplest method for weed control, chipping, also referred to as hoeing, involves the physical removal of the entire serrated tussock plant from the ground using a hoe or mattock. The soil is then removed from the roots and the plant is left exposed to dry out, so as to prevent regrowth. If chipped whilst in flower, the plant must be destroyed by fire in order to prevent the spread of seed (Osmond, et al., 2008). Treating infestations with fire does not kill all adult plants, though it does remove some biomass and destroys about 25% of the seed bank, as deeply buried seed will remain unaffected by fire (Osmond, et al., 2008). Burning also stimulates the mass germination of serrated tussock seeds (Osmond, et al., 2008), and so its best used only in combination with other control methods, such as using an appropriate herbicide or sowing desirable background pasture species. As explained in section 1.2.1.2 livestock will avoid eating mature plants but young seedlings can be somewhat be controlled in this manner, as they are slightly more palatable (Campbell and Vere, 1995).

1.2.2.2. Chemical control

The use of herbicides requires good knowledge of correct timing and application rates in order to be effective, and varies depending on the situation and density of serrated tussock. The main problem in using herbicide treatments is reinfestation of serrated tussock, from the seed in the soil, which is highly probable if the herbicide is used as the sole control method (Campbell, et al., 1998). The use of herbicides is associated with a number of problems, like possible side effects to the user and to desirable vegetation, and the high costs of purchasing the products. There is also a growing community awareness of the detrimental effects to the environment of the continual use of herbicides (Casonato, 2003). However the effectiveness of herbicides when controlling serrated tussock cannot be denied and their use has

dramatically increased over the last 30 years, with glyphosate and flupropanate being the two most commonly used (Campbell, et al., 1998).

1.2.2.3. Glyphosate

Glyphosate is a highly effective herbicide (Osmond, et al., 2008); it is fast acting and kills treated plants within 1 - 4 weeks after application. It is absorbed through the leaves and green stems without having any residual action and as such, ceases to work once it contacts the soil (Osmond, et al., 2008). One of its major drawbacks is that it is non-selective and kills off all plant species, leading to reduced ground cover (Campbell, et al., 1998). A reduced ground cover increases erosion and is an ideal environment for the germination of new serrated tussock plants (Miller, 1994). Therefore care is always needed when applying glyphosate on serrated tussock, but is often the best option if the intention is to sow a new pasture or crop (Osmond, et al., 2008). The effectiveness of glyphosate is influenced by environmental factors such as soil fertility, rainfall, and shadiness. Prior land use, such as grazing or burning and localised variants (accessions) of serrated tussock may also result in differences to glyphosate susceptibility (Campbell, et al., 1998, Campbell and Nicol, 2001)

1.2.2.4. Flupropanate

Flupropanate is another highly effective herbicide, but unlike glyphosate, flupropanate selectively kills serrated tussock (Campbell, et al., 1998). It is however, relatively slow acting, taking 3-12 months to kill mature plants, depending on weather conditions (Campbell, et al., 1998). Flupropanate is predominantly absorbed through the plant's roots and has a residual soil activity that can prevent serrated tussock from regrowing (Campbell and Vere, 1995). This residual lifetime varies depending on soil type and rainfall, but generally lasts 2 – 3 years, which allows desirable pasture species to increase in density, outcompeting and eventually ousting serrated tussock once the residual effect has gone (Viljoen, 1987). Presently flupropanate is the most effective and widely used herbicide for combating serrated tussock (Campbell and Vere, 1995)

1.2.3. Herbicide Resistance

Herbicide resistance is the ability of a plant to survive and reproduce following exposure to a dose of herbicide that would normally be lethal. Whilst herbicides are extremely effective in controlling serrated tussock, the excess or inappropriate use of herbicides can increase the chances of resistance developing. Herbicide resistance occurs as a result of random and infrequent mutations driven through selection; susceptible plants are killed while herbicide resistant plants survive and go on to reproduce without the competition from susceptible plants. If the herbicide treatment is repeated, resistant plants successfully reproduce and become dominant in the population (Stankiewicz, et al., 2001). In a potentially genetically diverse species, chemical selection pressures on some populations could have dire consequences. Herbicide-resistant weeds express the genetic variation required to evolve and escape chemical control (Stankiewicz, et al., 2001).

1.2.3.1. Serrated tussock resistance to flupropanate

Herbicides constitute the basis of the major control mechanisms used for serrated tussock, and in Australia, flupropanate has been used extensively and almost exclusively since the mid-1970s (Campbell and Nicol, 2001). Flupropanate resistance has been identified in a population of serrated tussock in Victoria (Noble, 2002). Serrated tussock plants suspected of being resistant to flupropanate were grown in a pot trial and treated with a range of flupropanate rates. The resistant serrated tussock survived application rates as high as 8 L/ha, which is four times the recommended rate used for controlling this species (Noble, 2002) There are now three separate confirmed instances of serrated tussock populations resistant to flupropanate in NSW and Victoria (McLaren, et al., 2008). The continued use of flupropanate over a 15–20 year period is the most likely cause of these resistant strains developing (McLaren, et al., 2008). In the long term, serrated tussock herbicide resistance will lead to its increased dominance as a weed and hence increased costs for land managers in the form of increased herbicide dosages and higher environmental pollution as a consequence.

1.2.4. Biological control

Research conducted over the past two decades led to the development of several strategies that utilise plant pathogens as weed control measures. Some of

Australia's most successful biological control projects have been against weeds originating from South America, including several species of cactus and tropical or subtropical aquatic plants, such as salvinia, water hyacinth, water lettuce and alligator weed (Briese, et al., 2000). It is a regulatory requirement that potential biocontrol agents are host-specific and pose a limited risk to non-target species (Morin, et al., 2006). If host specificity can be demonstrated under optimal conditions for disease development in the laboratory, then that strongly indicates that non-target species will not be affected in the wild (Berner and Bruckart, 2005). Fungal pathogens are increasing in their use as biological control agents (Te Beest, 1996). Using fungal pathogens in a biological programme would be an alternative to current control methods, which would be used as part of an integrated weed management scheme (Osmond, et al., 2008). In Australia, there have been some highly effective biological agents used for weed control, one example is the use of the a fungus, *Puccinia chondrillina* in controlling skeleton weed *(Burdon, et al., 1981)*. Previous attempts to control the weed by chemical and agronomic methods had proven unsuccessful (Burdon, et al., 1981). The use of *P. chondrillina* in this programme is an example of a successful *classical* biological control which stimulated interest in the use of fungi in an *inundative* programme, where the weed is controlled by an inoculation of host-specific indigenous fungal pathogens. Both methods require high specificity to the target weed and high effectiveness, with all biological control agents rigorously tested before any release into the environment (Shepherd, 1993).

1.2.4.1. Classical biological control

The classical approach in biological control is when an exotic organism, generally obtained from the weed's natural environment, is released into the target environment and allowed to self-perpetuate (Shepherd, 1993). Fungal pathogens used in successful biological control programmes are generally extremely effective due to their ability to reproduce and disperse amongst the target weed. The weed is thus continually controlled, but the introduction of the agent is generally irreversible due to the agent being almost impossible to eradicate (Harris, 1988)

1.2.4.2. Inundative biological control

In an inundative biological control programme, an aqueous solution made from the pathogen (also known as a mycoherbicide), is applied to the weed at specific locations and is not expected to provide control beyond the growing season or area

in which it is applied (Te Beest 1996). The application of the mycoherbicide generally occurs at the weed's early growth stages, with disease symptoms appearing after an appropriate inoculation period, and lesion development causing the plant to die without an epidemic occurring (Te Beest, 1996).

1.2.4.3. Biological control of serrated tussock

It is difficult to get approval for biological control agents for grasses, since the Poaceae family includes many agriculturally important cereal and pasture species (Briese and Evans, 1998).and therefore the relationship between serrated tussock and native tussock species has always been considered too close for there to be an exotic biocontrol agent with sufficient host specificity (Briese, et al., 2000). However, two events have now made the biological control of these grasses a more promising option. Firstly, taxonomic work has indicated a stronger separation between native Australian and South American species than previously thought (Briese and Evans, 1998) and secondly, surveys in Argentina have shown that there is a rich fungal flora associated with serrated tussock (Briese, et al., 2000). As a consequence, detailed surveys and research studies in Argentina (the country of origin of serrated tussock) identified three potential classical biological control pathogens for serrated tussock. Unfortunately, these biological control candidates were either not host specific, not sufficiently pathogenic to Australian accessions of the weed or their biology and life cycle could not be fully determined, precluding further work (Anderson, et al., 2006).
An inundative approach would therefore be the best, and potentially the only method used for developing a biological control for serrated tussock in Australia. As this approach uses indigenous fungus there is generally less risk than when using exotic organisms associated with the classical approach (Harris, 1988). The cost associated with this kind of approach is also significantly lower, due to a smaller list of things to test since the agent is already endemic. However, one disadvantage is the high costs associated with the formulation, application and constant upkeep of the mycoherbicide. Indeed, there have already been some fungi identified in Australia which cause disease in serrated tussock foliage and roots, namely *Zinzipegasa argentinensis*, *Dinemasporium sp*. and a *Fusarium sp*. (Hussaini, et al., 2000). But only very limited testing has been undertaken on these which did not include virulence testing on more mature serrated tussock plants or for host-specificity (Hussaini, et al., 2000). In 2003, *F. oxysporum* was again identified to be causing some dieback of small populations of serrated tussock in Victoria (Casonato,

2003). Plants inoculated with *F. oxysporum* were observed to have a visible white fungal infection and were rotting at the base. Many of the leaves of these plants were chlorotic (Casonato, 2003), but *F. oxysporum* was not found to be host specific and contributed to the death of numerous non-target grass species (Casonato, 2003).

1.2.5. Fusarium oxysporum

F. oxysporum is a common soil pathogen found globally, it can be extremely damaging and causes Fusarium wilt in carnations, tomatoes, sweet potatoes, cotton, radish, wheat, celery, bananas, peas, flax, eucalypts, strawberries, carrots, beans, asparagus, chickpeas and others (Leslie and Summerell, 2006). Indeed, there are many projects with the aim of containing detrimental *F. oxysporum* outbreaks. However, certain strains are very host-specific and have been used as biological controls for numerous weed species including leafy spurge, when a formulated strain of *F. oxysporum* reduced stand density of leafy spurge by 30% over a single season (Caesar, et al., 1999). *F. oxysporum* is the most widespread of the *fusarium* species and is observed in most soils around the world, be they arctic, tropical or desert, cultivated or not (Leslie and Summerell, 2006). *F. oxysporum* is also the most economically important species in the *Fusarium* genus given its numerous hosts and the level of loss that can result when it infects a plant (Leslie and Summerell, 2006). *F. oxysporums ability* to grow saprophytically in soil is one of the main components of its competitiveness (Alabouvette, et al., 2001). It is of great importance that work should be undertaken on *F. oxysporum* in order to determine if a certain strain exists for serrated tussock since this fungus can have strains that exhibit pronounced host specificity (Caesar, et al., 1999). The various strains of *F. oxysporum* are pathogenic to different plant species; there also exists non-pathogenic strains (Alabouvette, et al., 2001). Furthermore, this fungus may be more virulent in combination with other fungi, as pathogen mixture has previously been used successfully to control some weedy grass species (Chandramohan and Charudattan, 2001). There are also differences in accessions of serrated tussock within Australia (Casonato, 2003) and some may be more susceptible to infection than others.

1.3. Aims

1. To identify the pathogen causing dieback in serrated tussock populations.
2. To test the identified pathogen as potential bioherbicide for serrated tussock.

Chapter 2: Initial observations

2.1. Introduction

In 2009 large patches of Serrated Tussock in Tasmania were observed to be dying back for no apparent reason. After an investigation by the local Weeds Officer, samples of the affected weed were sent to the Victorian Department of Primary Industries where (amongst other pathogens) *F. oxysporum* was found to be present. It is the aim of this chapter to extend upon this investigation by performing a more detailed site survey of not only this area, but also a location in New South Wales near the Bathurst Lake region where a similar dieback event is occurring. During the investigation, several transects will be performed in order to make a quantitative assessment of both the health and extent of serrated tussock infestation in the regions. Furthermore, samples of plant material will be taken to ascertain the presence of any pathogens which may be causing the phenomenon.

2.2. Materials and methods

2.2.1. Site Surveys

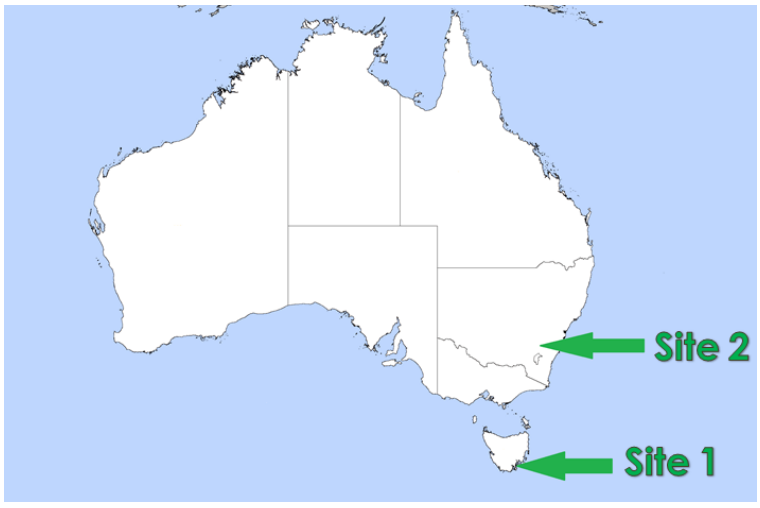

Figure 2 Locations of the reported locations of serrated tussock dieback

2.2.1.1. Site 1: Droughty Point Rd (TAS)

The initial visit to this site was undertaken by Jarrah Vercoe (Tasmanian Weeds Officer) who first identified dead and dying serrated tussock in August 2009. After consultation with the land owners it was determined that no control action had been undertaken in this area. During September 2010 before the inception of the project, the site was revisited by Karen Stewart and Bronwen Wicks (NSW Dept of Primary

Industries), during this visit, areas where pathogen was originally identified are still visible, but are now being re-infested by young serrated tussock. However, some isolated areas of plants were still affected and samples of these were sent to DPI Knoxfield and RMIT for identification and culturing during March 2011.

Figure 3 Photo taken near droughty point, TAS August 2009 showing a clear demarckation between the infected and healthy Serrated Tussock.

A detailed trip was planned for October to once again revisit the Tasmanian and perform detailed transects and get a better assessment of the area but due to unforeseen circumstances, this trip was cancelled.

Figure 4 Drought Point in September 2010, showing younger serrated tussock plants coming through after many of the older plants have died

Figure 5 Area of Serrated Tussock dieback near Lake Bathurst NSW

2.2.1.2. Site 2: Bathurst Lake (NSW)

In February 2011, just before the commencement the project, a second serrated tussock dieback event was observed, this time on a cattle farm near Bathurst Lake in remote NSW. The farmer, Hans Kazmaeir had areas of his property where no herbicide had been applied but serrated tussock was selectively and inexplicably dying amongst other grasses. Serrated tussock health and density was recorded at the site using four fixed 25 metre transects using a quarter metre quadrant. Measurements included serrated tussock density (%cover), serrated tussock health (0=healthy 9=dead), other grass density, broad leaf density, trash/bare ground density. GPS readings of affected areas were recorded. A second field trip to this property was undertaken on the 6th of April 2011.

2.2.1.3. Plant health Analysis

A scale of 0 to 9 was used to judge the health of the plants in this project, 0 being green and healthy and 9 being dead. Despite being a rather crude and qualitative scoring mechanism, the scores were repeatable and consistent across a species. It gets trickier to compare health between different species however.

2.2.2. Collection and Processing of Samples

Plant samples were received via express post from Karen Stewart from Tasmania on the 10th of March 2011 and immediately processed for fungal culturing. The samples from NSW were collected during a field trip in April 2011 During the site visit to NSW, several whole plant samples from the various transects were bagged and tagged and transported back to the RMIT laboratory for further processing and experimentation. At RMIT, the plant material was kept frozen, and processed rapidly as to minimise any contamination or degradation.

2.3. Results

2.3.1. NSW Site Survey

Table 1 Summary of transect data from Bathurst Lake NSW

Transect	Average % cover of				Average Serrated tussock health rating (lower is healthier)
	Broadleaf	Native Grass	Bare Ground	Serrated Tussock	
1	38.2%	59.6%	0.2%	2.0%	7.8 ±1.1
2	19.4%	13.5%	0.2%	66.9%	8.1 ±0.9
3	2.3%	11.3%	17.9%	68.5%	4.4 ±0.9
4	7.4%	58.0%	4.8%	29.8%	4.1 ±1.2

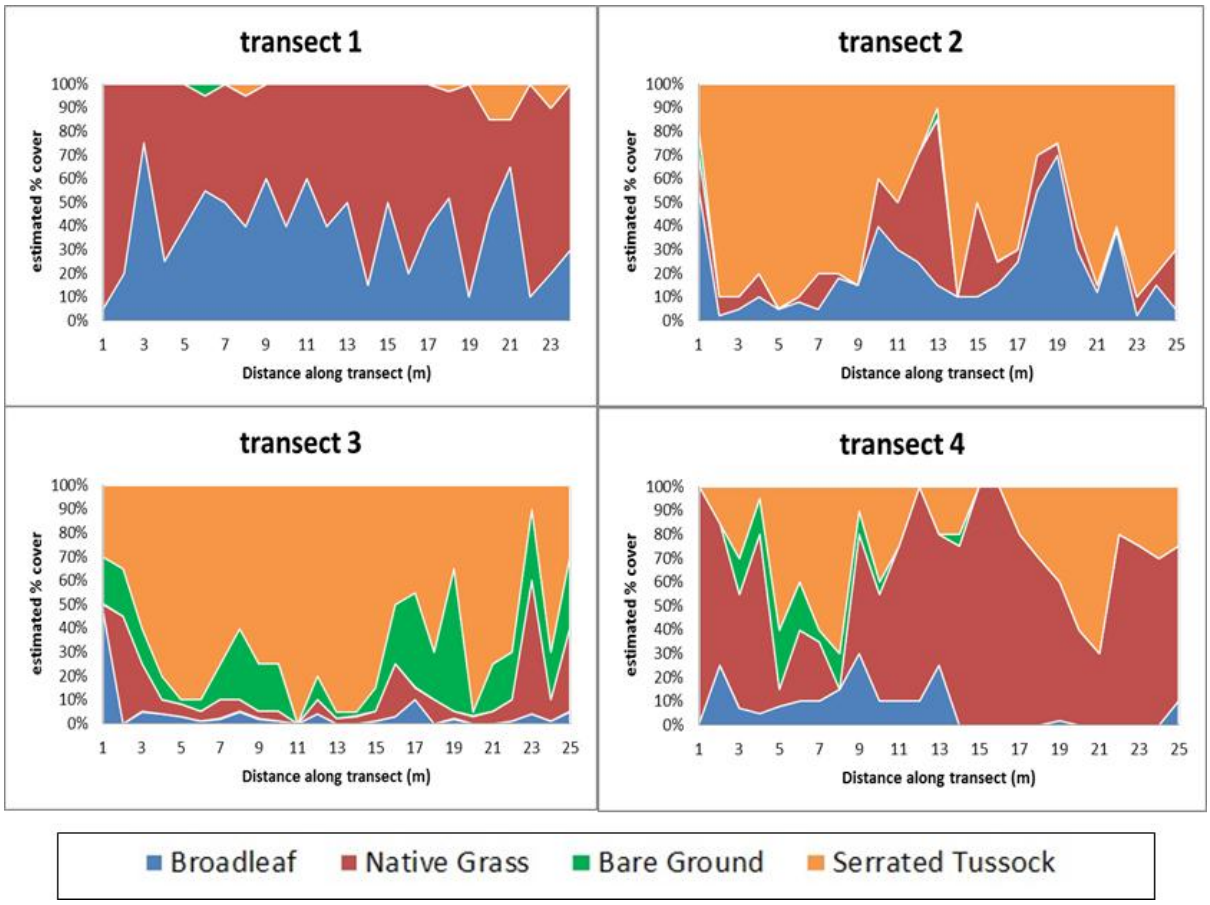

Figure 6 showing graphical representations of the transect data from NSW, the data shows the proportion of cover taken by each group in quadrant at that particular distance along the transect. We can see that the predominant category is inconsistent across the transects.

Transect 1: Details

GPS coordinates: 35.04551°S 149.4144°E
Serrated tussock here are sick and dying with a large proportion within category 7+, but there is also evidence of new of healthy shoots coming through. Using a hand lens, white fungal material can be seen at the roots of dead plants dead roots surrounding some healthy shoots. Dead seed head indicates plants died back during seeding(spring).

Transect 2

GPS coordinates: 35.04349°S 149.41401°E
Majority of dead serrated tussock and thistle, observations under field lens reveals serrated tussock has no white fungus in this area

Figure 7 A crown of serrated tussock found dead and rotten found amongst transect 2

Transect 3

GPS coordinates: 35.04312S 149.41339E
An area with much healthier looking serrated tussock. Many plants have dead centres and healthy surroundings.

Transect 4

GPS coordinates: 35.03669S 149.41360E

Transect 4 is located on a hilltop in the middle of a dried up lakebed. Serrated tussock in this area exhibits the white fungus, but a lot of the plants are very healthy. This area is very much isolated form the others.

Figure 8 despite the presence of this white fungal material at the base of many serrated tussock plants in Transect 4, the average health of serrated tussock plants were found to be higher than any other transect (see Table 1 Summary of transect data from Bathurst Lake NSW

2.3.2. Collection and Processing of Disease Samples

2.3.2.1. Tasmanian samples

The Tasmanian samples arrived via post on 10-03-2011. It was unclear if the sample was from one or two separate plants, so two sample ID's were created.

Table 2 Information of received samples

Sample ID	Location	Transect	Date	Observations
TAS1A	TAS	Droughty Point Road	060311	-
TAS2A	TAS	Droughty Point Road	060311	-

After the initial categorisation and surface sterilisations, the samples were then placed onto petri dishes containing V8+ agar. With the following plate replications: 1A,1B,1C,1D,1E,1F,1G,1H,1I,1J,1K,1L,1M,1N,1O,1P,1Q,2A,2B,2D,2E,2F,2G,2H,2I, 2J,2K,2L,2M,2N,2O,2P,2Q,2R.

2.3.2.2. NSW samples

Table 3 Information on collected samples

Sample ID	Location	Transect	Time	Date	Observations
T1	NSW	T1 (1)	0904	060411	
T2	NSW	T2	1100	060411	Rotten
T3	NSW	T3	1140	060411	
H1	NSW	T4 (1)	1215	060411	White fungi
H2	NSW	T4 (2)	1220	060411	
H3	NSW	T4 (3)	1255	060411	White fungi
T1(2)	NSW	T1 (2)	1448	060411	White fungi

2.4. Discussion

In this chapter a selection of sick and healthy plants were gathered from two different locations and prepared for analysis in the lab. It has been established that the dieback events are very clear (at least in NSW) and that something is causing this to occur two separate populations at the same time. Whether or not there is a pathogen involved at work is still questionable. After conversations with local farmers, the possibility that the recent wet weather may have influenced the dieback events by rotting the plants in waterlogged soil. This phenomenon is known as wet feet, and is known to kill some plants. But being wet doesn't always cause rotting, so a further possibility is that the pathogen is triggered by the environmental conditions caused by the recent moisture. It is also a possibility that the samples gathered merely contain ubiquitous soil born species from the area, and it would have be beneficial to

gather unrelated, healthy flora from the area in an attempt to document and eliminate the unimportant fungi.

In Table 1 we see that there are three very distinct areas from which samples were gathered. Any fungus cultured from sites with a large proportion of dead plants is likely to be entophytic. The extent of infestation at the NSW site is quite shocking, with vast swathes of land dominated with essentially a monoculture of the weed. A second trip to the site would be highly advantageous to gauge the change in this population over time, if indeed there is a pathogen at work, then it would be expected to be in decline.

Chapter 3: Pathogen isolation

3.1. Introduction

This Chapter aims to Isolate and identify the cause of the serrated tussock dieback as documented in Chapter 2. As Pathogenic fungi have been found attacking *N. trichotoma* in the plant's native habitat of Argentina and previous studies in Australia have identified fungi as potential biological control agents (Hussaini, et al., 1998), samples of the infected plant material will be plated out onto agar in order to isolate and identity any potentially pathogenic fungus. After this stage, a DNA analysis of the fungi grown in cultures was undertaken by Crop Health Services as commissioned by Dr. David McLaren. This will conclusively identify the fungal species. A further test was also to be undertaken, an attempt to observe the internal appearance of the collected plant material via sectioning. This would show whether or not the fungal hyphae are actually growing within the cell walls and damaging the plant.

3.2. Materials and methods

3.2.1. Fungal Culturing

The plant samples collected from NSW and those received from TAS underwent an initial preparation which involved removal of soil and other debris, the samples were then underwent surface sterilisation. This was performed in a laminar flow cabinet by submerging samples in a 2.5% sodium hypochlorite (NaOCl) solution for 5 min followed by two rinses of sterile distilled water for 2 min each. The purpose of surface sterilisation is to remove the inconsequential contaminants from the surface of the

samples, whilst keeping the internal fungal environment intact. It is a matter of trial and error about just how long to leave the sample in the sodium hypochlorite as too long can sterilise the entire sample. The bottom 1 cm of the stem were kept, and the rest discarded. These were then divided into three roughly 3 mm sections and tri-inoculated onto v8+ agar petri dishes and finally sealed with Parafilm. These were then placed in a growth room at 25c with a 12 hour photoperiod provided by Fluorescent tubes at 27 µmoles $m^{-2}s^{-2}$. After 2 weeks, 0.25cm^2 sections were cut out and placed upside down into freshly prepared v8+ agar plates in order achieve pure colonies consisting of a single species. A varying series of environmental conditions were employed to promote sporulation, such as altering, darkness, constant light, and inverting the plates.

Figure 9 Samples undergoing preparation for culturing

3.2.2. Microscopic Identification

Once the fungal cultures were sufficiently established, the dishes were opened and sticky tape was used to gather a sample of the colonies. The tape was then placed onto a glass slide stained with Bromothymol blue, then fixed with a slide cover. Petri dishes were then resealed with parafilm and placed back in the growth room. In an attempt at identification, the slides were examined for sporulation and categorisation to the closest genera was attempted using standard texts (Barnett and Hunter, 1972). Further references (web based) were used once the initial identification was made.

3.2.3. DNA Analysis

To extend upon the microscopic identification, some NSW plant samples were sent to Crop Health Services, where the samples underwent commercial culturing and

DNA analysis. This allowed a definitive determination of the fungal species present in the samples.

3.2.4. Internal Appearance

Cutting extremely thin sections of the plant samples and mounting them on slides will give an insight into the internal structure of the hyphae and how it interacts with the plant cells. Prior to cutting, the samples are embedded in a rigid fixative. This is achieved by immersing the sample in molten wax (paraffin), and then placed in a mold and allowed to harden which produces the "block". The block is then cut using a microtome into extremely thin slices of material, known as sections. The wax sections are then mounted on glass slides and stained and finally, examined under a microscope. Wax blocks were created from the selection of samples collected from TAS and NSW. Samples picked were H2, TAS1D, T12, T1, TAS2D, T2 T3, and H3 (See section 2.3.2).

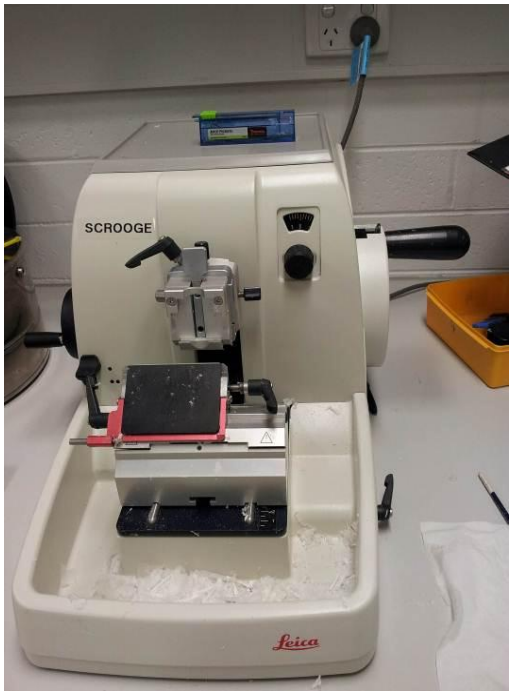

Figure 10 microtome used to cut sections

3.3. Results

3.3.1. Fungal Culturing

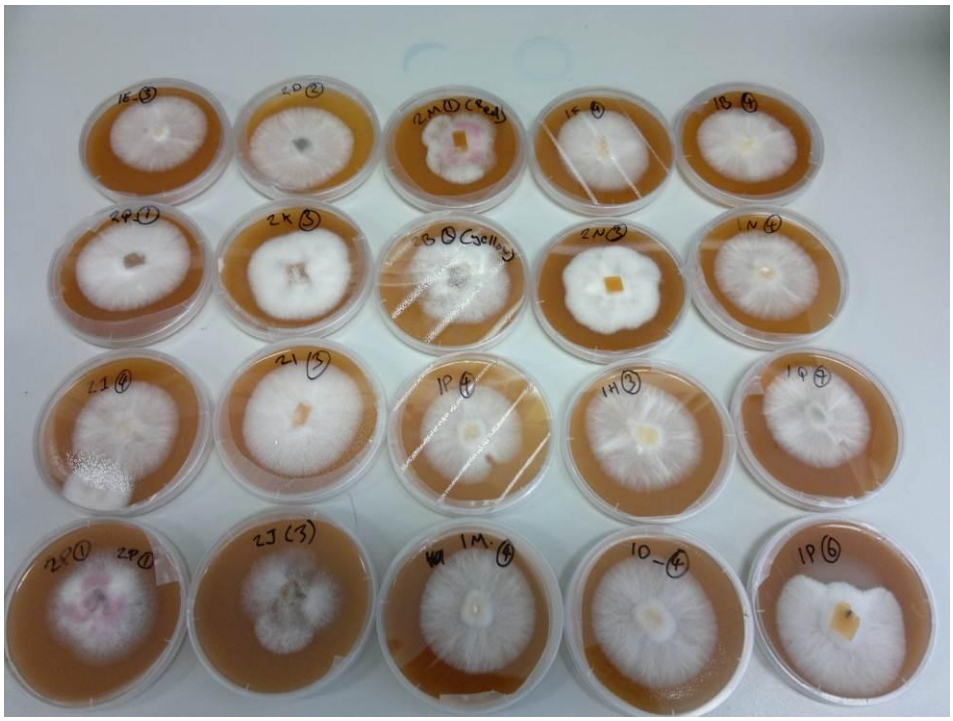

Figure 11 shows the uniformity of fungal isolates from the Tasmanian sample

After extensive examination of slides made from the fungal cultures, the following observations were made. In general, the cultures from Tasmania were very clean and had little diversity, in fact there were only two species observed: *Marasmius rotula*, and *Fusarium* sp. *Marasmius rotula* is from the Marasmiaceae family of mushrooms. It is commonly known variously as the pinwheel mushroom, it grows on decaying wood and leaves and obtains nutrients by decomposing dead organic matter (Kuo, 2004). Therefore it is hardly a likely candidate as a pathogen. The *Fusarium* sp, on the other hand is much more interesting as it does indeed have potential to be pathogenic, and has been used as a biological control in the past (see section 1.2.5). Unfortunately, the samples collected from NSW were far less straight forward, having many more species, and problems with mite and bacterial contamination. The increased complexity can be explained by the diversity of plant samples collected, ranging from healthy to dead and in the locations from which they were collected, over 4 transects, compared to the Tasmanian samples, which consisted of a single plant divided into multiple samples. Nevertheless, the following species were identified: Transect 1 contained a very low infestation of serrated tussock which was also very sick with a presence of a white fungal-like material visible at the bases of some of the plants. Fungi isolated from transect 1 were

Fusarium sp; *sordaria* sp, which are commonly found in the feces of herbivores (Kirk, et al., 2008); *trichoderma* sp which are present in all soils, and are the most prevalent culturable fungi and are opportunistic plant symbionts (Harman, et al., 2004). The only successful identification from transect 2 was a common environmental contaminant, *Apophysomyces* sp which is widely distributed in soil and decaying vegetation (Collier, et al., 1998). This is not surprising due to the large population of dead and rotting plants in this transect, it is also likely that any pathogen which may have killed the serrated tussock has moved on and been replaced with *Apophysomyces* sp and other saprophytic species. With its big population of mid-range healthiness serrated tussock, transect 3 appears to be the ideal place to isolate the pathogen in action, from this transect, *Fusarium* sp and *Tricoderma* sp were identified. The white fungal infected as observed in transect 1 was also seen in transect 4, but in this case no *Fusarium* sp was isolated, only the saprophytes *Cladosporium* sp, *Apophysomyces* sp, and *Trichoderma* sp. The tables containing identification data are located in section 7.2 and 7.3.

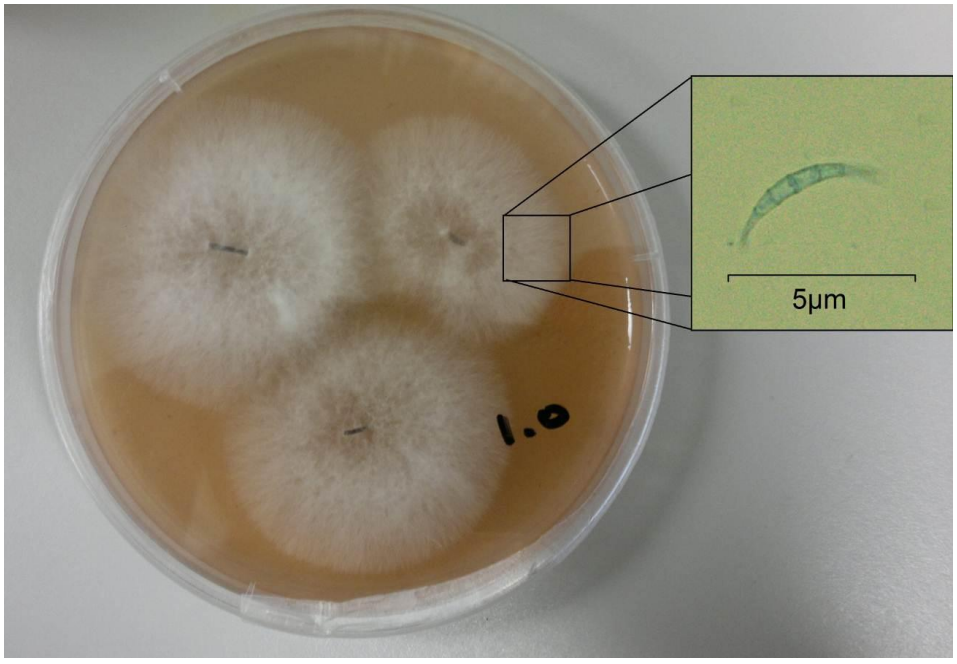

Figure 12 *Fusarium sp* spore observed from isolated fungi

3.3.2. DNA Analysis

After culturing the samples collected from NSW, the report from Crop Health Services stated that the *Fusarium* sp was identified as *Fusarium oxysporum*. This is collaborating evidence for the microscopic observations carried out in the previous section. It must be stated that the confirmed presence of F*. oxysporum* doesn't prove

that it is pathogenic, in this case. An interesting note is that *Phytophthora* was also isolated and identified as *Phytophthora cryptogea* which is another plant pathogen, but this wasn't found on any of the samples tested within section 3.3.1. The full report is attached in the appendix section 7.4

3.3.3. Internal Appearance

Unfortunately the plant material proved to be too fibrous to be able to section successfully, and the resulting sections were torn and shredded. This could have been due to the thick cellulose walls of the grass cells preventing the penetration of paraffin into the tissue. The blade was also damaged with each pass, hampering the ability to create an even ribbon. Possible solutions could be the use of a different block preparation method or pre-treating to soften the cells.

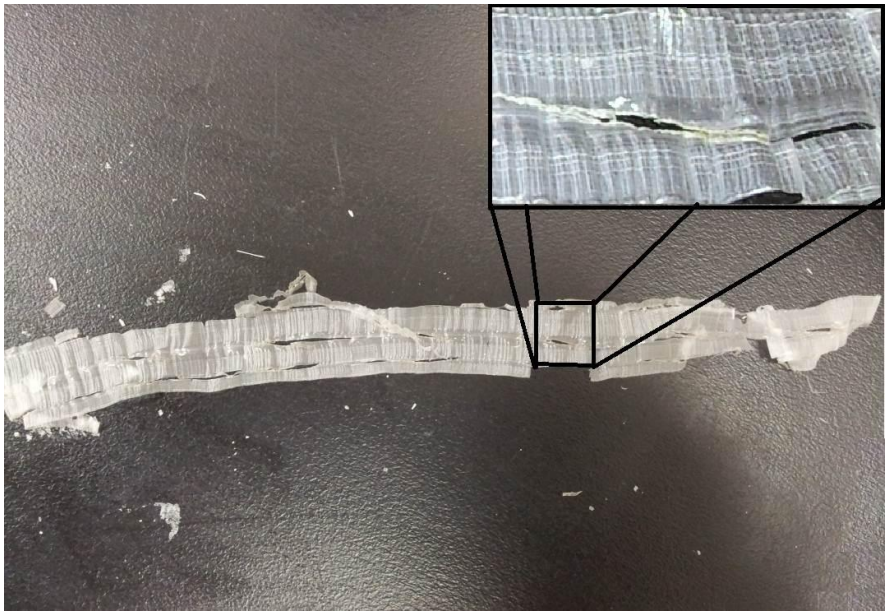

Figure 13 Shredded sections of plant material

3.4. Discussion

In this chapter, one of the most commonly isolated fungi species was *Fusarium* sp, and this was identified as *F. oxysporum* by DNA testing performed by Crop Health Services. This is an encouraging result as F*. oxysporum* has had a pathogenic history and some strains have been known to be host specific and used in biological control programs in the past (section 1.2.5). The fact that it was isolated amongst the healthier population of serrated tussock from NSW lends evidence to the fact that, in this case, F*. oxysporum* is not acting as a saprophyte. To have results from the

sectioning experiment would lend evidence to support this, but unfortunately this was not successful. There is also the clear the lack of any other potential pathogen isolated, so it appears that the next stage will be to test the pathogenicity of the isolated strain of F*. oxysporum* in controlled glasshouse trials. The fact that F*. oxysporum* was not isolated from samples collected from the second transect in NSW (lots of serrated tussock) indicates that strain of F*. oxysporum* is not acting as a saprophyte.

Chapter 4: Pathogenicity and host specificity

4.1. Introduction

In this chapter the both the virulence and host specificity of the *F. oxysporum* strain isolated in Chapter 3 will be assessed. A comparison of delivery methods will also be undertaken in order to determine the most effective way of infecting the plants. To that end, serrated tussock plants will be inoculated with a solution made from *F. oxysporum* spores in a controlled glasshouse environment and visual plant health will be recorded over time, and compared to untreated control plants. In addition to serrated tussock, 10 commercial and environmentally important species will also be inoculated to determine the host specificity the *F. oxysporum* strain, a very important factor when developing a biological control. A list of these species can be found in Table 6. Separate inoculums will be prepared for the *F. oxysporum* isolated from NSW and Tasmania, and the actual application of the inocula with occur in one of two ways, by spray or injection.

4.2. Materials and methods

4.2.1. Inoculum Preparation

Initially, two inoculums were to be made, one for each strain of *F. oxysprum* (TAS and NSW), however it was necessary to make up two solutions of the Tasmanian inoculum due to a miscalculation in the volume required. So, there were three different solutions, and less than ideally, each solution had a different concentration of spores. The specifics of these inoculums can be found in Table 4 Summary of inoculums. The inocula were produced by scraping the surface of the fungal culture with a spatula moistened in sterile 0.1% Tween 20. Scraped colonies were dissolved in distilled water for 30 minutes with a magnetic stirrer and the resulting solution was

then filtered through a coarse sieve in order to remove clumps of hyphae and other excess material. After determining the concentration of the spores in solution, 1 mL of Tween was added in order to help homogenisation and aid in the adhesion of spores onto plant surfaces. The spray inoculation was performed initially, with the intention that the same inoculation solution would be used for the injection process but there was insufficient inoculum remaining so a new batch had to be made up. Unfortunately this second solution had a lower concentration than the original one. The concentration of the inoculum was determined by calculating the volume of solution was visible within a known area under the microscope, then counting visible spores. This was repeated and the results were averaged. The tables and calculations are presented in section 7.5

Table 4 Summary of inoculums

Inoculum	Fungal origin	Application method	Glasshouse number	Concentration (spores/mL)
1	TAS	Spray	1	2.9E+05
2	TAS	injection	1	1.4E+05
3	NSW	injection	3	8.3E+05
control	-	-	2	-

4.2.2. Application Methods

The plants were infected in one of two ways, by spray or injection. This was done to gain insight into the optimal delivery method of the pathogen. For the spray injections a typical water mister was used. Before using it, it was calibrated to find out how much volume was delivered. It was found that 3 sprays from approximately 10 cm delivered 3.5 ml. Spraying was performed at a distance of 10 cm directly towards the base of the plant inside a 1 m^3 Perspex cage. The cage minimised spray lose due to wind, and also helped prevent contaminating other plants.

An equal volume of inoculum was used during the injection method, in which an auto pipette was used to inject 3.5 mL of inoculum directly onto the base of the plant. After undergoing inoculation, the plants were completely covered with plastic bags, thus they were maintained in approximately 100% relative humidity for 24 hours at which time the bags were removed.

Figure 14 Dr David McLaren with the freshly inoculated plants. The plastic bags keep the humidity optimal during the first 24 hours, after which they are removed

4.2.3. Test Species

It has been shown that serrated tussock varies quite significantly according to region (Casonato, 2003), so it is to this end that 10 different accessions (samples collected from different locations) of serrated tussock will be tested. The different accession and their origins are listed in Table 5. Unfortunately, one of the accessions 'Rokeby' is crossed out because there were insufficient plants to test, so 9 accessions were included in the experiment, not 10. The 10 important agricultural and environmental species that are to be used in the host specificity testing are shown in Table 6. Each species and each serrated tussock accession will have five replicate plants treated and assessed for F*. oxysporum* impacts. The initial germination and subsequent care of the plants was undertaken by Julio Bonilla (DPI). At the time of testing, any dead leaves were removed and the plants were approximately 6 months old.

Table 5 Serrated tussock provenances

State	Provenance	ID
Victoria	Bulburn Rd , Werribee	V2
Victoria	Rocklands	V1G
Victoria	Bungleeltap Rd, Mt. Wallace	V4
Victoria	Missen, Rowsley Valley (Flupropanate resistant)	Missen
Victoria	Birdsnest, St Albans (Flupropanate susceptible)	Birdsnest
~~Tasmania~~	~~Rokeby~~	~~n\a~~

NSW	Armidale	Armidale
NSW	Goulburn	Goulburn
NSW	Abercrombie	Abercrombie
NSW	Bathurst Lake	Bathurst Lake

Table 6 Important agricultural and environmental species.

Common name	**Botanical name**
Kangaroo grass	*Themeda triandra*
Oats	*Avena sativa*
Perennial Ryegrass	*Lolium perenne*
Wheat	*Triticum aestivum*
Spear grass	*Austrostipa scabra*
Double jointed spear grass	*Austrostipa bigeniculata*
Lobed needle grass	*Nassella charruana*
Snow grass	*Poa labillardieri*
Tall fescue	*Festuca arundinacea*
Phalaris	*Phalaris aquatica*

4.2.4. Glasshouse conditions

The glasshouse trials were undertaken at the Department of Primary Industries facility in Frankston, Victoria. The NSW inoculated plant were in glasshouse 1, control plants in glasshouse 2 and the plants inoculated with the NSW fungi were kept in glasshouse 3. This was to minimise contamination, but does introduce some variability of environment between trials. See Table 4 Summary of inoculums for a summary. In glasshouses 1 and 2 watering was achieved via automatic spraying every 2 days, but in glasshouse 3, water was dripped directly into the pots, which is a more efficient watering mechanism. Despite this, the glasshouses were automatically maintained with a temperature of 20-25^{0}c and a relative humidity of 60-80%. Each bench within the glasshouse was a replication so in each glasshouse 5 benches were used. The plants were placed on these benches in no particular order. Plants were not rotated after their initial placement.

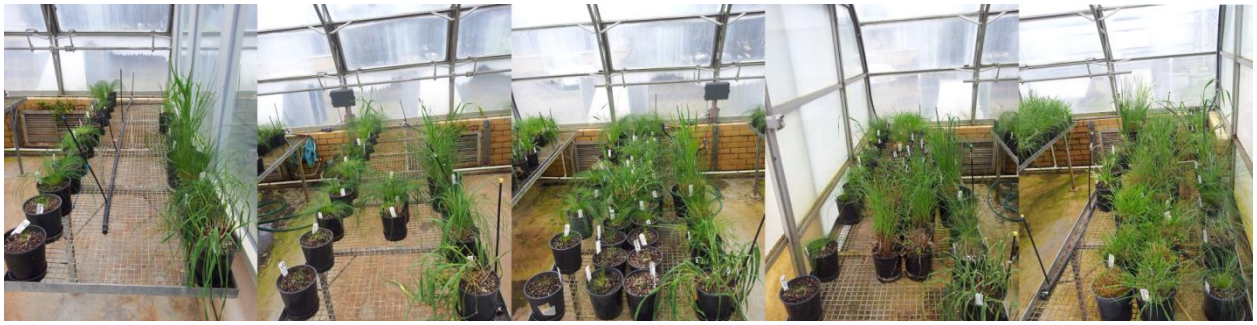

Figure 15 Five tables containing the same species within one glasshouse make up the 5 replicates

4.2.5. Glasshouse trial 1: TAS fusarium

This trial was initiated in July 2011, using the fungi isolated from Tasmanian cultures. Both spray and injection methods were used, but a different inoculum was used for each method, the injected inoculum had a spore concentration of 1.4E+05 spores per mL whilst the spray inoculum had a concentration of 2.9E+05 spores per mL. This is not ideal because it prohibits a direct comparison between the two techniques. Plant health was recorded at least once per week over a two month period, using the same procedure as outlined in section 2.2.1.1. This however, expanded to monitoring the non-target species as well which was less consistent than for serrated tussock, but is still a useful method of measuring change in health over time.

4.2.6. Glasshouse trial 2: NSW Fusarium

This trial will allow the comparison between the virulence of the two of *F. oxysporum* isolations. Due to an insufficient amount of viable sporulation in the NSW cultures gathered in section 2.3.2.2, freeze dried spores of the NSW *F. oxysporum* isolated by Crop Health Services were thawed, cultured, and prepared. Conditions were kept as close as possible to that of the first glasshouse trial with the differences described in section 4.2.4. The final exception is that only one inoculation method was used. This was due to insufficient plants, in fact, for some species the samples were cannibalised from the control group, leaving only 3 control replicates in some cases. Dates commenced and terminated. As with the Tasmanian trial, plant health will be assessed according to the method in section 2.2.1.1. The NSW inoculum had a concentration of 8.3E+05 spores per mL, which higher than either of the Tasmanian inoculums. Once again- 3.5 mL was used, injected directly onto the plant bases.

4.2.7. Controls

The control group was located in glasshouse 2 and underwent no inoculation. The purpose of this group was to have plants that are in identical conditions as the test samples to be a point of reference when comparing with the plants that underwent inoculation. The only significant difference in conditions was the fact that the control group didn't undergo the 'bagging' from section 4.2.2 but this should not have a great deal of effect on plant health. There were initially 5 replicates of each species and serrated tussock accession, until some plants were taken for use in the second inoculation. There was only one control group and it was in both of the other trials. When assessing plant health, the control samples were always checked first to avoid contaminating them.

4.3. Results

All results shown in this section, except for Figure 16, use the 0-9 scale as used previously on the field trials as explained in section 2.2.1.1 the assessment of the non-target species will be more qualitative, but nevertheless provide a useful tool to track plant health in the experiment. The error bars on the graphs indicate standard error.

4.3.1. Pathogenicity

The results shown in Figure 17 indicate that serrated tussock plants inoculated with the Tasmanian *F. oxysporum* (both methods) have more damage after the test period than both the NSW *F. oxysporum* inoculated plants and the control samples, which have equivalent damage. When comparing the two differing inoculation methods with the Tasmanian *F. oxysporum*, we can see in Figure 16 that the average health of all serrated plants declines at the same rate, signifying that there are no significant benefits for one method over the other. However, the spray method had a much higher spore concentrations than the injection method so perhaps the advantages of a more efficient application technique cancelled out the disadvantages of a lower quality inoculum. Overall the damage inflicted on serrated tussock is only minor, in no way threatening to kill the plant- indeed near the conclusion of the trial, even the plants with the higher damage ratings of 4 or 5 were beginning to go to seed. The slope of the lines in Figure 16 suggests that plant health would continue

to decline, and only a longer period of testing would confirm this, and whether the health of the control and test samples will equalize.

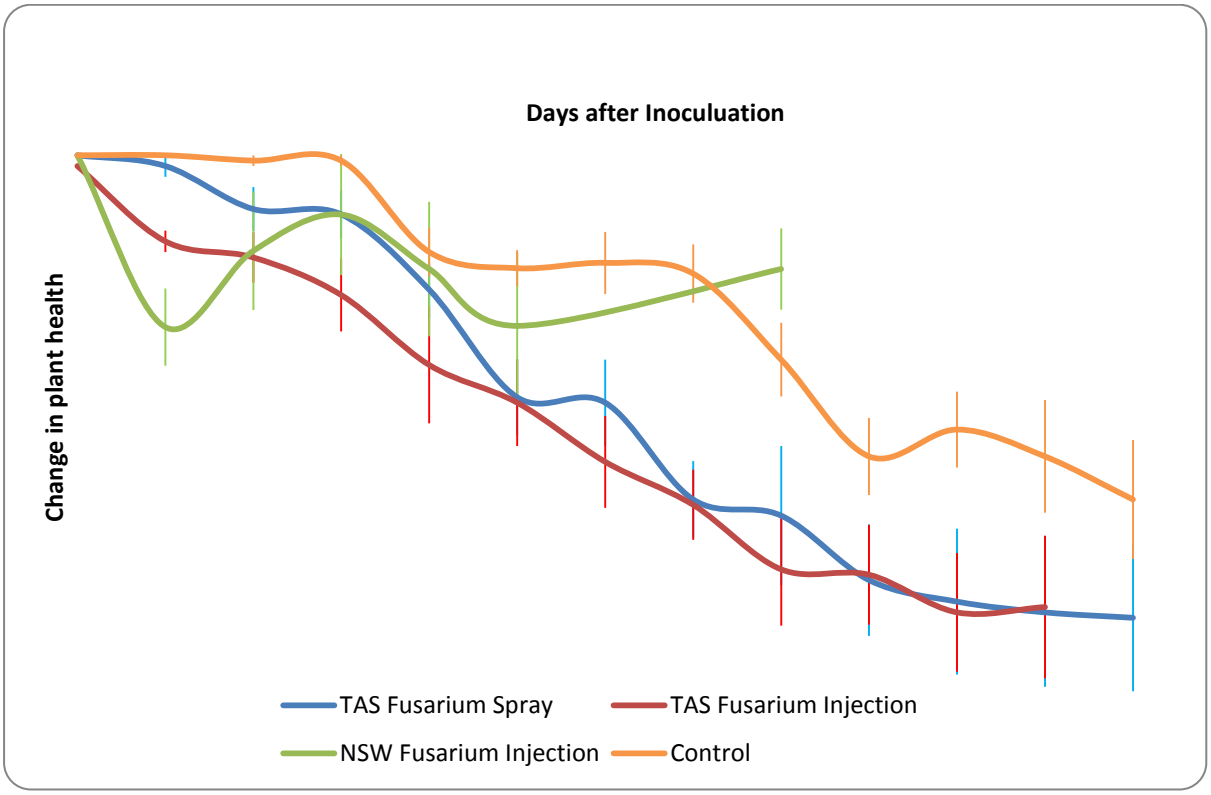

Figure 16 the average health data for serrated tussock over the duration of the trial, the error bars indicate the standard error for each value. In this graph, the health of each plant at the beginning of the trial was designated as being equal to zero and subsequent values in health were calculated by taking change in health (i.e. current observation – last observation) and adding it to the last observation. A negative slope indicates a decline in plant health, and vice versa. The bars indicate Standard error.

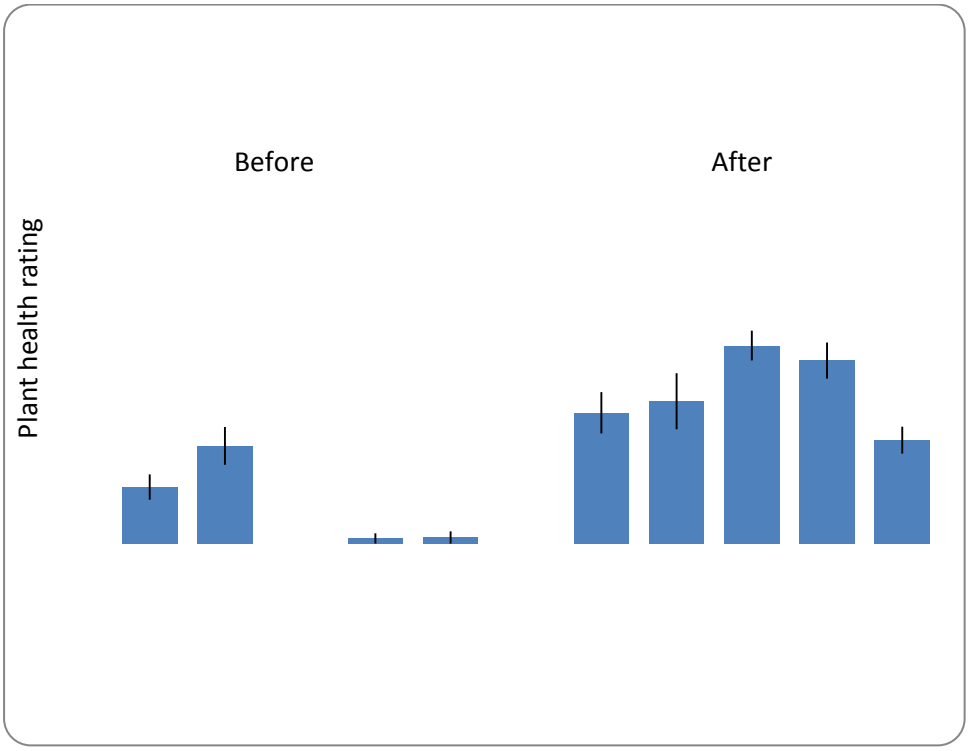

Figure 17 compares average serrated tussock health data before and 41 days after inoculation. 41 days was chosen as the end point, because both inoculation trials went for at least this long, the Tasmanian trial ended at 66 days. The bars indicate Standard error.

4.3.1.1. Tasmanian inoculum

The following are the individual results for each providence of serrated tussock, the general trend is that plants began the trial with damage scores less than one, indicating that they are very healthy and green, and ended the trail only slightly more damaged, with average scores no higher than 3 or 4, which could possibly be attributed with natural changes in the plant as the weather gets hotter, or a slight pathogenic effect from the pathogen, though this is perhaps unlikely as the control samples have a very similar damage score, tho is the majority of cases this is less than the inoculated scores. Maybe combinations of factors are responsible for the health decline.

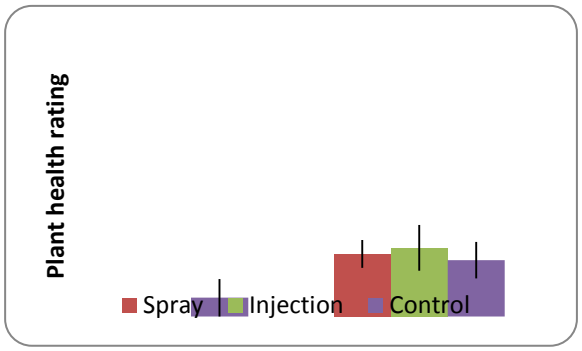

Figure 18 Serrated Tussock (Goulburn)

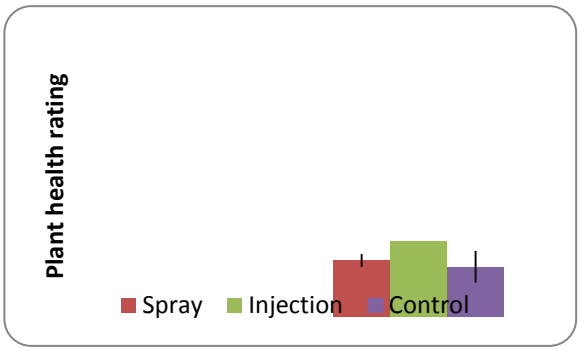

Figure 19 Serrated Tussock (Missen)

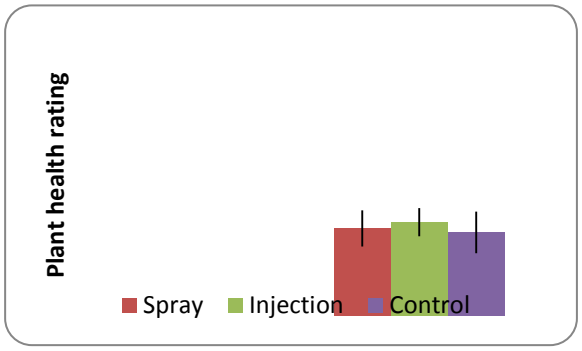

Figure 20 Serrated Tussock (V1G)

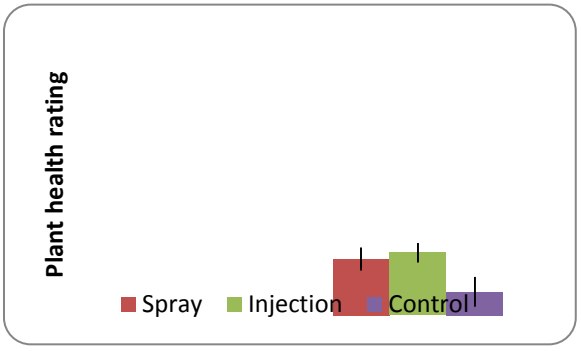

Figure 21 Serrated Tussock (Birdsnest)

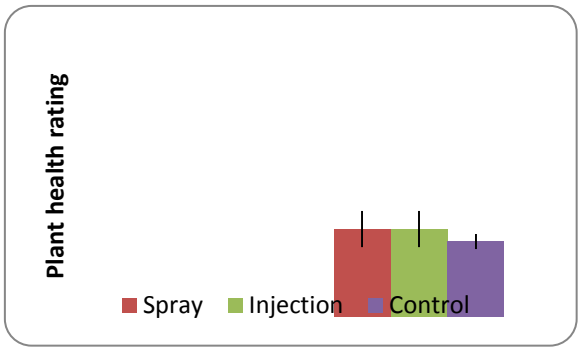

Figure 22 Serrated Tussock (T1)

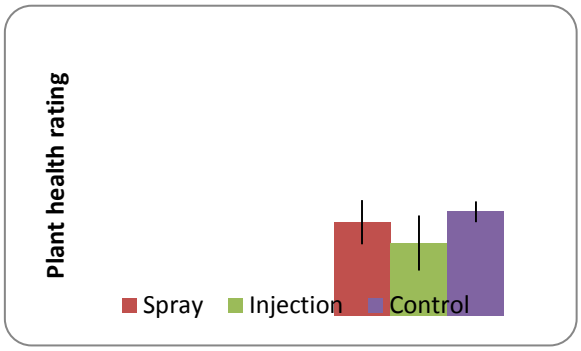

Figure 23 Serrated Tussock (Abercrombie)

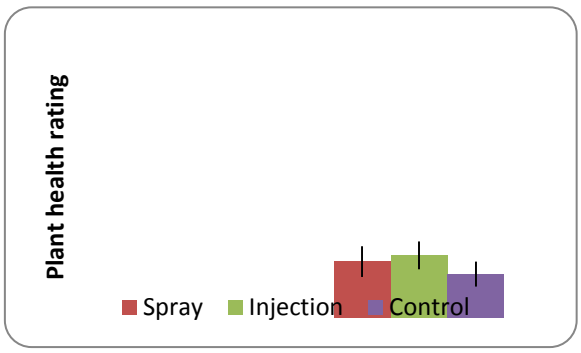

Figure 24 Serrated Tussock (V8)

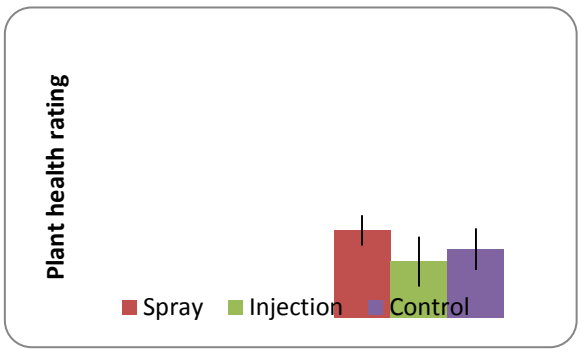

Figure 25 Serrated Tussock (V4)

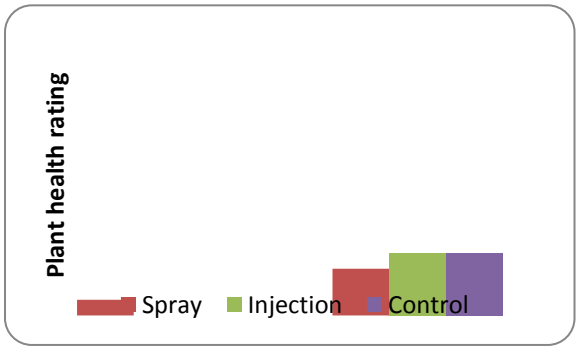

Figure 26 Serrated Tussock (Armidale)

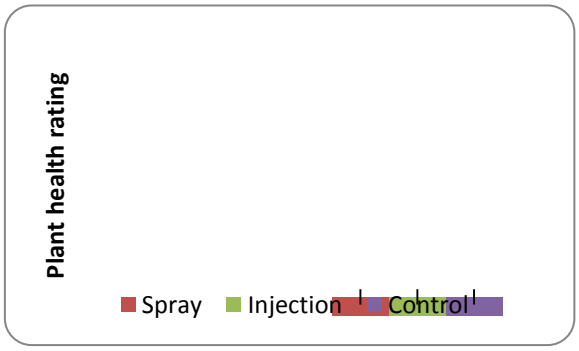

Figure 27 Serrated Tussock (Bathurst Lake)

4.3.1.2. NSW inoculum

The results for the second inoculation trial are very similar to the first except that the initial health of the plants was lower because the experiment was started later in the project, as all plants exhibited a decline in health over time. In this trail, there is only one method of inoculation used, injection. And we can see no tangible difference between the control and inoculated samples at the conclusion of the trial.

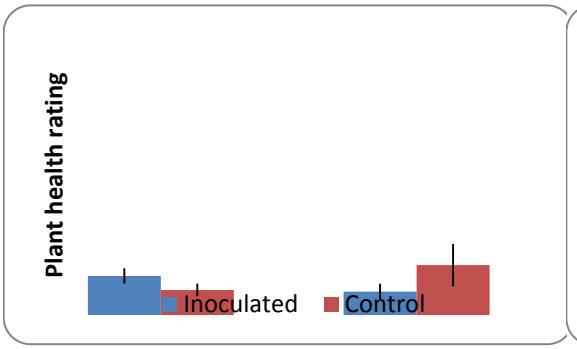

Figure 28 Serrated Tussock Goulburn

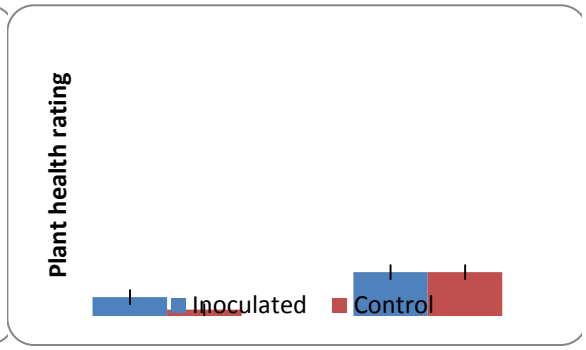

Figure 29 Serrated Tussock Missen

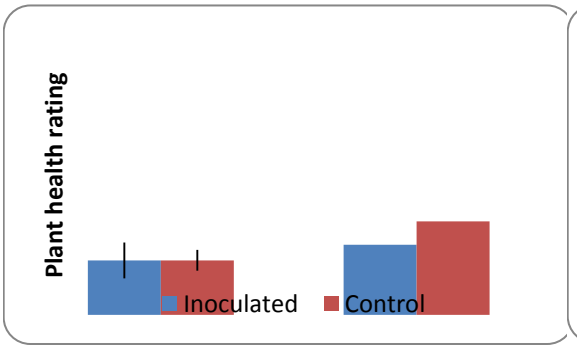

Figure 30 Serrated Tussock V1G

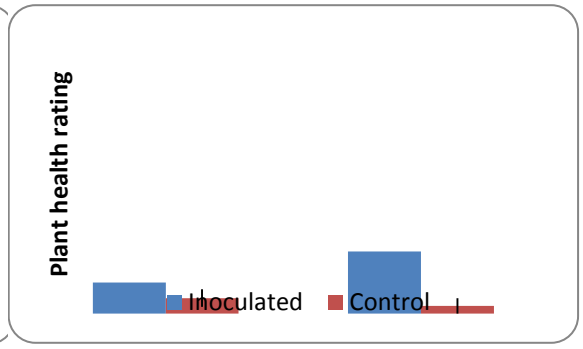

Figure 31 Serrated Tussock (Birdsnest)

4.3.1.3. Tasmanian Inoculum

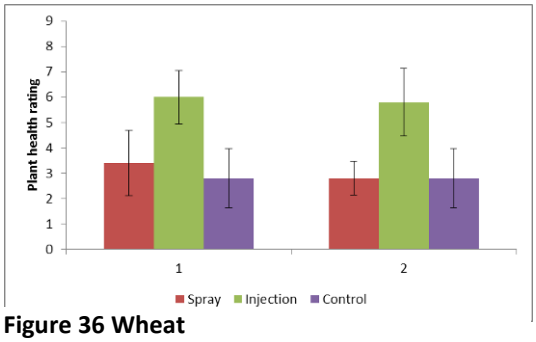

Figure 36 Wheat

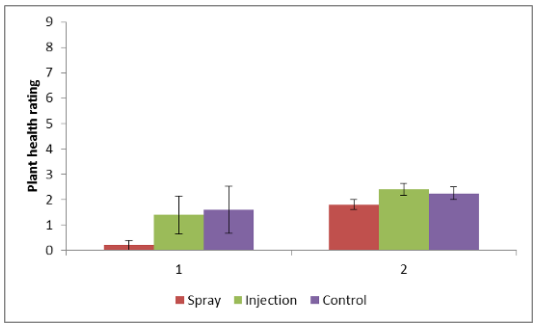

Figure 37 Lobed needle grass

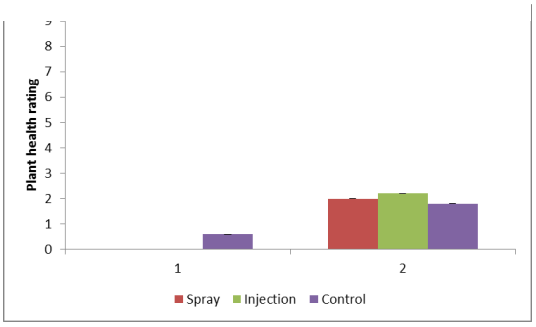

Figure 38 Kangaroo Grass

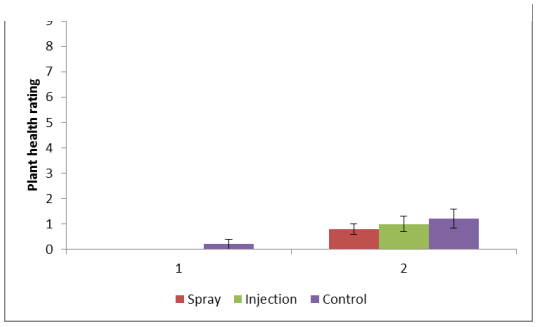

Figure 28 Rough spear grass

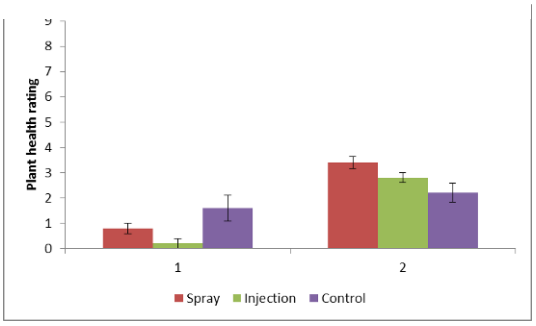

Figure 40 Tall Fescue

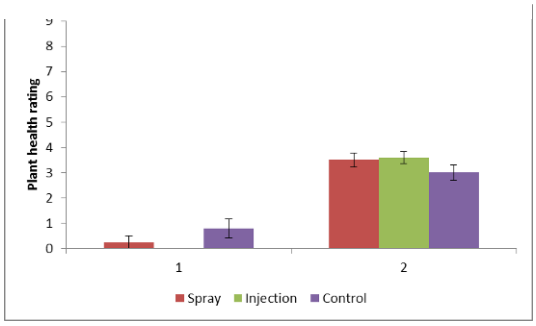

Figure 41 Tall speargrass

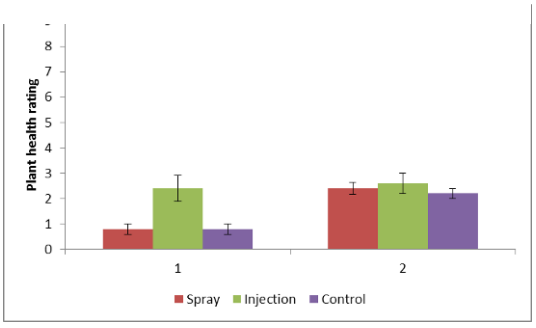

Figure 42 Phalaris

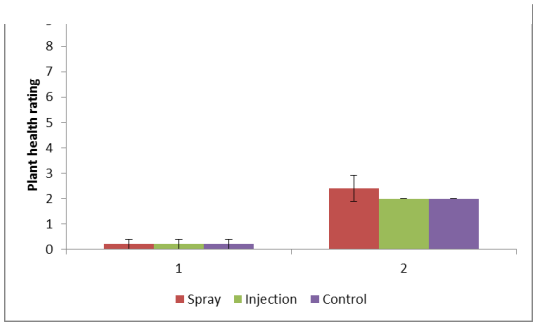

Figure 43 Perennial Ryegrass

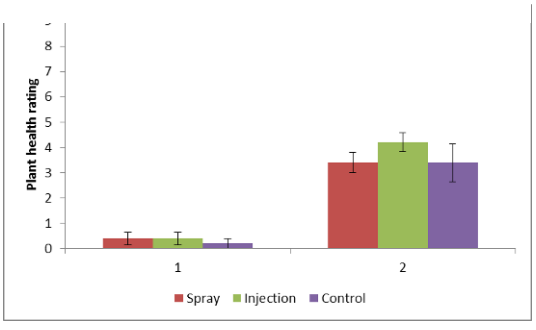

Figure 44 Large Tussock Grass

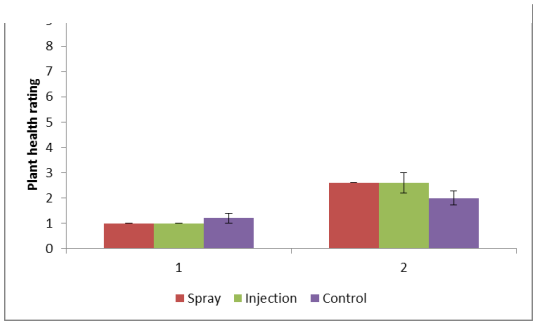

Figure 45 Oats

4.3.1.4. NSW inoculum

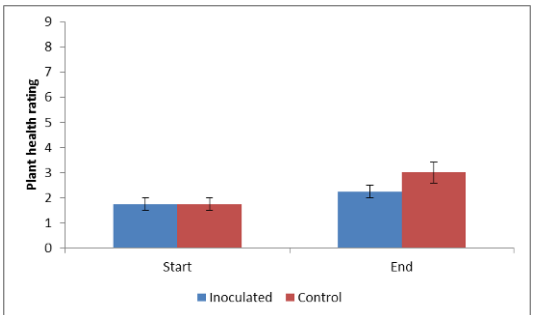

Figure 46 Lobed needle grass

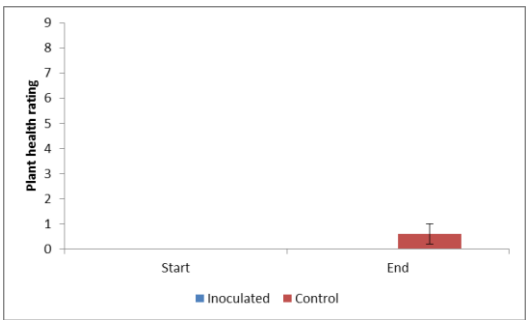

Figure 47 Kangaroo Grass

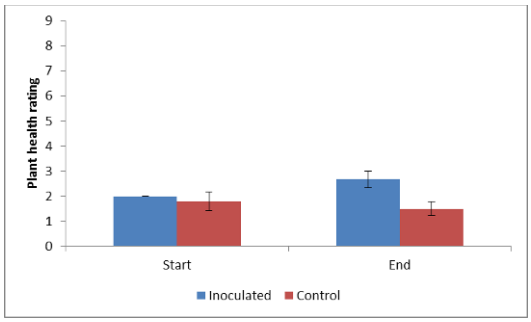

Figure 48 Rough spear grass

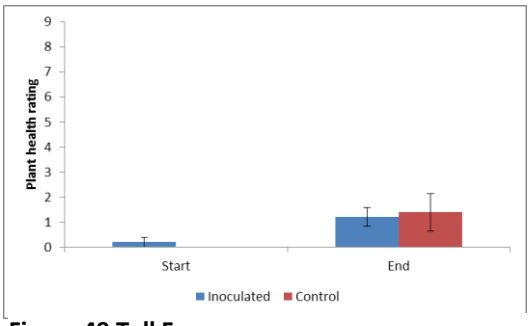

Figure 49 Tall Fescue

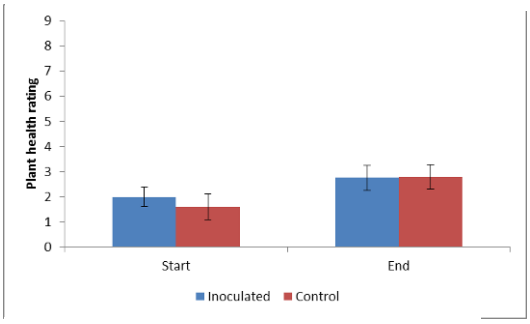

Figure 50 Tall speargrass

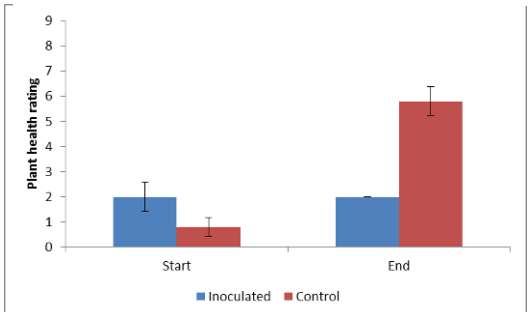

Figure 51 Phalaris

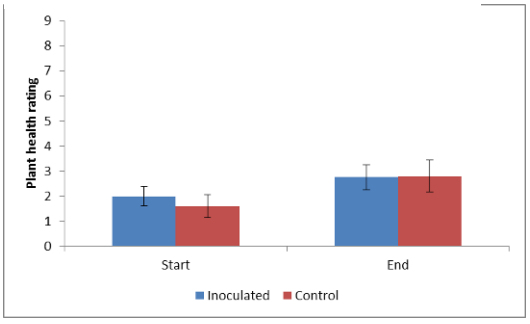

Figure 52 Perennial Ryegrass

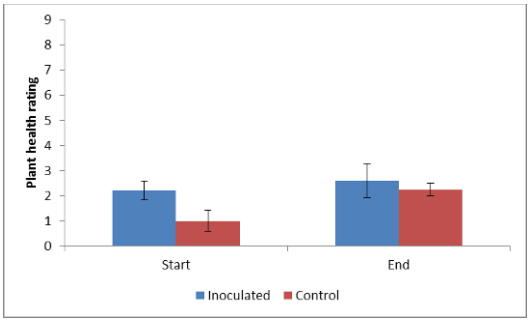

Figure 53 Large Tussock Grass

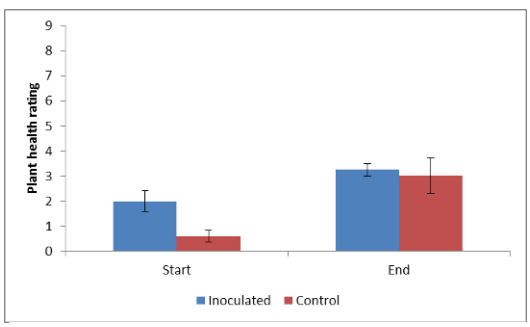

Figure 54 Oats

4.4. Discussion

So, after 66 days and 41 days respectively the plants inoculated with the Tasmanian and NSW isolates of *F. oxysporum* didn't perish, or even show signs of fungal infection such as rotting at the base as described in previous *F. oxysporum* inoculations (Casonato, 2003) or like from the field study in section 0. However, it must be said that running the trial for a longer time would have been ideal as in other experiments, symptoms took necrotic streaks and marginal chlorosis appeared 90 days after inoculation (Mathisa Thevar Ramasamy Thevar, 2008). And indeed, even though appears that the *fusarium* failed to inflict significant damage infect to any of the plants, the fact is there is a small but significant decline in health of the Tasmanian infected serrated tussock as compared to the controls. Perhaps these differences can be attributed to differences in test conditions i.e., greenhouse 1 was more crowded, with less water applied per plant, and greenhouse 3 had the most space and had the more efficient watering mechanism NSW. The discrepancies between the trials is regrettable, but was unavoidable at the time. However, in figure 9 we can see that the injection and spray methods using the TAS *fusarium* are highly correlated, this validates the use of only the injection method for the NSW *fusarium* in that experiment. The NSW inoculum doesn't appear to have had any effect the health of serrated tussock which this could have been due to a myriad of factors, such as shorter trial time, different greenhouse, later in the season, experimental error, etc. All these things factors confound the variables and make it hard to assign causation.

Chapter 5: Post Inoculation DNA Analysis

5.1. Introduction

The results from the glasshouse trials were non dramatic, with minimal damage observed across the variety of species, methods, and inoculums. This suggests that the pathogen either wasn't virulent, or just didn't infect the plants. So, to investigate this, samples were taken from a selection of the inoculated and control samples at the conclusion of the glasshouse trial in order to determine if the *F. oxysporum* inoculums actually infected the plants. If not, then perhaps the tests will reveal if the damage observed in section 4.3 was caused by the presence of another fungal pathogen.

5.2. Materials and methods

5.2.1. Sample Collection

The following plant material from both glasshouse trials was collected; serrated tussock V1G (NSW inoculum), Perennial Ryegrass (TAS inoculum), and Oat (TAS inoculum). And also the respective control samples. So, six samples in total were taken. The samples were taken from the base of the plant, minimising as much as possible any significant damage. The samples then underwent surface sterilisation and were prepared in a similar way to section 3.2.

5.2.2. DNA extraction

Samples were crushed in a mortar and pestle in the presence of liquid nitrogen, and then the DNA was extracted from the plant samples using a DNeasy Plant Mini Kit (Qiagen), with the procedure in the handbook followed exactly. Extracted DNA was then quantified by electrophoresis along with the molecular weight marker GeneRuler™ 100 bp DNA Ladder on a 1.4% agarose gel in TBE buffer at 100 V for 60 min. The gel was then stained in a solution containing ethidium bromide (1 µg/mL). Products were visualised on a transilluminator and recorded on a Gel Doc system (Biorad, USA) with Quantity One software (results shown in Figure 55)

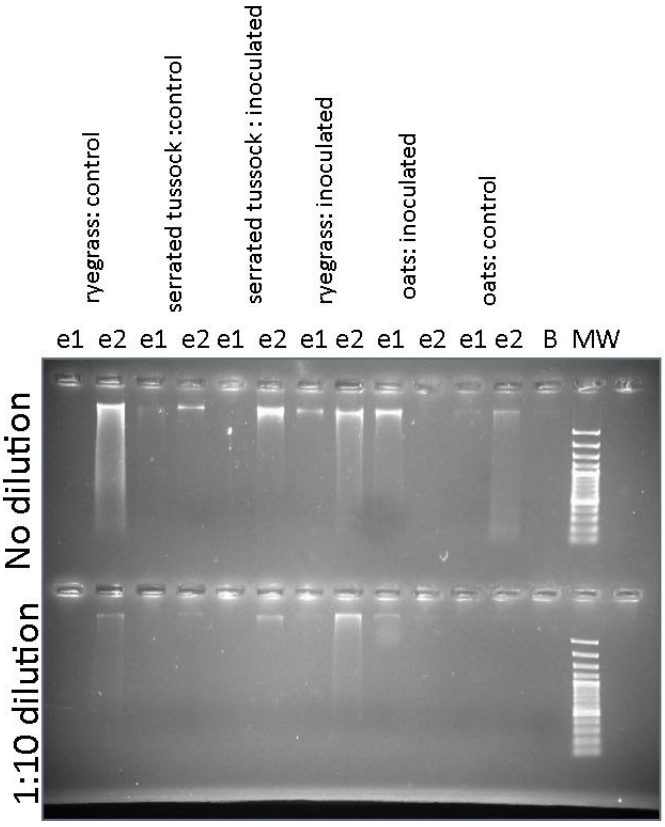

Figure 55 quantification of extracted DNA the top set of well is with dilution, whilst the bottom row has a 1:10 dilution factor. Also, e1 and e2 refer to eluent 1 and eluent 2 when extracting the DNA.

5.2.3. PCR

The polymerase chain reaction (PCR) is used to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The ITS region was amplified with the fungal-specific primer IT2F, and ITS4. The PCR reaction was performed in 50 μL reaction volumes. A negative control was used in the reaction, which contained no DNA. Reactions were performed in a Thermal Cycler using the following protocol:

- One cycle of denaturation at 94 ^{o}c
- 35 cycles of:
    - Denaturation at 94 ^{o}c for 10 min
    - Annealing at 55 ^{o}c for 30 s
    - Extension at 72 ^{o}c for 1 min
- Final extension at 72 ^{o}c for 10 imin

Products were separated by electrophoresis along with the molecular weight marker GeneRuler™ 100 bp DNA Ladder on a 1.4% agarose gel in TBE buffer at 100 V for 60 min. The gel was then stained in a solution containing ethidium bromide (1 μg/mL). Products were visualised on a transilluminator and recorded on a Gel Doc system (Biorad, USA) with Quantity One software (results in Figure).

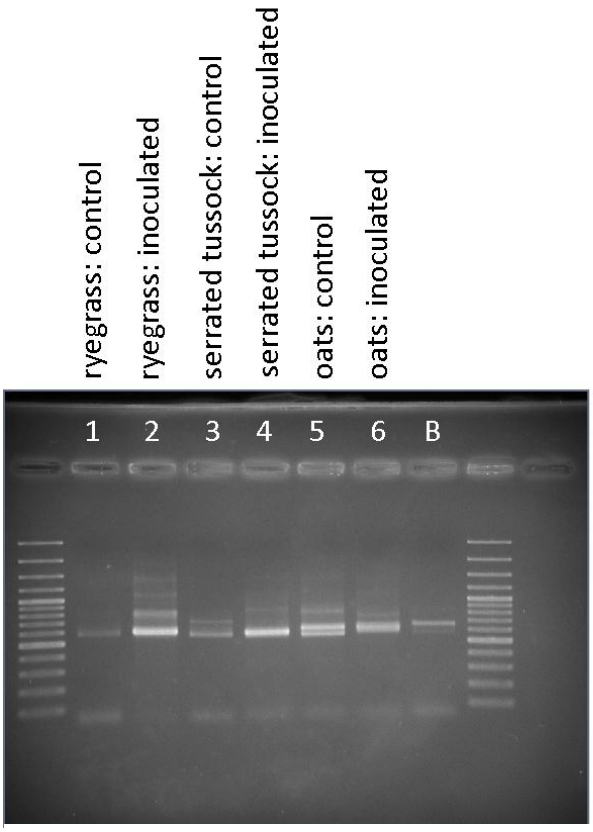

Figure 56 PCR Results

Figure shows that lanes 3,4 and 7 appear to have the same band. These are the wells in which plant material inoculated with the *fusarium* solution were injected, so these samples were sent off for sequencing in the next section in order to determine which fungal species is common to these inoculated specimens. Lane 6 contains two closely spaced bands, indicating that there is more than one type of DNA present. An attempt to separate and sequence each of these was unsuccessful as even when run on 2% agarose gel, the bands remain too close to cut apart.

5.2.4. RFLP

Restriction fragment length polymorphism (RFLP) involves fragmenting the DNA sample with restriction enzymes. The DNA sample is digested, and the resulting restriction fragments are then separated by length via electrophoresis, the PCR products amplified in section 5.2.3 were digested using the three restriction enzymes:

- HhaII
- HinfI
- HaeIII

All restriction digestions were incubated at 37°C for 2 h then separated by electrophoresis along with a molecular weight marker, on a 1.4% agarose gel in TBE buffer at 100 V for 60 min. The gel was then stained in a solution containing ethidium bromide (1 µg/mL). Products were visualised on a transilluminator and recorded on a Gel Doc system (Biorad, USA) with Quantity One software. After adding the fragments from Figure 29, products 2, 4, and 6 were judged to contain only one type of DNA, these products were the inoculated samples, so these were puffed and sent for sequencing in the next section.

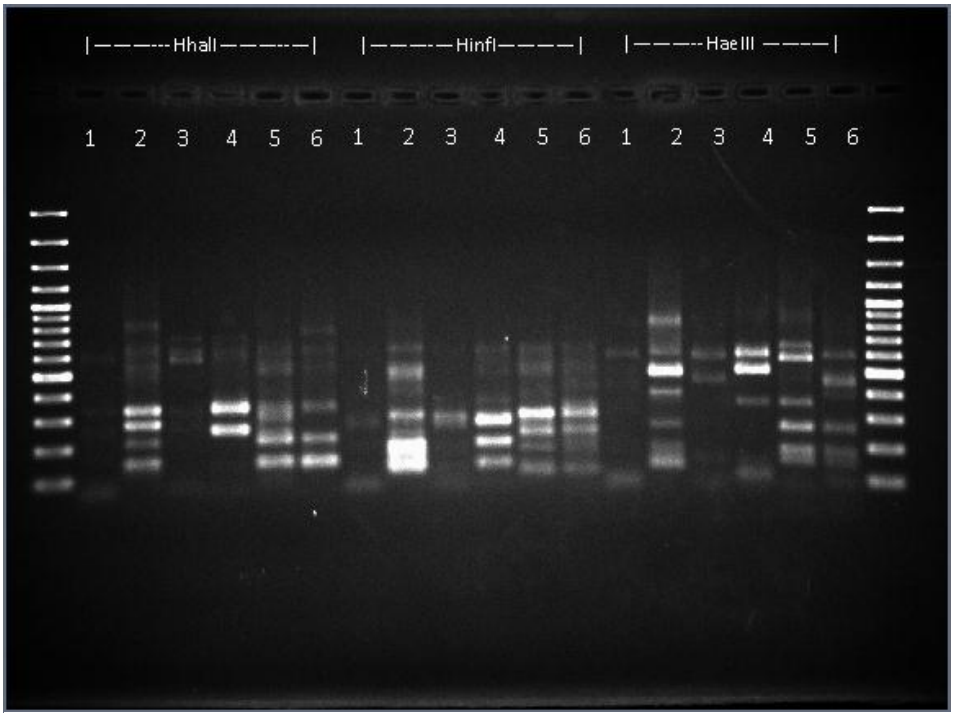

Figure 29 RFLP gel showing the DNA restriction fragments created after digestion with the 3 enzymes. On the far left and right lanes the GeneRuler 100 bp DNA Ladder was used.

5.2.5. Sequencing

PCR products 2, 4, and 6 from section 5.2.3 were purified in preparation for sequencing according to the method detailed out in the QIAquick PCR Purification Kit Protocol. The purified product was precipitated in a single direction using the ITS1F primer. The reaction underwent the following PCR reaction cycle:

- Initial denaturation 96 ^{o}c for 1 min
- 25 cycles of

- Denaturation at 96 °c for 10 sec
- Annealing at 50 °c for 5 sec
- Extension at 60 °c for 4 mins

Products were removed and cleaned using an ethanol-precipitation protocol. After complete removal of ethanol by evaporation overnight, the purified DNA was delivered to Micromon (the DNA Sequencing Facility at Monash University) for sequencing.

5.3. Results

The resulting sequences are located in the appendix section 7.6. From looking at the sequences, the RC2 file was a nice clean sequence with a single DNA signal, whilst the RC4 file was messier, but still contained one DNA signal, the last one however, RC6, was very messy, and likely had two or more DNA signals, so this data must be treated as unreliable. Displayed below are a list of the top ten NCBI database blast search results for each of the sequences obtained from Micromon., Curiously, none of the samples sent for sequences contained any *F. oxysporum* DNA, which is unexpected.

5.3.1. Inoculated serrated tussock (RC4)

Table 7 Top 10 NCBI BLAST search results for fungal DNA isolated from Inoculated serrated tussock. The DNA isolated from this plant was likely one of the following entophytes and other miscellaneous fungi, it is likely that none of these matches are responsible for damaging the health of the plant.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF504424.1	Uncultured endophytic fungus	315	315	47%	2.00E-82	99%
EF504417.1	Uncultured endophytic fungus	315	315	47%	2.00E-82	98%
JN032516.1	Uncultured fungus clone	309	309	47%	8.00E-81	98%
JN033464.1	Cladosporium ramotenellum	309	309	47%	8.00E-81	98%
JF432971.1	Uncultured fungus clone	309	309	47%	8.00E-81	98%
JF691038.1	Uncultured Capnodiales clone	309	309	47%	8.00E-81	98%
JF691013.1	Uncultured Capnodiales clone	309	309	47%	8.00E-81	98%
AB622945.1	Uncultured fungus	309	309	47%	8.00E-81	98%
AB622943.1	Uncultured fungus	309	309	47%	8.00E-81	98%
JF311953.1	Davidiella macrospora strain	309	309	47%	8.00E-81	98%

5.3.2. Inoculated ryegrass (RC2)

Table 8 Top 10 NCBI BLAST search results for fungal DNA isolated from inoculated ryegrass. The sequence obtained from the inoculated ryegrass sample was of average quality- that is, there was only one DNA signal present, but the quality and length of the sequence is poor. The closest matches are quite good however, all at 99%. Microdochium bolleyi is a plant pathogen, and is possibly responsible for the fluctuating health observed in the glasshouse trial- it is likely that the plant already possessed this fungus before inoculation.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AM502261.1	Microdochium bolleyi	944	944	97%	0	99%
GU566298.1	Microdochium bolleyi	942	942	97%	0	99%
AJ279477.1	Microdochium sp.	942	942	97%	0	99%
HQ630981.1	Microdochium sp.	939	939	97%	0	99%
AM502265.1	Microdochium bolleyi	939	939	97%	0	99%
AJ279485.1	Microdochium sp.	933	933	96%	0	99%
GU566262.1	Microdochium bolleyi	931	931	97%	0	99%
FN391313.1	Uncultured fungus	929	929	96%	0	99%
FN391312.1	Uncultured fungus	929	929	96%	0	99%
FN391311.1	Uncultured fungus	929	929	96%	0	99%

5.3.3. Inoculated oats (RC6)

Table 9 Top 10 NCBI BLAST search results for fungal DNA isolated from inoculated Oats. The Max ident values are very low, even on the top match – so it is unlikely that the sequence was very clean. The closest matching species listed here contain a selection of entophytes and saprophytes

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
GQ999266.1	Uncultured fungus clone	675	675	65%	0	97%
HQ631051.1	Pleosporales sp.	656	656	67%	0	95%
HQ630983.1	Phaeosphaeriopsis sp.	647	647	67%	0	95%
HQ608029.1	Ascomycota sp.	645	645	65%	0	96%
HQ631018.1	Phaeosphaeria sp.	645	645	67%	0	95%
EF060651.1	Pleosporales sp.	636	636	65%	6.00E-179	95%
EF505542.1	Uncultured endophytic fungus	628	628	63%	1.00E-176	96%
HM537063.1	Fungal endophyte	627	627	65%	3.00E-176	95%
HQ649992.1	Pleosporales sp	621	621	64%	2.00E-174	95%
HQ649991.1	Pleosporales sp.	621	621	64%	2.00E-174	95%

5.4. Discussion

DNA testing would have been a fine tool for detecting the presence of the *F oxysporum* in the test plants, but in this case, insufficient samples were taken and with too few replicates to come to any full conclusions. The selection of entophytes is probably just typical 'background' fungal ecosystems for these plants, but without more samples it is hard to make a definitive statement. The fact that none of the DNA turned out to be from *F oxysporum* can indicate that either the isolated strains are not pathogenic or that the inoculation methods were inadequate for infection. Or another option is that the glasshouse conditions were not optimal for the fungus to become pathogenic.

Regarding the problems with the PCR, i.e. the smearing of bands, perhaps this could have been mitigated by diluting the PCR product. And the band in the blank was probably caused by contamination of one of the constituents used in the method. Repeating the procedure would very likely yield better results, but in the end- time was too short.

Figure 30 Orange fungal infection observed on ryegrass before inoculation, possibly the *Microdochium bolleyi* as detected in DNA analysis.

Chapter 6: General discussion

6.1. Research Outcomes

It has been shown that from both dieback locations, it was possible to isolate *F. oxyspoum*. And that there was a white fungal infection observable on plants within the dieback zone in NSW. The presence of this has been associated with F. oxysporum in the past (Casonato, 2003). But the strains of F. oxysporum tested in

the glasshouse trials have proven to be insufficiently pathogenic to kill serrated tussock within the time frame and conditions of this experiment. It has also been shown that it is insufficiently pathogenic to kill the native and beneficial species tested, It is almost as if the conditions in the glasshouse are just not ideal for this fungus, and that there is something located at the sites which can trigger pathogenicity, more specifically *fusarium* has had documented cases of only becoming pathogenic in the presence of other fungi (Chandramohan and Charudattan, 2001) perhaps even one of the other isolated species from section 3.3.1. And perhaps that could be the basis of future trials, as could running the glasshouse trial for a longer time which would allow the pathogen greater time to work, however the DNA result showed that *F. oxysporum* wasn't present on the inoculated species after 41 days anyway, so an extended testing period would perhaps be futile. It must be noted that the DNA tests were far from exhaustive and the pathogen could have been lost in the extraction process, or just not present on the particular group of plants sampled.

So what caused the dieback if not *fusarium oxysporum*? Well more work is required in the field to undertake extensive observations and sample gathering. Maybe there is no pathogen after all, for we have seen in the glasshouse trials that the plants health declined over time, including the controls so other factors may be responsible for this. One explanation for the decline (to some extent) is experimental bias. For example, if the observer is actively looking for a change in a plant, then he or she is more likely to find it. A way to combat this is to mix in the control and inoculated plants, and label them in an obscure manner so the observer doesn't know which were inoculated or not and expect (or hope for) them to show symptoms.

6.1.1. Future directions

With the weed rapidly spreading and with diminishing returns on control methods, serrated tussock is becoming a problem fast out of control. It is therefore a worthy cause to investigate new approaches to tackling the problem, and in this paper I have attempted to build on the past works in the development of a successful biological control for this noxious weed. However, once again, serrated tussock has not only survived but thrived, and gone on to produce the spring seed when faced with another potential pathogen. It is worth noting however, that the experimental techniques employed here are not without error and that the phenomenon causing the dieback in the field sites is still unsolved, so additional examination of these

plants is advised as samples gathered in Chapter 2 may have not taken the particular pathogen, and the *F. oxysporum* species tested here was but merely a saprophyte which attacks the rotting plant material and may just be symptomatic of the true pathogen.

Chapter 7: Appendix

7.1. Transect data

Table 10 transect 1

Distance (m)	% cover of				Serrated tussock health rating
	Broadleaf	Native Grass	Bare Ground	Serrated Tussock	
1	5	95	0	0	
2	20	80	0	0	
3	75	25	0	0	
4	25	75	0	0	
5	40	60	0	0	
6	55	40	5	0	
7	50	50	0	0	
8	40	55	0	5	9
9	60	40	0	0	
10	40	60	0	0	
11	60	40	0	0	
12	40	60	0	0	
13	50	50	0	0	
14	15	85	0	0	
15	50	50	0	0	
16	20	80	0	0	
17	40	60	0	0	
18	52	45	0	3	6
19	10	90	0	0	
20	45	40	0	15	8
21	65	20	0	15	8
22	10	90	0	0	
23	20	70	0	10	8
24	30	70	0	0	
total %	38.2%	59.6%	0.2%	2.0%	
average					7.8

Table 11 transect 2

Distance (m)	% cover of				Serrated tussock health rating
	Broadleaf	Native Grass	Bare Ground	Serrated Tussock	
1	55	15	10	20	9
2	2	8	0	90	9
3	5	5	0	90	8
4	10	10	0	80	8
5	5			95	9
6	8	2		90	9
7	5	15		80	7
8	18	2		80	9
9	15			85	8
10	40	20		40	8
11	30	20		50	9
12	25	45		30	9
13	15	70	5	10	9
14	10			90	7
15	10	40		50	7
16	15	10		75	8
17	25	5		70	6
18	55	15		30	8
19	70	5		25	8
20	30	10		60	9
21	12	3		85	7
22	38	2		60	8
23	3	7		90	8
24	15	5		80	8
25	5	25		70	9
total %	19.4%	13.5%	0.2%	66.9%	
average					8.1

Table 12 transect 3

Distance (m)	% cover of				Serrated tussock health rating
	Broadleaf	Native Grass	Bare Ground	Serrated Tussock	
1	47	3	20	30	5
2		45	20	35	3
3	5	20	15	60	5
4	4	6	10	80	4
5	3	5	2	90	4
6	1	4	5	90	5
7	2	8	15	75	3
8	5	5	30	60	5
9	2	3	20	75	3
10	1	4	20	75	5
11				100	5
12	4	6	10	80	5
13		2	3	95	5
14		3	2	95	5
15	1	4	10	85	6
16	3	22	25	50	4
17	10	5	40	45	4
18		10	20	70	3
19	2	3	60	35	4
20		3	2	95	5
21		5	20	75	4
22	1	9	20	70	6
23	4	56	30	10	3
24	1	9	20	70	5
25	5	35	30	30	4
total %	2.3%	11.3%	17.9%	68.5%	
average					4.4

Table 13 transect 4

Distance (m)	% cover of				Serrated tussock health rating
	Broadleaf	Native Grass	Bare Ground	Serrated Tussock	
1	1	99			
2	25	60		15	4
3	7	48	15	30	6
4	5	75	15	5	5
5	8	7	25	60	6
6	10	30	20	40	4
7	10	25	5	60	5
8	15		15	70	5
9	30	50	10	10	4
10	10	45	5	40	3
11	10	65		25	4
12	10	90			
13	25	55		20	3
14		75	5	20	4
15		100			
16		100			
17		80		20	2
18		70		30	4
19	2	58		40	2
20		40		60	5
21		30		70	5
22		80		20	3
23		75		25	6
24		70		30	3
25	10	65		25	4
total %	7.4%	58.0%	4.8%	29.8%	
average					4.1

7.2. Fungal culturing of Tasmanian samples

Table 14 NSW Fungal identification

Tasmanian Sample			
Culture	**identified species**	**Culture**	**identified species**
1A	unidentified	2A	unidentified
1B	*Marasmius rotula*	2B	*Fusarium* sp
1C	*Marasmius rotula*	2D	unidentified
1D	unidentified	2E	unidentified
1E	*Fusarium* sp	2F	unidentified
1F	*Marasmius rotula*	2G	unidentified
1G	*Marasmius rotula*	2H	unidentified
1H	*Marasmius rotula*	2I	*Marasmius rotula*
1I	*Marasmius rotula*	2J	*Fusarium* sp
1J	*Marasmius rotula*	2K	*Fusarium* sp
1K	*Marasmius rotula*	2L	*Marasmius rotula*
1L	unidentified	2M	*Fusarium* sp
1M	*Marasmius rotula*	2N	*Fusarium* sp
1N	unidentified	2O	*Marasmius rotula*
1O	unidentified	2P	*Fusarium* sp
1P	*Fusarium* sp	2Q	unidentified
1Q	*Marasmius rotula*	2R	unidentified

7.3. Fungal culturing of NSW samples

Table 15 Tasmanian Fungal identification

NSW Transect 1		NSW Transect 2		NSW Transect 3		NSW Transect 4	
Culture	**identified species**	**Culture**	**identified species**	**Culture**	**identified species**	**Plate ID**	**identified species**
T1(2)a	no fungal growth	T2a	no fungal growth	T3a	Fusarium sp	H1a	no fungal growth
T1(2)b	*Fusarium* sp	T2b	mites and unidentified	T3b	Trichoderma sp	H1b	unidentified
T1(2)c	*Trichoderma* sp	T2c	no fungal growth	T3c	unidentified	H1c	unidentified
T1(2)e	*Fusarium* sp	T2d	no fungal growth	T3d	*Fusarium* sp and *Tricoderma* sp	H1d	*Trichoderma* sp
T1a	*Sordaria* sp and *Fusarium* sp	T2d	no fungal growth	T3e	unidentified	H1e	no fungal growth
T1b	no fungal growth	T2e	no fungal growth			H2a	unidentified
T1c	*Trichoderma* sp	T2f	*Apophysomyces* sp			H2b	*Cladosporium* sp
T1d	*Fusarium* sp					H2c	*Cladosporium* sp
T1e	*Sordaria* sp					H2d	unidentified
						H2e	unidentified
						H2f	no fungal growth
						H2g	no fungal growth
						H3a	contaminated and unitentified
						H3b	unidentified
						H3c	*Apophysomyces* sp
						H3d	no fungal growth
						H3e	no fungal growth
						H3f	unidentified
						H3g	unidentified

7.4. Report from crop health services

Bathurst Lake 15-02-2011
Test(s):
Fungal Culturing (first test)
Extended microscopic examination

The fungi identified from the February samples were *F. oxysporum, Rhizoctonia* sp. and *Mucor* sp.
Results:
Serrated Tussock samples submitted were checked for fungal and pathogens. Fungi isolated from fungal culturing of the plant material are as follows:
Fusarium spp., one of them probably is F. oxysporum,
Some Rhizoctonia and Mucor, the soil tested positive for Phytophthora

CROP HEALTH SERVICES DNA
Phytophthora and Fusarium cultures x. AG/152l45, submitted for further identification

The Fusarium is *F. oxysporum*.
The Phytophthora is Phytophthora cryptogea

7.5. Inoculum spore count

Table 16 Spore count data for inoculum 1

TAS spray (inoculum 1)		
repetition	1	2
volume counted	0.05	0.05
area	spores counted	
1	0	2
2	0	2
3	1	0
4	0	2
5	0	0
6	0	0
7	5	1
8	0	1
9	2	0
10	1	4

Table 17 Spore count data for inoculum 2

TAS injection (inoculum 2)		
repetition	1	2
volume counted	0.05	0.05
area	spores counted	
1	1	2
2	0	0
3	0	0
4	1	0
5	1	0
6	1	3
7	0	0
8	0	0
9	0	0
10	1	0

Table 18 Spore count data for inoculum 3

NSW injection (inoculum 3)										
repetition	1	2	3	4	5	6	7	8	9	10
volume counted	0.03	0.02	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.03
area	spores counted									
1	5	1	6	2	1	4	2	0	1	0
2	4	1	0	2	1	1	4	1	4	0
3	4	4	2	4	0	3	2	2	4	1
4	2	3	0	2	1	3	2	0	2	1
5	2	3	2	0	0	2	3	1	0	2
6	5	2	0	1	2	3	4	3	1	2
7	1	4	2	2	0	1	1	5	3	0
8	1	4	3	2	0	5	3	0	2	1
9	1	2	0	3	0	1	2	0	3	1
10	2	1	2	4	0	0	4	3	0	3

Table 19 Calculations of spore count

inoculum	1	2	3
spores per area	1.05	0.5	1.92
areas per coverslip*	13,828	13,828	13,828
spores per coverslip	14519.4	6914	26549.76
volume under coverslip (mL)	0.05	0.05	0.032
spores per mL	2.9E+05	1.4E+05	8.3E+05

***areas per coverslip:**

Coverslip area

22mm x 22mm = 484 mm^2

22,000,000 µm x 22,000,000 µm

=484,000,000 $µm^2$

Area of spore count:

35* x 100µm x 100µm

=35000 $µm^2$

*35 is the number of grid squares displayed on the monitor (of 100µm x 100µm size)

Number of count area's per coverslip

484,000,000/35000= 13,828 areas per slip

7.6. DNA sequences

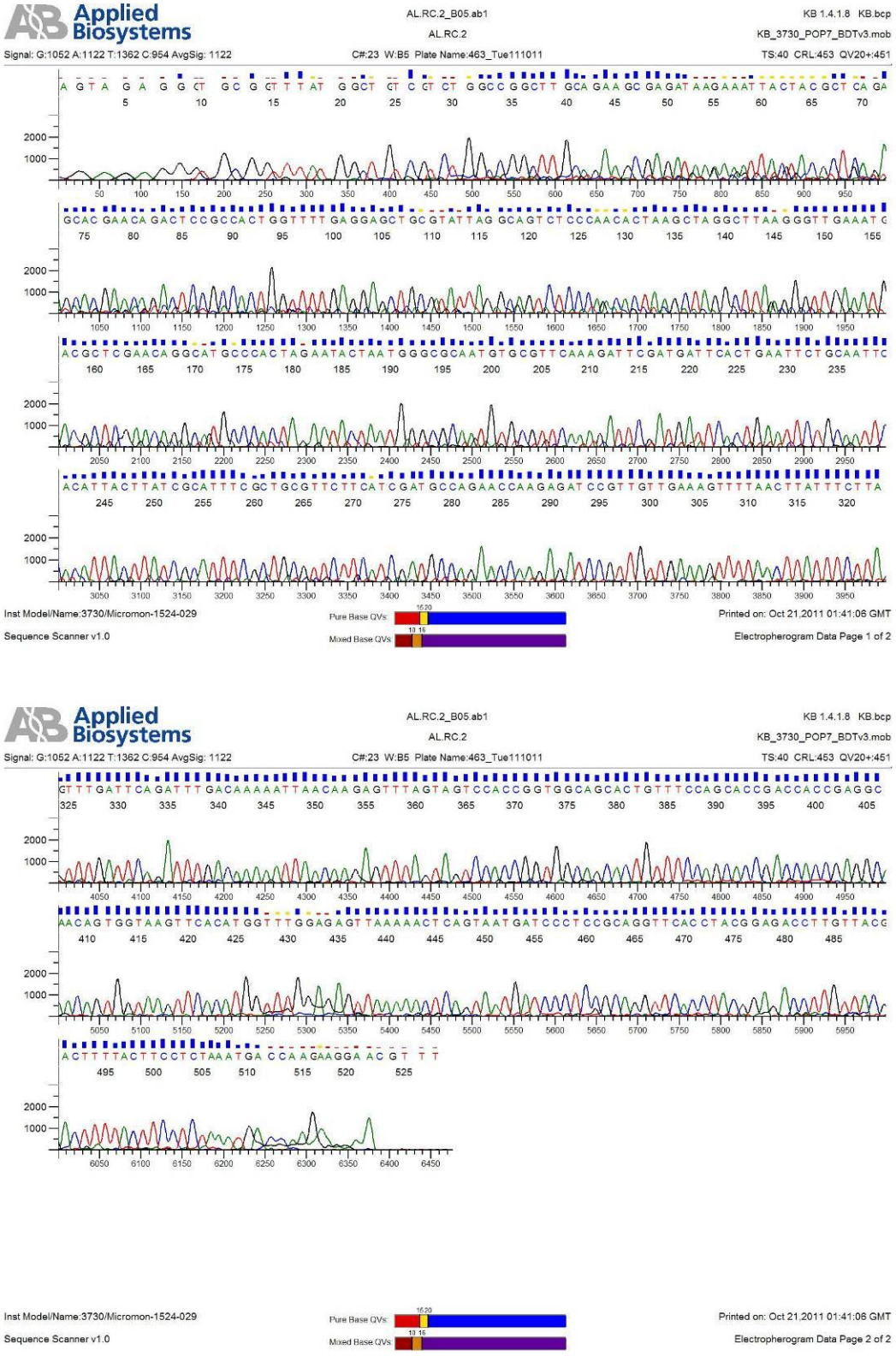

Figure 59 INOCULATED SERRATED TUSSOCK

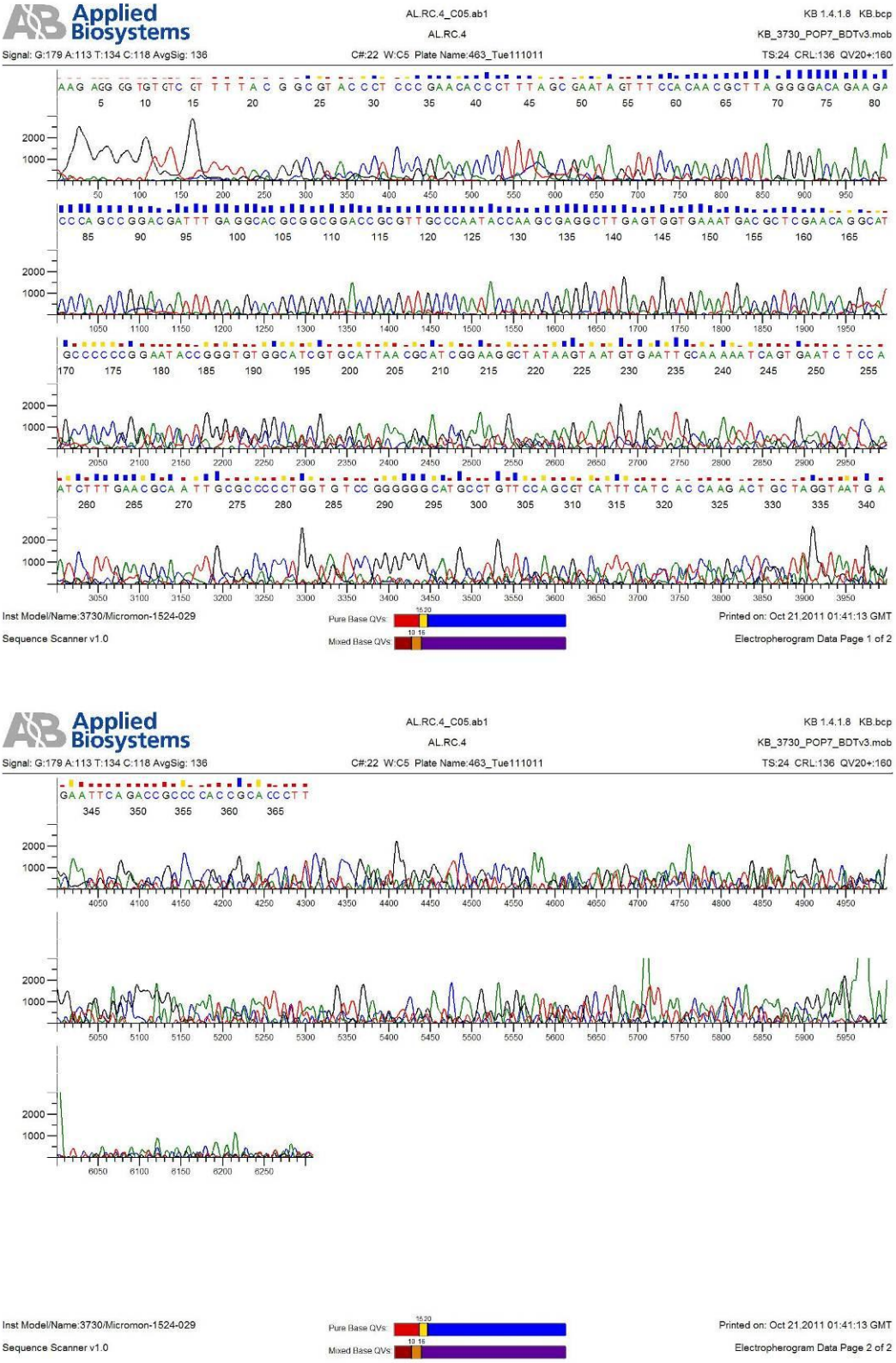

Figure 60 INOCULATED RYEGRASS

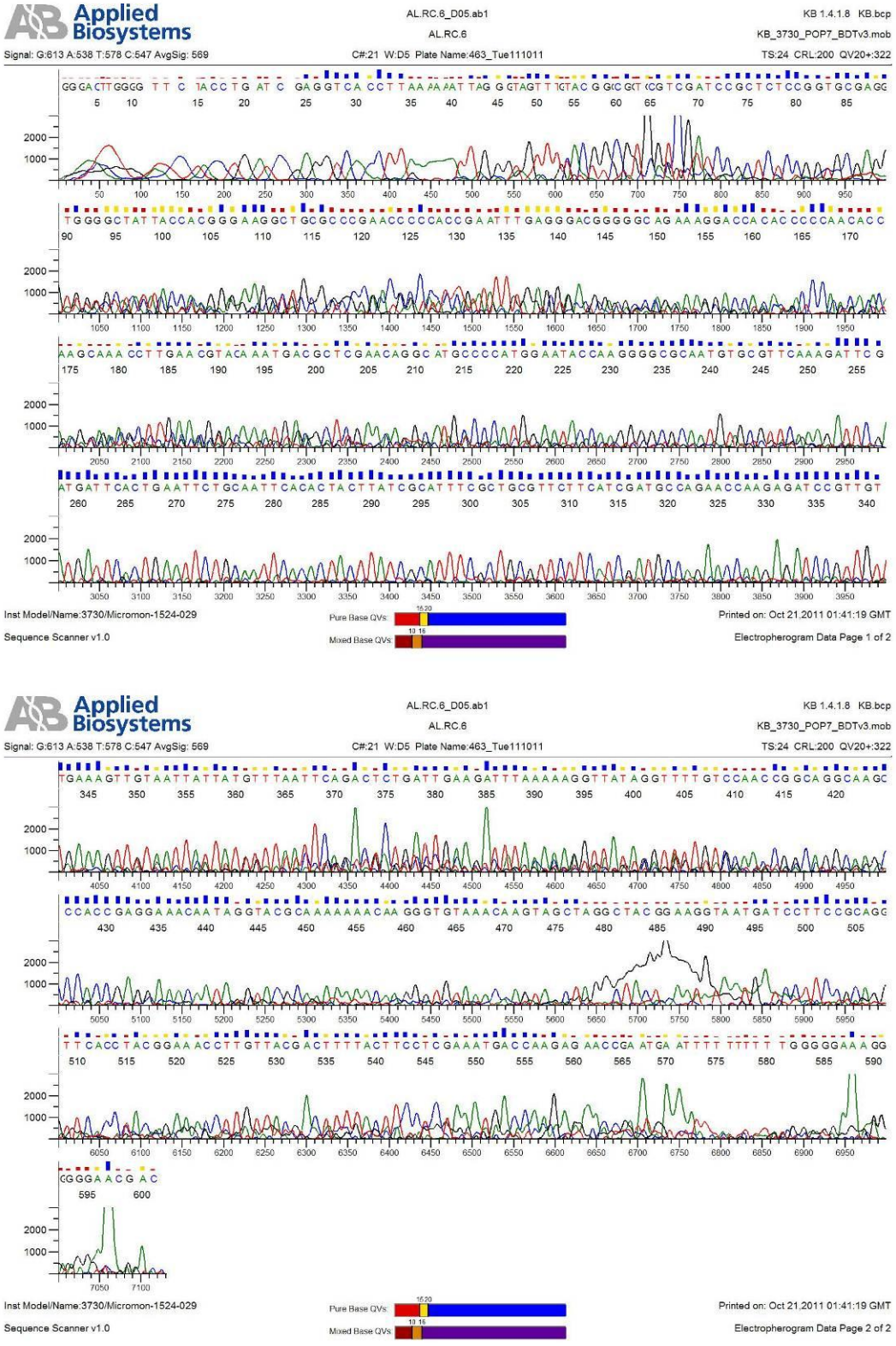

Figure 61 INOCULATED OATS

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