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Prepared by: Associate Professor Vic Galea
The University of Queensland

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Preliminary investigation of prickly acacia (*Acacia nilotica*) dieback

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Abstract

Prickly acacia (*Acacia nilotica*) is a weed of national significance (WONS) which significantly impacts on the grazing industry across northern Australia. A dieback phenomenon has been reported to occur in some locations where this woody weed exists. 150 fungal isolates were collected from field sampling of dieback-affected prickly acacia plants. The majority (70%) of these were found to mainly belong to the genus *Botryosphaeria*. Insect damage was also associated with dieback symptoms in the field, while anecdotal information suggested that climate and location were contributing factors. Laboratory and glasshouse testing of these isolates found that the most promising agents belonged to the genus *Botryosphaeria* which were able to both kill seedlings and induce dieback symptoms in juvenile trees. These preliminary studies have provided a firm platform for ongoing studies that seek to develop prickly acacia dieback into a management tool for use in the grazing industry.

Executive summary

Prickly acacia (*Acacia nilotica*) is a weed of national significance (WONS) which significantly impacts on the grazing industry across northern Australia. A dieback phenomenon has been reported to occur in some locations where this woody weed exists.

A key element of this research project was a field study conducted in July 2010, during which several locations between Julia Creek and Richmond in northern Queensland were visited to investigate and collect material from sites with prickly acacia infestations. Particular effort was made to locate sites where active dieback could be found, and to collect information and samples from sites where it had previously been active.

The field work phase of this study provided an opportunity to capture useful biological and anecdotal information about the dieback phenomenon. Although large-scale active dieback was not observed during the study, significant evidence was captured to provide an understanding of past historical events. Dieback in prickly acacia appears to be linked to climatic events, and may be influenced by site-specific factors such as soil type and drainage. It is also influenced by the activity of insect pests such as locusts, stem borers and twig girdlers.

A range of fungi were isolated from affected plants. Approximately 150 isolates were made from field collected material, of which 70% belonged to the genus *Botryosphaeria*.

Many of these fungi were found to be capable of killing seedlings under laboratory conditions, and some are also capable of causing significant infection and dieback symptoms under glasshouse conditions. The most effective isolates were those of the genus *Botryosphaeria*.

The study has therefore indicated significant potential for harnessing some of these fungal isolates as bioherbicides to induce dieback symptoms in healthy prickly acacia plants. Is has also provided a sound basis for ongoing studies on research questions associated with this disease model. These studies will seek a better understanding of the disease mechanism and will also conduct field trials using inoculation methods currently being used in the research of dieback in parkinsonia (*Parkinsonia aculeata*).

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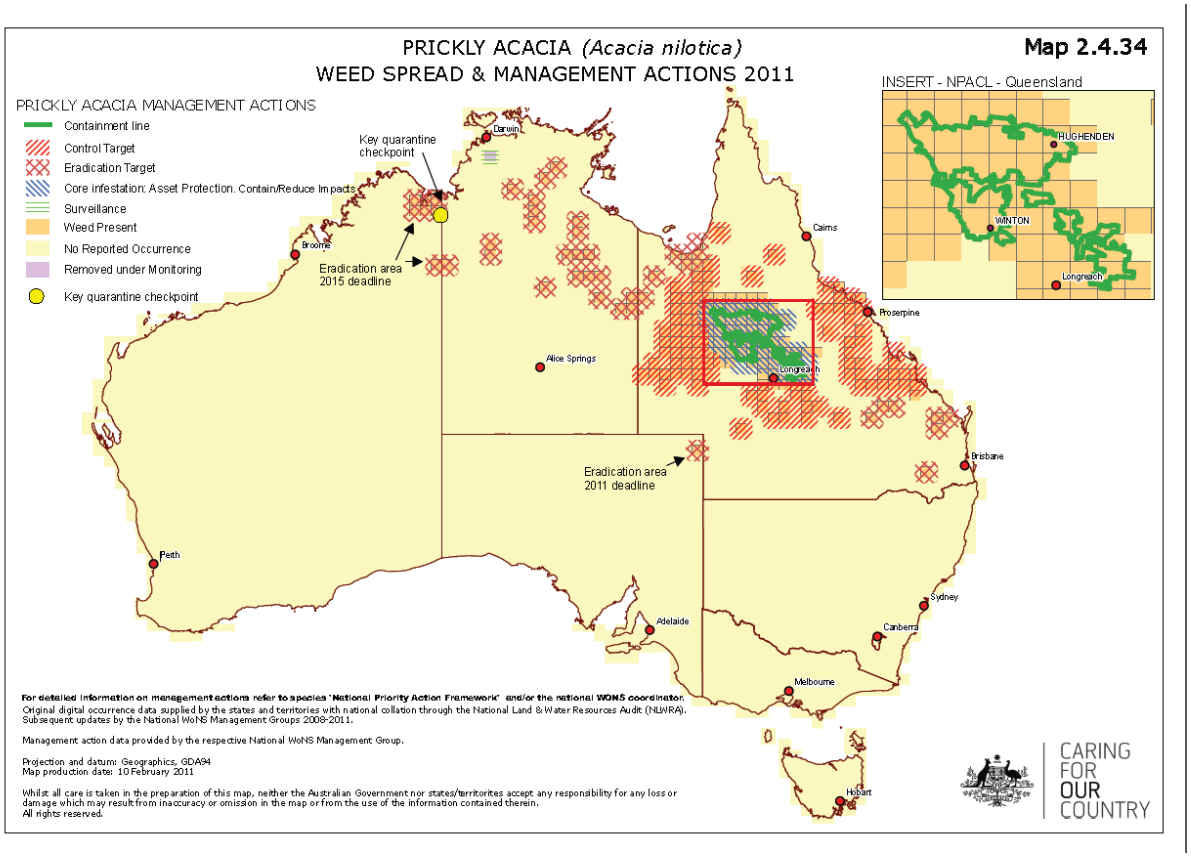
1. Background

Prickly acacia (*Acacia nilotica* ssp. *indica* (Benth.)) is one of the most harmful environmental weeds in Australia in terms of invasiveness, potential for spread and socio-economic and environmental impacts. It has ranked at seven under the category of *Weeds of National Significance* (Thorp & Lynch 2000). Upon introduction from the Indian Subcontinent in the late nineteenth century (Dhileepan et al. 2008 ; Wardill et al. 2005) it has already been established over vast areas of western Queensland including the Mitchell grass lands and coastal belts (Kriticos et al. 2006; March 2004). The core infestation of prickly acacia was estimated in 2004 to cover over 6 million hectares (March 2004). Under present climatic scenarios of Australia, it has the potential to invade a vast area of 50 million hectares including the Mitchell grass downs which is far greater than the current coverage (Kriticos et al. 2006; March 2004; DEEDI 2009). The map below indicates the current distribution and status of prickly acacia across northern Australia.



Current distribution of prickly acacia in Australia (National Land & Water Resources Audit (NLWRA) & National WoNS Management Groups 2011) http://www.weeds.org.au/WoNS/pricklyacacia/

The adverse environmental, ecological and economic effects broadly include competition with desirable grass resulting in reduced pasture production, increasing soil erosion, increase mustering costs, maintenance cost of bore drains and restricting stock movement, reduce access of stock to water and damage to vehicle tyres (March 2004; DEEDI 2009). It also clogs waterways, competitively utilises water from rivers, creeks and bore drains, obstructs native flora and uptakes nutrients from the soil (Sutherland 2011; DEEDI 2009). Besides ecological threats, it is also considered one of the most harmful weeds to Australian industries and economy. Each year it costs landholders nearly $4-9 million in terms of reduced beef and wool production, control costs and other difficulties (March 2004). Traditional control measures are often not practical or affordable given the extensive areas involved. For example, more than $200 million is required for its control in the Southern Gulf catchment only (Sutherland 2011).

Presently this weed is generally controlled by mechanical means (pulling, cutting etc.) and chemical (application of herbicides) methods (Parsons & Cuthbertson 2001; Spies & March 2004). Such control measures are often inadequate and often expensive as they have little impact on soil seed bank reduction and require repeated follow-up actions (Byrne & Ford 2004; Chris & Moloney 2004; Dhileepan et al. 2006; Magnussen 2004; Spies & Reddie 2004). Therefore, the classical bio-control approach is considered potentially the most effective, economic and sustainable option against this weed (Dhileepan et al. 2006).

Research seeking bio-control agents for prickly acacia was initiated in 1980's (Dhileepan et al. 2008 ; Lockett & Palmer 2003), and a number of promising insects believed to have potential for bio-control of prickly acacia were introduced into Australia (Mackey 1996; Senaratne et al. 2006). Only two of these agents established in the wild, but their performance in terms of seed bank reduction and uniformity of establishment were unsatisfactory (Dhileepan et al. 2008 ; Dhileepan et al. 2006; Palmer 1996; Palmer et al. 2007; Radford et al. 2001).

Due to poor success of insect oriented bio-control programs, other suitable options need to be investigated. Application of fungi as bio-control agents for this weed could be a valuable alternative. Exploitation of fungal dieback phenomenon of prickly acacia around north-western Queensland (Galea 2011; March 2009; DEEDI 2009) may provide an efficient, sustainable and cost-effective tool to control this noxious invader. Apart from a single report of dieback in prickly acacia in northwest India caused by the fungal pathogen *Botryodiplodia theobromae* (Dhileepan et al. 2010), there appears to be no other record of this kind of pehonomenom in this woody weed species..

However in a broader sense, much is known about fungal mediated dieback in woody plants. Among the different groups of fungi the members of the family Botryosphaeriaceae are reported to cause die back and canker in more than 100 genera of economically important woody trees and shrubs across the globe (Bush 2009; Mehl et al. 2010; Perez et al. 2010; Shah et al. 2010; Slippers et al. 2010; Vajna 2010; Begoude et al. 2011; Bertetti et al. 2011; Chen et al. 2011; Heath et al. 2011; McDonald & Eskalen 2011). Besides dieback and canker, Botryosphaeriaceae fungi are also reported to be associated with other disease symptom like leaf spot (Jayakumar et al. 2011). In Australia, Botryosphaeriaceae die back is generally observed in a wide range of perennial trees and shrubs like mango (Sakalidis et al. 2011), acacias (Wingfield et al. 2011), eucalypts (Pavlic et al. 2008; Slippers et al. 2009; Taylor et al. 2009), baobabs (Pavlic et al. 2008), peppermint (Dakin et al. 2010), grapevine (Qiu et al. 2008) and pine (Golzar & Burgess 2011). Often these diseases are linked to environmental stressors such as drought low soil fertility, soil compaction, loss off stopsoil or leaf litter, high temperatures and injury through insect attack (Nihlgard 1985; Gerrish et al. 1988; Tomlinson 1993; Davis et al. 2002; Jurskis & Turner 2002; Mills 2006; Ogburn & Alber 2006; Sinkkonen 2008; Mehl et al. 2010; Hoffmann et al. 2011). .

This research was therefore conducted to describe the process of dieback found in natural populations of this weed host as it occurs in Australia and to identify the fungal isolates with potential for bio-control of prickly acacia.

2. Project objectives

The project objectives were to:

- Survey populations of prickly acacia across northern Australia to locate natural occurrences of dieback
- Document, photograph and develop an understanding of the symptoms associated with dieback in prickly acacia
- Collect samples of dieback affected prickly acacia plants and conduct laboratory isolations of causative fungal agents
- Identify, to species level, the organisms associated with dieback affected plants
- Conduct glasshouse pathogenicity tests to select fungi with potential for further testing under field conditions.

3. Methodology

3.1 Field work

A field trip to Northern Queensland was conducted between 26th and 29thof July, 2010 to investigate a range of sites where Prickly acacia was located in relatively high density and where dieback may be active or have been known to have occurred historically. Samples collected from plants were taken as 20 cm stem lengths cut with a hand saw or pruning shears. In addition, at some sites drill shavings were taken using a cordless electric drill with a 10 mm drill bit. Field notes were made of observed symptoms and interview notes with station managers / owners were recorded.

Field notes:

Julia Creek DPI Reserve – This site is just north of the township of Julia Creek. Some prickly acacia plants were found showing signs of stress and there was evidence of plants re-shooting. Cut stems revealed ashy internal staining. Two sets of samples were collected JC01 and JC02.

Cross section of prickly acacia stem showing internal staining of wood

Garomna Station – manager, Nigel Simmons, provided evidence of past dieback events. Bore drains had also been treated with Diuron herbicide. Pushing and chaining of prickly acacia had also been done on parts of the property. Plants were showing signs of stress near bore drains and there was also insect damage evident on some plants. Cut stems did

not reveal significant internal staining of wood. Four samples sets taken (GS01, GS02, GS03 & GS04).

A line of prickly acacia trees on a bore drain with a dieback affected tree in the foreground – Garomna Station

Nelia Downs Station – manager, Robert Hacon. Trees on parts of this property were extremely stressed due to a combination of dry conditions and insect damage. Locusts were present in large numbers defoliating trees and causing bark wounds. Stem borers also were active. Significant stem staining was apparent, but localised to insect wound sites. Healthy (control) site was also investigated near a waterhole. Three sample sets taken (NDS01, NDS02 & NDS03).

Dieback affected prickly acacia tree on Nelia Downs Station on a severely water stressed site

Alick Creek Crossing – located on Punchbowl to Julia Creek Rd. Several trees by roadside showed striking dieback-like symptoms. Some trees had basal water shoots (suckers) indicating recovery after loss of main stem, a symptom typical of dieback in parkinsonia.

Prickly acacia plant showing removal of water shoots – Alick Creek site

Internal staining was apparent in some trees. The site contained a mixture of killed, semi-killed and apparently healthy prickly acacia trees. Stem and drill shavings were collected from 2 locations (AC01 & AC02).

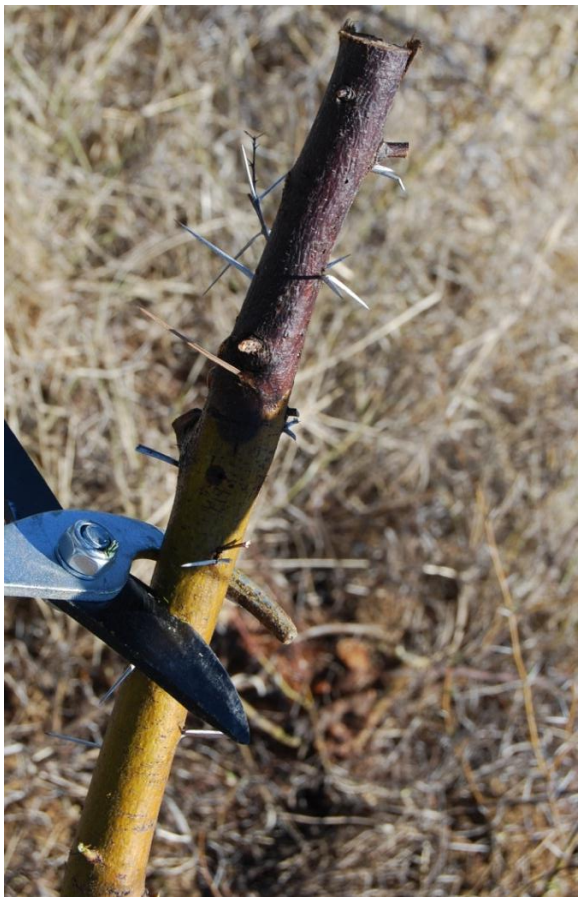
Use of cordless drill to remove samples of internal wood from a prickly acacia tree trunk – Alick Creek site

Lindfield Station – Location of a previous significant dieback event. A very large patch of prickly acacia appears to have been previously killed by dieback with little evidence of new plant recruitment at this site. Adult trees have possibly been dead for at least 5 years. At least 30 ha affected. Adjacent area has apparently healthy trees indicating a sharp demarcation between affected and un-affected zones. Possibly a drainage or soil type boundary. Dead trees appear to be in a basin. Internal wood of severely affected (but living) trees indicated staining. Stem and drilling samples were taken (MDS01).

Site of major dieback event at Lindfield Station.

Internal staining of wood from dieback affected prickly acacia at Lindfield Station

Proa Station – manager, Duncan Fysh. At this location, evidence of the relationship between insect damage and dieback in drought affected prickly acacia plants was apparent. Whole plants were excavated and dissected to show that shoot dieback could be associated with wounding caused by a combination of stem boring and twig girdling insects and fungal organisms. Blackened shoot tips were apparent in some plants.



Stem blackening as a result of localised dieback – Proa Station

Thorough investigation of dieback affected plants indicated that wood staining was localised to areas where insect damage was present. Observations from landholder were that dieback was associated with drought events. Possible explanation is that water stressed plants succumb to higher levels of insect damage predisposing them to fungal infections which kill off the plant. Samples were collected.

Wyangarie Station – David & Jane Carter. David grew up on this property, and has seen waves of dieback occur, often in conjunction with wet years. His observations are that dieback is more prevalent on low sites (heavier soils) and not apparent on ridge (stony) country.

Some plants near a bore drain were observed to have dieback symptoms. This drain had been treated with Diuron two years previously. Other plants close to the drain were un-affected. Plants were found to have stem lesions which did not appear to be caused by insect damage. Investigation below the surface indicates damage to the conductive tissues. Four sets of samples were collected.

Stem lesions on dieback affected plants near bore drain – Wyangarie Station

Internal staining of stem in dieback affected plant – Wyangarie Station

20 mile reserve – Richmond town common, Mark MacDonald, Richmond Shire Council. Observations by Mark MacDonald are that dieback is more prevalent in Flinders grass country (ashy soils) and not Mitchell grass country (heavy clays). Often found in depressions where water sits.

Investigation of dieback affected plants found that internal staining of wood is not systemic throughout whole plant, but limited to areas of insect damage. In one location, plants have produced water shoots as a response to dieback of main stem.

Samples were collected from three locations.

Significant area of dieback affected prickly acacia – 20 mile reserve

Toorak Station – formerly DEEDI research station. Trees in creek bed showed signs of severe dieback and partial recovery. Water shoots were apparent and samples were collected.

Prickly acacia stem with lateral damage to cambial layer showing internal staining of wood –Toorak Station.

3.2 Isolation of fungi from collected samples

149 isolates (Appendix 1) were recovered from dieback stem pieces collected from nine locations in North Queensland (Figure 1). Tissue fragments made by drilling into surface sterilised stem pieces with flame sterilised drill bits were collected in sterile Petri dishes before being aseptically transferred to either ½ strength Potato Dextrose Agar (1/2 PDA), Malt Extract Agar (MEA), V8 Juice Agar (V8A) or Oatmeal Agar (OA) plates. To inhibit bacterial growth the media were amended with Penicillin (Sigma®, Penicillin G sodium salt) at 0.12 g/ 400 ml (300 ppm) and Streptomycin (Sigma®, Streptomycin sulfate salt) at 0.08 g/ 400 ml (200 ppm) (Commonwealth Mycological Institute 1983; Waller et al. 1997). Plates were incubated at 25^{o}C and sub-cultures were made onto the same media. Only one sub-culture was taken from the initial isolation plates unless there were distinct morphological differences observed on a plate then each variant was sub-cultured.

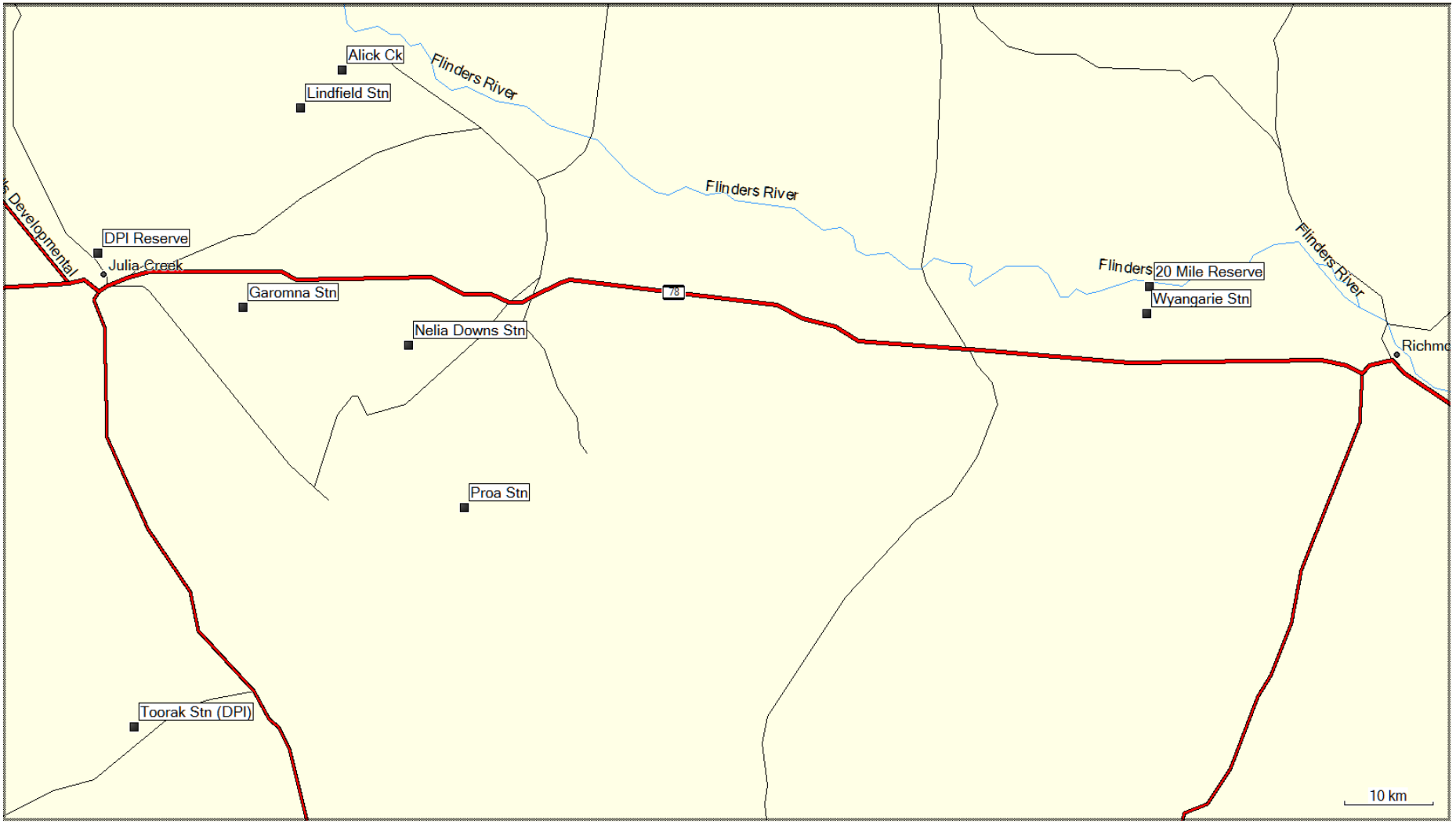


Figure 1 Location of the collecting sites in north Queensland.

3.3 Seedling assay

The seedling assay was conducted in the plant science laboratory of the University of Queensland during April to June 2011 following the method outlined by Toh (2009) and Toh et al. (2012 Submitted).

3.3.1. Collection and preparation of seeds

Prickly acacia seeds were removed from mature pods collected by Mr Nathan March (DEEDI) from Caerphilly Station near Charters Towers, QLD. Seeds were dried for 16 hours at 35^{o}C in a drying oven before being stored in an air-tight container at 4^{o}C.

3.3.2. Preparation of inoculum

Inoculum was also prepared according to the procedure outlined by Toh (2009) and Toh *et al* (2012 Submitted). French White millet (*Panicum milliaceaum*) seeds were rinsed twice, soaked for 24 hours in deionised water, rinsed again and approximately 10 mL transferred to 30mL plastic McCartney tubes. Tubes were autoclaved at 121^{o}C for 20 minutes and again for a second time 24 hours later. When the sub-cultured fungi had grown sufficiently, a 10mm x 10mm section of the culture was cut from the growing colony margin and transferred aseptically into a McCartney tube containing previously autoclaved millet. Fungi were grown within the sealed tubes at 25°C and shaken every 48hrs to achieve even distribution. When the substrate was totally colonized the inoculum was dried by replacing the tube cap with sterile tissue paper held in place by an elastic band and then placing the tubes over dried silica gel under laboratory vacuum for 48-72hrs. Dried tubes were resealed with the original caps and stored at 4°C before use. The negative control was un-inoculated autoclaved millet.

3.3.3. Seed pre-germination

Prickly acacia seeds were pre-germinated to ensure the utilization of viable and healthy seedlings in the subsequent experiments. At first, the required number of clean, insect free and uniform seeds was selected. As stated by Palani *et al* (1995) prickly acacia seeds generally have exogenous dormancy and don't germinate until triggered by water penetrating the hard seed coat. To facilitate water absorption and subsequent germination the selected seeds were clipped to remove the hard seed coat at the embryo end using a nail clipper. Within the sterile working area of a laminar flow cabinet, clipped seeds were soaked for 5 minutes in 2% Sodium hypochlorite (NaOCl) and washed repeatedly with sterile deionised water to remove any debris and traces of NaOCl. Seeds were then transferred to clean plastic trays each containing two pieces of moist sterile filter paper, covered with a lid

and incubated in a darkened incubator at 25°C which is the optimum temperature for germination of prickly acacia seed (Mackey 1998). Germination trays were checked and watered with sterile deionised water if necessary to ensure sufficient moisture was provided. After three to four days incubation, most seeds were germinated with the root radicle appearing and extending over half the length of the seed.

3.3.4. Isolate screening (seedling assay)

First stage laboratory screening

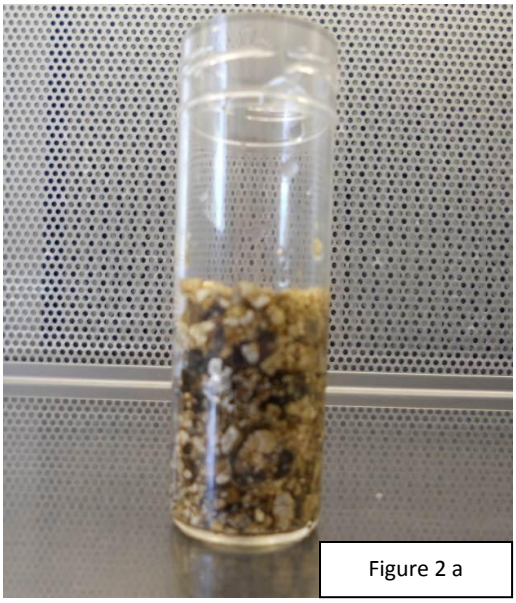
A preliminary screening of 149 isolates was conducted to identify aggressive isolates with high potential for use as bio-control agents for further study. Plastic McCartney tubes (80 mm-external height, 25 mm-internal diameter & with a 4 mm drainage hole in the base) were filled with 15mL vermiculite (Grade#3) and then autoclaved with sterile deionised water (Figure 2 a). Within the sterile working area of a laminar flow cabinet one pre-germinated prickly acacia seed was placed in each tube using sterile forceps (Figure 2 b) and manipulated to ensure the radicle pointed down. Inoculation was conducted by placing three colonised millet grains adjacent to the radicle of a pre-germinated seed which was then covered with around 10 mm of autoclaved vermiculite (Figure 2 c-d). After that the tubes were placed on a rack and sterile water was added to ensure sufficient moisture for seedling growth and re-activation of the dried inoculum. Excess water was drained out through the hole at the bottom of the tube. Afterwards the tubes were transferred to an incubation enclosure consisting of a black plastic screen over a laboratory bench with fluorescent lighting on a 12 hr day / 12 hr night cycle at 25^{o}C. Each isolate was replicated twice.

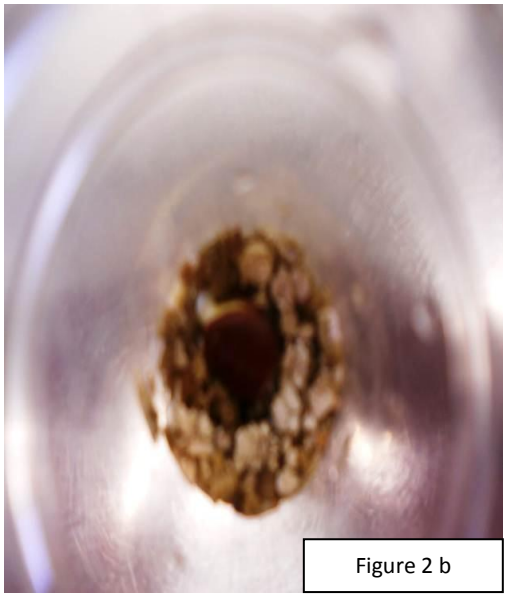
Second stage laboratory screening

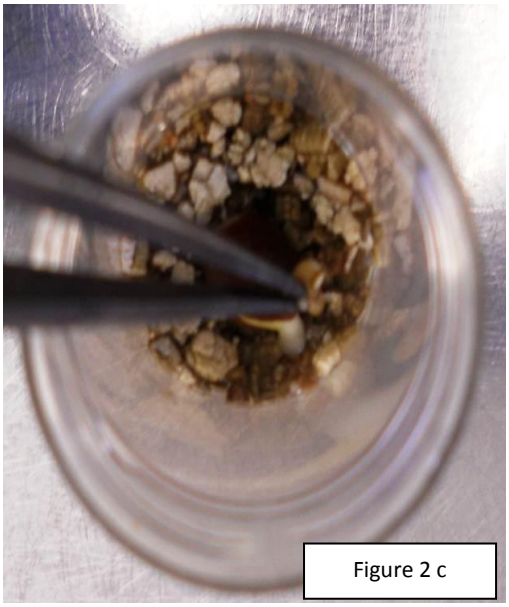
44 fungal isolates were screened for a second time with ten replications for each following the method described by Toh (2009) and Toh et al. (2011 submitted). Based on the result of preliminary screening and culture morphology, 41 isolates were selected from the pathogenic group and three isolates were selected randomly from the non-pathogenic group as control. The pre-germinated seeds were inoculated in McCartney tubes following the technique described above. The tubes were placed on elevated drainage mesh platforms (gutter guard glued to a 10mm thick PVC plastic ring) in transparent plastic incubation box (Décor®, 1.75 L containers with lids) with a 6 mm ventilation hole in each side. Each incubation box contained sixteen plants. Ten were treated with a single test isolate, three were positive controls (inoculated with isolate NT039 *Lasiodiplodia pseudotheobromae*) and three were negative controls (inoculated with sterile millet). Both positive and negative controls were consistently included in each incubation box as a check that experimental

conditions were appropriate for infection (positive control) and also appropriate for seedling growth. Background levels of infection due to natural contamination of prickly acacia seed would also be detected by the negative control treatment.

The incubation boxes were randomly arranged in an incubation chamber operating at 25°C for 16 days with daily 12-hour-light period and with watering every fourth day. Pathogenicity assessment was carried out on completion of the required incubation period.







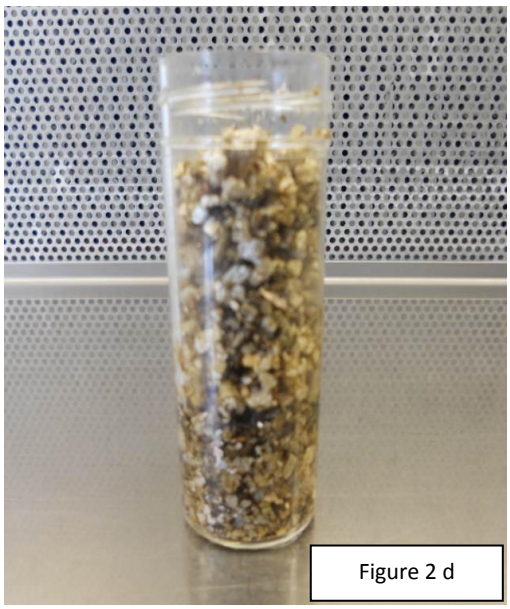


Figure 2 Inoculation technique in seedling assay, a: McCartney tubes with approx. 15 mL vermiculite, b: prickly acacia seed placed on vermiculite (radicle downward), c: inoculation with colonised millet grains and d: inoculated seed covered with vermiculite

Pathogenicity assessment

First stage laboratory screening

Preliminary screening for pathogenicity was done at fourteen days after inoculation. Seedlings were removed from the tubes and washed to remove vermiculite. The seedlings were then critically assessed and divided into two categories viz. diseased and healthy. Symptoms observed with different isolates were also recorded (Appendix 2).

Second stage laboratory screening

In the second stage of the seedling assay, pathogenicity assessment was carried out at sixteen days after inoculation. Similarly, seedlings were removed from the tubes and washed to remove vermiculite. Shoots and roots were examined separately using a more detailed disease scale. Shoots were classified into three categories A) non-emerged; B) emerged but showing disease symptoms; and C) emerged & symptomless (healthy). At the same time roots were classified into the following groups: A) no root; B) adventitious roots only; C) tap root only and D) tap root and secondary roots.

Cleaning and disinfection

After each experiment, equipment was generally rinsed with water, soaked in warm water containing Pyroneg detergent, rinsed again, air dried, sprayed with 5% NaOCl solution, rinsed with deionised water, and finally air dried. Remaining plant material was disposed of through the UQ Biological Waste Handling System (Biohazard Bins).

3.4 Glasshouse assay

The glasshouse assay was conducted during July to November 2011 in the glasshouse of the University of Queensland using one year old juvenile prickly acacia plants in pots.

3.4.1. Growing juvenile trees

Prickly acacia seeds were pre-germinated following the technique described earlier. Pre-germinated seeds were transferred to seedling trays containing standard potting media (UC mix) collected from the UQ Gatton nursery and placed in the glasshouse for two weeks with automatic watering. On 18 July 2010, each seedling was planted in 1L pots containing potting mix and sent to UQ Gatton nursery for growing on. On 29 July 2011, the pots containing the plants were moved from nursery and arranged in a glasshouse bay. Drip irrigation was provided three times a day for one minute. A total of 36 pots were used in this experiment.

3.4.2. Preparation of inoculum

Inocula of seven fungal isolates of various pathogenicity (chosen based on results of the previously described seedling assay) and another isolate NT039 (isolated from parkinsonia) were prepared following the procedure outlined earlier.

3.4.3. Experimental design and treatments:

The experiment consisted of 9 treatments with 4 replications following a randomized block design. The treatments were as described in Table 1 below.

Table 1 Treatments applied in glasshouse assay experiment

Treatment (Isolates)	**Relative aggressiveness (in seedling assay)**	
	Shoot infection	**Root Damage**
AN028	Very strong	Very strong
AN036	Very strong	Very strong
AN063	Weak	Moderate
AN108	Very strong	Very strong
AN110	Very strong	Very strong
AN122	Very strong	Very strong
AN123	Strong	Very strong
NT039 (Positive Control)	Very strong	Very strong
Autoclaved millet (Negative Control)	-	-

3.4.4. Inoculation

Plants were inoculated following the modified stem inoculation technique outlined by Diplock et al. (2006) and Wong (2008). Using an alcohol sterilized 4.5 mm drill bit mounted in a cordless drill, a 3-4 mm deep hole was made in the stem 10 cm above the soil level (Fig. 3 a-b). Using a sterilized forceps, five millet seeds from each fungal isolate were placed into the hole in each plant (Fig. 3 c). The inoculated wound was then capped with a sealant (Selleys™ No More Gaps Multipurpose Gap Filler - White, Fig. 3 d). The forceps and drill bit were sterilized by dipping in alcohol between each inoculation.

3.4.5. Disease evaluation

Individual plants placed in front of a white background were evaluated fortnightly based on eye-estimation of percentage leaf cover relative to a healthy (negative control) plant, leaf pigmentation, presence or absence of lesions and lesion length (if present). Leaf cover was evaluated using a 1-6 rating scale where 1 = 0-10 % leaf cover, 2 = 11-25 % leaf cover, 3 = 26-50 % leaf cover, 4 = 51-75 % leaf cover, 5 = 76-90 % leaf cover and 6 = 91-100 % leaf cover. Again, for evaluation of leaf pigmentation, a 1-5 scale was used where 1 = 100 % green, 2 = 75 % green, 3 = 50 % green, 4 = 25 % green and 5 = 0 % green. Presence and absence of lesion were marked by '+' and '-' respectively and length of lesions (when present) was measured in cm.

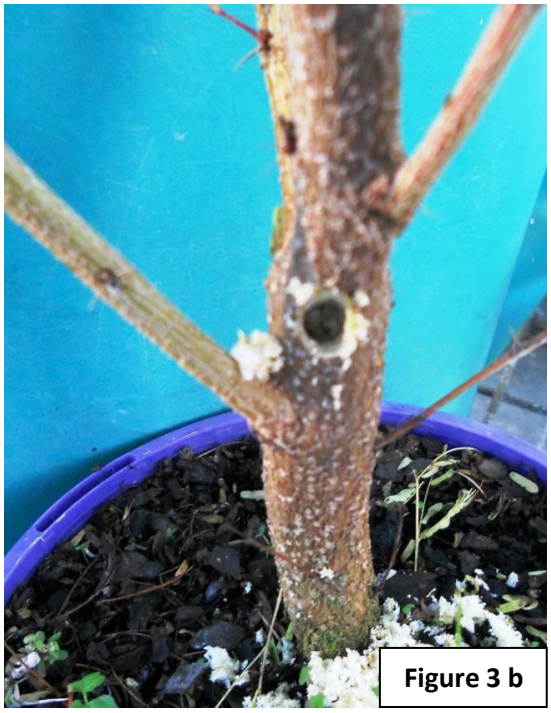


Figure 3 Inoculation of prickly acacia plants in the glasshouse, **a**: drilling the inoculation hole, **b**: inoculation hole, **c**: placing the inocula and **d**: the inoculation hole capped with sealant after inoculation.

3.5 Identification of fungal isolates

Pure isolates of fungi prepared from Prickly acacia stem and drilling materials collected from field work in northern Queensland were prepared for identification by growing on sterile V8 juice broth at 25^{o}C in until sufficient mycelia was produced.

Mycelial mats were then harvested, rinsed in sterile deionised water and placed in small plastic centrifuge tubes, placed in a freezer at -4^{o}C for at least 12 hours, then transferred to a freeze drying unit. Freeze dried fungal samples were then sent to Dr Andrew Bissett at CSIRO Plant Industry labs in Canberra for DNA Sequence Identification.

Genomic DNA was extracted from the isolates using Mo-Bio Ultraclean Microbial DNA Isolation Kit and the internal transcribed spacer (ITS) regions PCR amplified using fungal specific primers (Martin and Rygiewicz 2005). The ITS is the most widely sequenced DNA region for fungal identification providing the means to rapidly identify the target fungal endophytes. The amplified ITS fragments were then sequenced using standard cloning and sequencing methods. The DNA sequences were then compared with data on the BLAST (Basic Local Alignment Search Tool) database by Dr Ken Goulter to determine the identification of each isolate.

4. Results

4.1 First stage laboratory screening

Based on the incidence of disease symptoms in the preliminary screening the fungal isolates were grouped into two categories viz. pathogenic (developed symptoms in one or both seedlings) and non-pathogenic (didn't develop symptoms in any seedlings). Among the 149 isolates 54 isolates were found to be pathogenic and the rest were non-pathogenic to prickly acacia seedling (Table 2 and Appendix 2). Among these 54 pathogenic isolates 41 manifested differential culture morphologies and, therefore, were selected for the next stage of screening. Conversely, the culture morphology of AN005, AN009, AN011, AN012, AN014, AN021 AN025, AN059, AN077, AN096, AN113 and AN119 were common to at least one of the other pathogenic isolates so discarded. Isolate AN060 was lost due to contamination.

Table 2 Grouping of the isolates based on preliminary seedling assay

Isolates					
Pathogenic		**Non-pathogenic**			
AN005*	AN077*	AN001	AN041	AN085	AN126
AN009*	AN080	AN002	AN043	AN086	AN129
AN011*	AN083	AN003	AN044	AN087	AN132
AN012*	AN091	AN004	AN045	AN088	AN133
AN014*	AN092	AN006	AN046	AN089	AN135
AN021*	AN094	AN007	AN047	AN090	AN137
AN025*	AN096*	AN008	AN048	AN093	AN139
AN028	AN097	AN010	AN050	AN095	AN140
AN029	AN098	AN013	AN051	AN099	AN141
AN030	AN102	AN015	AN052	AN100	AN142
AN032	AN108	AN016	AN055	AN101	AN145
AN035	AN110	AN017	AN058	AN103	AN146
AN036	AN113*	AN018	AN061	AN104	AN148
AN042	AN119*	AN019	AN064	AN105	AN149
AN049	AN120	AN020	AN066	AN106	
AN053	AN122	AN022	AN067	AN107	
AN054	AN123	AN023	AN068	AN109	
AN056	AN127	AN024	AN069	AN111	
AN057	AN128	AN026	AN070	AN112	
AN059*	AN130	AN027	AN072	AN114	
AN060*	AN131	AN031	AN073	AN115	
AN062	AN134	AN033	AN074	AN116	
AN063	AN136	AN034	AN078	AN117	
AN065	AN138	AN037	AN079	AN118	
AN071	AN143	AN038	AN081	AN121	
AN075	AN144	AN039	AN082	AN124	
AN076	AN147	AN040	AN084	AN125	

* indicates the pathogenic isolates discarded prior to the second stage of screening

4.2 Second stage laboratory screening

In this stage, the fungal isolates were grouped into sixteen different categories based on their pathogenicity to shoots and roots (Table 3). A common rating scale was used to score incidence of foliar infection or root damage as follows: weak = 0-30 %, moderate= 31-50 %, strong = 51- 80 % and very strong = 81-100 %. Among the 44 isolates tested, 11 isolates namely AN028, AN036, AN042, AN049, AN053, AN071, AN108, AN110, AN114, AN120 and AN122 were found to have very strong pathogenicity toward both shoots and roots of prickly acacia seedlings. In contrast, seven isolates viz. AN076, AN083, AN092, AN098, AN134, AN138 and AN143 were classified as weak in terms of their pathogenicity to both shoots and roots of prickly acacia seedlings.

The remaining isolates manifested an intermediate range of pathogenicity from moderate to strong. General symptoms included non-emergence, severe leaf yellowing, development of necrotic spots on both upper and lower surface of the leaf, leaf tip necrosis, cankerous lesions in the collar region and stunted growth. Root development was also hampered by several isolates. Instead of a normal and healthy root system, a number of isolates resulted in various abnormalities such as the development of adventitious roots without any taproot, development of taproot lacking secondary roots etc. In addition, root development was completely hindered by many isolates. Brown to black root lesions were also caused by a few isolates. Incidence of shoot infection ranged from 10 to 100% and incidence of root damage ranged from 0 to 100% among different isolates (Appendix 3).

Table 3 Grouping of the isolates based on incidence of shoot infection and root damage in the seedling assay

		Shoot infection			
		Very Strong (81-100 %)	Strong (51- 80 %)	Moderate (31-50 %)	Weak (0-30 %)
Root damage	Very Strong (81-100 %)	AN028 AN036 AN042 AN049 AN053 AN071 AN108 AN110 AN114 AN120 AN122 NT039	AN123 AN127 AN128 AN131	-	AN130
	Strong (51- 80 %)	AN032 AN075	AN029 AN030 AN065 AN102	AN144 AN148	AN136
	Moderate (31-50 %)	-	AN035 AN078	AN097	AN147
	Weak (0-30 %)	AN054 AN057	AN080 AN091 AN094	AN056 AN062 AN063	AN076 AN083 AN092 AN098 AN134 AN138 AN143

4.3 Glasshouse assay

Table 4 represents the change in the health status of juvenile prickly acacia plants over a period of 56 days after inoculation (DAI). Regular defoliation resulting in gradual decrease of leaf cover was observed (Figure 4) in plants inoculated with all isolates. However, it was most prominent in plants inoculated with AN028, AN036, AN108, AN110, AN122 and NT039 (positive control). By contrast, the isolates AN063 and AN123 resulted in a little change of average leaf cover of the plants. At 14 DAI, a very small amount of defoliation (nearly 5%) was reported from the negative control but it remained unchanged over the period of observation.

Plants were also observed for change of leaf colour. Yellowing, leaf and twig necrosis were observed as general symptoms in the inoculated plants. With a few exceptions such as AN110 and AN122, the isolates didn't vary significantly in foliar discolouration caused, and the change was irregular.

No lesions or change in stem colour was observed around the inoculation point (Figure 5) until 28 DAI. After that period, sudden changes such as the initiation of dark brown lesions (Figure 6) and excretion of sap around the inoculation site (Figures 7, 8 & 9) were noticed in plants inoculated with isolates AN028, AN036, AN108, AN110, AN122 and NT039 (positive control). In contrast, the negative control and other two isolates viz. AN063 and AN123 didn't develop any lesions around the inoculation point (Figure 5).

Table 4 Health status of glasshouse grown prickly acacia plants recorded over a period of 56 days after inoculation with various fungal isolates.

Isolates	Leaf Cover (%)				Leaf Pigmentation (% green leaf)				Lesion Height (cm)			
	14 DAI	28 DAI	42DAI	56DAI	14 DAI	28 DAI	42DAI	56DAI	14 DAI	28 DAI	42DAI	56DAI
AN028	95.00	86.25	82.50	71.25	93.75	87.50	88.75	92.50	0.00	0.00	0.31	0.75
AN036	92.50	85.00	72.50	62.50	92.50	93.75	90.00	87.50	0.00	0.00	0.62	1.00
AN063	95.00	91.25	90.00	90.00	96.25	98.75	97.50	95.00	0.00	0.00	0.00	0.00
AN108	91.25	81.25	72.50	60.00	95.00	92.50	85.00	95.00	0.00	0.00	1.68	2.12
AN110	92.50	78.75	62.50	47.50	88.75	80.00	71.25	67.50	0.00	0.00	2.00	2.57
AN122	91.25	76.25	65.00	55.00	86.25	82.50	88.75	76.25	0.00	0.00	0.25	0.37
AN123	93.75	90.00	88.75	86.25	96.25	96.25	97.50	95.00	0.00	0.00	0.00	0.00
NT039	92.50	76.25	60.00	55.00	87.50	88.75	96.25	90.00	0.00	0.00	0.87	1.13
Negative control	95.00	95.00	95.00	95.00	100.00	100.00	100.00	100.00	0.00	0.00	0.00	0.00

* DAI = days after inoculation.

Figure 4 Visual comparison of overall appearance among negative control and two isolates. Left side, healthy plant; centre and right side, wilted and defoliated plants

Figure 5 Close view of inoculation point of negative control plant. Note lack of lesion at inoculation point (arrow)

Figure 6 Close view of inoculation point of plant inoculated with isolate AN108. Note brown lesion and sap extrusion at inoculation point (arrow)

Figure 7 Close view of inoculation point of plant inoculated with isolate AN110. Note blackened lesion and sap extrusion at inoculation point (arrow)

Figure 8 Close view of inoculation point of plant inoculated with isolate AN036. Note darkened lesion at inoculation point (arrow)

Figure 9 Close view of inoculation point of plant inoculated with isolate NT039 (positive control). Note extensive production of wound sap (arrow)

4.4 Identification of fungal isolates

Of the 150 isolates submitted, 125 were successfully identified to genus and or species level Table 5). Among these, 76 isolates were identified as *Botryosphaeria mamane*, and a further 12 as being in the same genus (70% of successfully identified samples belonged to this genus).

The remaining 30% of identified isolates were spread around 16 other species. *Pseudofusicoccum vioaceum* (of which there were 8 identifications) belongs to the same family as the genus *Botryosphaeria*.

Table 5 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data – summary of data in Appendix 4

Fungal Species name	No. of times isolated
Botryosphaeria mamane	76
Botryosphaeria sp.	12
Pseudofusicoccum vioaceum	8
Aureobasidium sp	5
Paecilomyces sinenis	4
Phaeobotryosphaeria citrigena	4
Asteromella pistaciarum	3
Pleurostoma ootheca	2
Rhytidhysteron rufulum	2
Alternaria alternata	1
Aspergillus niger	1
Cladosporium sp.	1
Cochliobolus lunata	1
Curvularia pseudorobusta	1
Cytospora sp.	1
Exserohilum sp.	1
Paecilomyces formosus	1
Pyrenochaetopsis microspora	1
Total	125

5. Discussion/Conclusion

The field work phase of this study provided an opportunity to capture both useful biological and anecdotal information about the dieback phenomenon in prickly acacia. Although large scale active dieback was not observed during this study, significant evidence was captured to provide an understanding of past historical events. Dieback in prickly acacia appears to be linked to climatic events, possibly influenced by site specific factors such as soil type and drainage. It is also influenced by the activity of insect pests such as locusts, stem borers and twig girdlers.

Detailed investigation of affected plants showed that a range of fungi can be isolated from such plants. Approximately 150 isolates were made from field collected material, of which 70% belonged to the genus *Botryosphaeria* and an additional 6% belonging to a different genus in the same family.

Many of these fungi were found to be capable of killing seedlings under laboratory conditions, and some are also capable of causing significant infection and dieback symptoms under glasshouse conditions. The most effective isolates were those of the genus *Botryosphaeria*. This is the same group of fungi implicated in the dieback process in parkinsonia (*Parkinsonia aculeata*) indicating potential similarities in the way this disease mechanism operates, and can potentially be harnessed for both species.

The outcomes of this research indicate that there is potential for harnessing some of these fungal isolates as bioherbicides to induce dieback symptoms in healthy prickly acacia plants.

These preliminary studies have provided a firm platform for ongoing studies that seek expand the research questions associated with this disease model. This work will utilise the culture bank to expand the range of laboratory and glasshouse studies to develop a better understanding of the disease mechanism. Field trials will also be conducted using inoculation methods currently being used in the research of dieback in parkinsonia (*Parkinsonia aculeata*).

Future directions for research on this disease system should specifically include:

- More detailed evaluation of a broader set of fungal isolates for their ability to infect prickly acacia plants under glasshouse conditions
- Establishment of major field trials to examine the effectiveness of selected isolates to infect prickly acacia in core infestation areas

- Sampling of prickly acacia plants from areas not covered in the original field survey for additional isolates associated with naturally occurring dieback
- Detailed genetic evaluation of isolates of *Botryosphaeria mamane* to improve understanding of this key group
- Collection of healthy prickly acacia plants for isolation and identification of other endophytic fungi which could also be associated with this woody weed species
- Further development of successful isolates with the view of producing a bioherbicide for control of prickly acacia.

6. Appendices

Appendix 1 List of isolates from prickly acacia (*Acacia nilotica* ssp. *indica*)

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN001 AN002 AN003 AN004 AN005	20_ mile Reserve	29/7/2010	#3	54K 0695632 7713945
AN006 AN007 AN008 AN009 AN010 AN011	Wyangarie Station	28/7/2010	#1	54K 0695246 7710925
AN012 AN013 AN014 AN015	20_ mile Reserve	29/7/2010	#1	54K 0695632 7713945
AN016 AN017 AN018 AN019 AN020 AN021 AN022 AN023 AN024 AN025 AN026 AN027	DPI Station	29/7/2010	#1	54K 0580533 766634

Appendix 1 List of isolates from prickly acacia (Acacia nilotica ssp. indica) - Continued

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN028 AN029 AN030 AN031 AN032 AN033 AN034 AN035 AN003	Wyangarie Station	28/7/2010	#3	54K 0695246 7710925
AN037 AN038 AN039 AN040 AN041 AN042 AN043 AN044 AN045 AN046		28/7/2010	#2	

Appendix 1 List of isolates from prickly acacia (Acacia nilotica ssp. indica) - Continued

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN047 AN048 AN049 AN050	Proa Station	28/7/2010	#3	54K 0617948 7690322
AN051 AN052 AN053 AN054 AN055 AN056 AN057 AN058 AN059 AN061			#2	54K 0617948 7690322
AN062			#4	
AN063 AN064 AN065 AN066 AN067 AN068 AN069 AN070 AN071 AN072	Lindfield Station	27/7/2010	#1	54K 0599749 7734448
AN073 AN074	Alick Creek Crossing		#3	54K 0604444 7738559

Appendix 1 List of isolates from prickly acacia (Acacia nilotica ssp. indica) - Continued

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN075 AN076 AN077 AN078 AN079 AN080 AN081 AN082 AN083 AN084 AN085 AN086	Garomna Station	27/7/2010	#1	54K 0593116 7712508
AN087 AN088	Nelia Downs Station		#2	54K 0611726 7708206
AN089 AN089 AN090 AN091 AN092 AN093 AN094	DPI Reserve	26/7/2010	#2	54K 0576728 7718519
AN095 AN096 AN097 AN098 AN099 AN100 AN101	DPI Reserve	26/7/2010	#2	54K 0576728 7718519

Appendix 1 List of isolates from prickly acacia (Acacia nilotica ssp. indica) - Continued

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN102 AN103 AN104 AN105 AN106 AN107 AN108	Garomna Station	27/7/2010	#3	54K 0593116 7712508
AN109 AN110 AN111 AN112 AN113 AN114 AN115 AN116 AN117	DPI Reserve	26/7/2010	#3	54K 0576728 7718519
AN118 AN119 AN120 AN121	DPI Station			54K 0580533 766634
AN122 AN123 AN124 AN125 AN126 AN127 AN128 AN129 AN130 AN131	20 mile Reserve	29/7/2010	#2*	54K 0695632 7713945
AN132	DPI Station			54K 0580533 766634
AN133	20_ mile Reserve			54K 0695632 7713945

Appendix 1 List of isolates from prickly acacia (Acacia nilotica ssp. indica) - Continued

Isolate	Site	Date Collected	Stem #	GPS Coordinates
AN134 AN135 AN136	DPI Station	29/7/2010	#1*	54K 0580533 766634
AN137 AN138 AN139 AN140	Alick Creek Crossing	27/7/2010	#2*	54K 0604444 7738559
AN141	20_mile Reserve	29/7/2010	#3*	54K 0695632 7713945
AN142	Proa Station	28/7/2010	#2*	54K 0617948 7690322
AN143 AN144	DPI Reserve	29/7/2010	#2*	54K 0576728 7718519
AN145 AN146 AN147 AN148			#1*	
AN149	Lindfield Station	27/7/2010	#1*	54K 0599749 7734448
AN150	Seed Contaminant UQ	05/7/2010		

Missing: AN060 was lost due to contamination

* represent cultures originating from field drillings made during the field survey and collected and stored in small zip-lock plastic bags.

Appendix 2 Preliminary screening of prickly acacia (*Acacia nilotica* ssp. *indica*) isolates

Isolates	Symptoms	
	Seedling#1	**Seedling#2**
AN001	Healthy	Healthy
AN002	Healthy	Healthy
AN003	Healthy	Healthy
AN004	Healthy	Healthy
AN005	Diseased (No roots)	Healthy
AN006	Healthy	Healthy
AN007	Healthy	Healthy
AN008	Healthy	Healthy
AN009	Healthy	Diseased (Hypocotyl rot)
AN010	Healthy	Healthy
AN011	Healthy	Diseased (Hypocotyl lesion)
AN012	Healthy	Diseased (No roots)
AN013	Healthy	Healthy
AN014	Healthy	Diseased (Hypocotyl rot)
AN015	Healthy	Healthy
AN016	Healthy	Healthy
AN017	Healthy	Healthy
AN018	Healthy	Healthy
AN019	Healthy	Healthy
AN020	Healthy	Healthy
AN021	Healthy	Diseased (No roots)
AN022	Healthy	Healthy
AN023	Healthy	Healthy
AN024	Healthy	Healthy
AN025	Diseased (Hypocotyl rot)	Healthy
AN026	Healthy	Healthy
AN027	Healthy	Healthy
AN028	Diseased (No roots)	Diseased (Weak roots)
AN029	Healthy	Diseased (Pre-emergent damping-off)
AN030	Diseased (No roots)	Diseased (No roots)
AN031	Healthy	Healthy
AN032	Healthy	Diseased (Pre-emergent damping-off)

Appendix 2 Preliminary screening of prickly acacia (*Acacia nilotica* ssp. *indica*) isolates - Continued

Isolates	Symptoms	
	Seedling#1	**Seedling#2**
AN033	Healthy	Healthy
AN034	Healthy	Healthy
AN035	Healthy	Diseased (Blackened roots)
AN036	Healthy	Diseased (Stunted, tap root lesion)
AN037	Healthy	Healthy
AN038	Healthy	Healthy
AN039	Healthy	Healthy
AN040	Healthy	Healthy
AN041	Healthy	Healthy
AN042	Healthy	Diseased (Dark taproot lesion)
AN043	Healthy	Healthy
AN044	Healthy	Healthy
AN045	Healthy	Healthy
AN046	Healthy	Healthy
AN047	Healthy	Healthy
AN048	Healthy	Healthy
AN049	Healthy	Diseased (Pre-emergent damping-off)
AN050	Healthy	Healthy
AN051	Healthy	Healthy
AN052	Healthy	Healthy
AN053	Diseased (Chlorotic shoot)	Diseased (Distorted seedling)
AN054	Healthy	Diseased (Pre-emergent damping-off)
AN055	Healthy	Healthy
AN056	Diseased (Stunted, no roots)	Diseased (Hypocotyl rot)
AN057	Diseased (No roots)	Diseased (No roots)
AN058	Healthy	Healthy
AN059	Diseased (Stunted, root lesion)	Healthy
AN061	Healthy	Healthy
AN062	Healthy	Diseased (Pre-emergent damping-off)
AN063	Healthy	Diseased (Stunted, no tap root)
AN064	Healthy	Healthy
AN065	Diseased (Dark tap root)	Diseased (Dark tap root)

Appendix 2 Preliminary screening of prickly acacia (*Acacia nilotica* ssp. *indica*) isolates - Continued

Isolates	**Symptoms**	
	Seedling#1	**Seedling#2**
AN066	Healthy	Healthy
AN067	Healthy	Healthy
AN068	Healthy	Healthy
AN069	Healthy	Healthy
AN070	Healthy	Healthy
AN071	Diseased (No roots)	Healthy
AN072	Healthy	Diseased (Hypocotyl rot)
AN073	Healthy	Healthy
AN074	Healthy	Healthy
AN075	Healthy	Diseased (Pre-emergent damping-off)
AN076	Diseased (No tap root)	Diseased (Stunted)
AN077	Healthy	Diseased (Small taproot lesion)
AN078	Healthy	Healthy
AN079	Healthy	Healthy
AN080	Diseased (Hypocotyl lesion)	Healthy
AN081	Healthy	Healthy
AN082	Healthy	Healthy
AN083	Diseased (Pre-emergent rot)	Diseased (Stunted)
AN084	Healthy	Healthy
AN085	Healthy	Healthy
AN086	Healthy	Healthy
AN087	Healthy	Healthy
AN088	Healthy	Healthy
AN089	Healthy	Healthy
AN089	Healthy	Healthy
AN090	Healthy	Healthy
AN091	Diseased (No roots)	Diseased (Pre-emergent damping-off)
AN092	Healthy	Diseased (Hypocotyl rot)
AN093	Healthy	Healthy
AN094	Diseased (Stunted)	Diseased (Stunted)
AN095	Healthy	Healthy
AN096	Diseased (No roots)	Healthy

Appendix 2 Preliminary screening of prickly acacia (*Acacia nilotica* ssp. *indica*) isolates - Continued

Isolates	**Symptoms**	
	Seedling#1	**Seedling#2**
AN097	Healthy	Healthy
AN098	Diseased (Taproot lesion)	Healthy
AN099	Healthy	Healthy
AN100	Healthy	Healthy
AN101	Healthy	Healthy
AN102	Diseased (No roots)	Diseased (No roots)
AN103	Healthy	Healthy
AN104	Healthy	Healthy
AN105	Healthy	Healthy
AN106	Healthy	Healthy
AN107	Healthy	Healthy
AN108	Diseased (Hypocotyl lesion)	Healthy
AN109	Healthy	Healthy
AN110	Healthy	Diseased (Pre-emergent damping-off)
AN111	Healthy	Healthy
AN112	Healthy	Healthy
AN113	Healthy	Diseased (Pre-emergent damping-off)
AN114	Healthy	Healthy
AN115	Healthy	Healthy
AN116	Healthy	Healthy
AN117	Healthy	Healthy
AN118	Healthy	Healthy
AN119	Healthy	Diseased (Pre-emergent damping-off)
AN120	Diseased (Stunted)	Diseased (Stunted)
AN121	Healthy	Healthy
AN122	Healthy	Diseased (Pre-emergent damping-off)
AN123	Healthy	Diseased (Stunted, cotyledon lesion)
AN124	Healthy	Healthy
AN125	Healthy	Healthy
AN126	Healthy	Healthy
AN127	Healthy	Diseased (Hypocotyl lesion)
AN128	Healthy	Diseased (Stunted, hypocotyl lesion)

Appendix 2 Preliminary screening of prickly acacia (*Acacia nilotica* ssp. *indica*) isolates - Continued

Isolates	**Symptoms**	
	Seedling#1	**Seedling#2**
AN129	Healthy	Healthy
AN130	Diseased (Weak plant)	Healthy
AN131	Diseased (Stunted)	Healthy
AN132	Healthy	Healthy
AN133	Healthy	Healthy
AN134	Diseased (Dark tap root)	Diseased (Hypocotyl lesion)
AN135	Healthy	Healthy
AN136	Diseased (Hypocotyl lesion)	Diseased (Stunted, hypocotyl lesion)
AN137	Healthy	Healthy
AN138	Healthy	Diseased (No roots, hypocotyl lesion)
AN139	Healthy	Healthy
AN140	Healthy	Healthy
AN141	Healthy	Healthy
AN142	Healthy	Healthy
AN143	Healthy	Diseased (Hypocotyl lesion)
AN144	Diseased (Stunted)	Healthy
AN145	Healthy	Healthy
AN146	Healthy	Healthy
AN147	Diseased (Hypocotyl root)	Healthy
AN148	Healthy	Healthy
AN149	Healthy	Healthy

Missing: AN060 was lost due to contamination

Appendix 3 Effect of selected fungal isolates on overall health of prickly acacia seedlings

Isolate	Shoot		Root	
	Diseased (%)	Healthy (%)	Diseased (%)	Healthy (%)
AN 28	100	0	100	0
AN 29	70	30	60	40
AN 30	70	30	70	30
AN 32	100	0	70	30
AN 35	60	40	40	60
AN 36	100	0	90	10
AN 42	100	0	90	10
AN 49	100	0	90	10
AN 53	100	0	100	0
AN 54	100	0	20	80
AN 56	50	50	10	90
AN 57	90	10	30	70
AN 62	50	50	20	80
AN 63	50	50	10	90
AN 65	80	20	80	20
AN 71	90	10	90	10
AN 75	100	0	70	30
AN 76	20	80	0	100
AN 78	70	30	40	60
AN 80	60	40	10	90
AN 83	20	80	10	90
AN 91	70	30	10	90
AN 92	30	70	30	70
AN 94	60	40	30	70
AN 97	50	50	50	50
AN 98	30	70	30	70
AN 102	80	20	80	20
AN 108	100	0	100	0

Appendix 3 Effect of selected fungal isolates on overall health of prickly acacia seedlings

Isolate	Shoot		Root	
	Diseased (%)	Healthy (%)	Diseased (%)	Healthy (%)
AN 110	100	0	100	0
AN 114	100	0	100	0
AN 120	100	0	100	0
AN 122	100	0	100	0
AN 123	80	20	100	0
AN 127	80	20	100	0
AN 128	60	40	100	0
AN 130	30	70	90	10
AN 131	80	20	100	0
AN 134	30	70	30	70
AN 136	10	90	60	40
AN 138	10	90	10	90
AN 143	20	80	20	80
AN 144	40	60	60	40
AN 147	30	70	40	60
AN 148	50	50	80	20
Control (-)	0	100	0	100
Control (+)	100	0	100	0

Appendix 4 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data

Isolate #	Anamorph	Telomorph
AN001		*Botryosphaeria mamane*
AN002		*Botryosphaeria mamane*
AN003	*Scytalidium sp*	*Aureobasidium* sp
AN004		*Botryosphaeria mamane*
AN005		*Botryosphaeria mamane*
AN006		*Botryosphaeria mamane*
AN007		*Botryosphaeria mamane*
AN008		*Botryosphaeria mamane*
AN009		*Botryosphaeria mamane*
AN010		*Botryosphaeria mamane*
AN011		*Botryosphaeria mamane*
AN012	*Asteromella pistaciarum*	
AN013		
AN014	*Asteromella pistaciarum*	
AN015	*Asteromella pistaciarum*	
AN016		*Botryosphaeria* sp.
AN017		*Botryosphaeria mamane*
AN018		*Botryosphaeria mamane*
AN019		*Botryosphaeria* sp.
AN020		*Botryosphaeria mamane*
AN021		*Botryosphaeria mamane*
AN022		*Botryosphaeria mamane*
AN023		*Botryosphaeria mamane*
AN024		*Botryosphaeria mamane*
AN025		*Botryosphaeria mamane*
AN026		*Botryosphaeria sp.*
AN027		
AN028		
AN029		
AN030		*Botryosphaeria mamane*

Appendix 4 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data

Isolate #	Anamorph	Telomorph
AN031	Paecilomyces formosus	
AN032		*Botryosphaeria mamane*
AN033		*Botryosphaeria mamane*
AN034		
AN035		*Botryosphaeria mamane*
AN036		
AN037		*Botryosphaeria mamane*
AN038		*Botryosphaeria mamane*
AN039		
AN040		*Botryosphaeria mamane*
AN041		*Botryosphaeria mamane*
AN042		*Botryosphaeria* sp.
AN043		*Botryosphaeria mamane*
AN044		*Botryosphaeria* sp.
AN045		*Botryosphaeria mamane*
AN046		*Botryosphaeria mamane*
AN047		
AN048	*Pseudofusicoccum vioaceum*	
AN049		*Botryosphaeria mamane*
AN050		*Botryosphaeria mamane*
AN051	*Pseudofusicoccum vioaceum*	
AN052	*Pseudofusicoccum vioaceum*	
AN053		*Botryosphaeria mamane*
AN054	*Pseudofusicoccum vioaceum*	
AN055	*Pseudofusicoccum vioaceum*	
AN056		
AN057	*Pseudofusicoccum vioaceum*	
AN058	*Pseudofusicoccum vioaceum*	
AN059		
AN061	*Pseudofusicoccum vioaceum*	
AN062		

Appendix 4 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data

Isolate #	Anamorph	Telomorph
AN063	*Cladosporium* sp.	
AN064		*Botryosphaeria mamane*
AN065		*Botryosphaeria* sp.
AN066		Unidentified ascomycete endophyte
AN067		*Botryosphaeria mamane*
AN068		*Botryosphaeria* sp.
AN069		*Botryosphaeria mamane*
AN070		*Botryosphaeria mamane*
AN071		*Botryosphaeria mamane*
AN072		*Botryosphaeria mamane*
AN073		Unidentified ascomycete endophyte
AN074		
AN075		*Botryosphaeria* sp.
AN076		*Botryosphaeria* sp.
AN077		*Botryosphaeria mamane*
AN078		*Botryosphaeria mamane*
AN079		*Botryosphaeria mamane*
AN080		*Botryosphaeria mamane*
AN081		*Botryosphaeria mamane*
AN082		*Botryosphaeria mamane*
AN083		*Botryosphaeria mamane*
AN084		*Botryosphaeria mamane*
AN085		*Botryosphaeria mamane*
AN086		*Botryosphaeria mamane*
AN087		*Botryosphaeria mamane*
AN088		*Botryosphaeria mamane*
AN089		*Botryosphaeria mamane*
AN090		*Botryosphaeria mamane*
AN091		*Botryosphaeria mamane*
AN092		*Botryosphaeria mamane*

Appendix 4 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data

Isolate #	Anamorph	Telomorph
AN093		*Botryosphaeria* sp.
AN094		*Botryosphaeria mamane*
AN095		*Botryosphaeria mamane*
AN096		*Botryosphaeria mamane*
AN097		*Botryosphaeria mamane*
AN098		*Botryosphaeria mamane*
AN099		*Botryosphaeria mamane*
AN100		*Botryosphaeria mamane*
AN101		*Rhytidhysteron rufulum*
AN102		*Botryosphaeria mamane*
AN103		*Botryosphaeria mamane*
AN104		
AN105		*Botryosphaeria mamane*
AN106		*Botryosphaeria mamane*
AN107		*Botryosphaeria mamane*
AN108		*Botryosphaeria mamane*
AN109		*Botryosphaeria* sp.
AN110		*Botryosphaeria* sp.
AN111		*Botryosphaeria mamane*
AN112		*Botryosphaeria mamane*
AN113		*Botryosphaeria mamane*
AN114		*Botryosphaeria mamane*
AN115		*Botryosphaeria mamane*
AN116		*Botryosphaeria mamane*
AN117		*Botryosphaeria mamane*
AN118	Curvularia lunata	*Cochliobolus lunata*
AN119		*Botryosphaeria mamane*
AN120	Exserohilum sp.	
AN121	*Rhizopus oryzae* (probable contaminant)	
AN122	*Alternaria alternata*	
AN123	*Paecilomyces sinenis*	
AN124	*Curvularia pseudorobusta*	*Cochliobolus* sp

Appendix 4 Identification of fungal isolates by Anamorph (asexual form) and or Telomorph (sexual form) based on DNA analysis data

Isolate #	Anamorph	Telomorph
AN125	*Paecilomyces sinensis*	
AN126		
AN127	*Paecilomyces sinensis*	
AN128	*Scytalidium* sp	*Aureobasidium* sp
AN129	*Scytalidium* sp	*Aureobasidium* sp
AN130	*Scytalidium* sp	*Aureobasidium* sp
AN131	*Paecilomyces sinenis*	
AN132		
AN133		
AN134		
AN135	*Pleurostomomorpha ootheca*	*Pleurostoma ootheca*
AN136	*Pleurostomomorpha ootheca*	*Pleurostoma ootheca*
AN137	*Scytalidium* sp	*Aureobasidium* sp
AN138		*Botryosphaeria mamane*
AN139		
AN140	*Paecilomyces sinenis*	
AN141		*Rhytidhysteron rufulum*
AN142		
AN143	*Scytalidium* sp	*Aureobasidium* sp
AN144	*Pyrenochaetopsis microspora*	
AN145	*Cytospora* sp.	
AN146		*Phaeobotryosphaeria citrigena*
AN147		*Phaeobotryosphaeria citrigena*
AN148		*Phaeobotryosphaeria citrigena*
AN149		*Phaeobotryosphaeria citrigena*
AN150	Aspergillus niger	

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