

## A preliminary investigation of prickly acacia dieback (*Acacia nilotica* ssp. *indica*)

Ahsanul Haque<sup>1</sup>, Victor Galea<sup>1</sup>, Ken Goulter<sup>1</sup>, Andrew Bissett<sup>2</sup> and Riëks D. van Klinken<sup>3</sup>

<sup>1</sup> School of Agriculture and Food Sciences, The University of Queensland, Gatton, Qld 4343, Australia

<sup>2</sup> CSIRO Plant Industry, Clunies Ross Street, Black Mountain Laboratories, Acton ACT 2610, Australia

<sup>3</sup> CSIRO Ecosystem Sciences, PO Box 2583, Brisbane Qld 4001, Australia

(ahsanul.haque@uqconnect.edu.au)

**Summary** Prickly acacia (Mimosaceae: *Acacia nilotica* ssp. *indica* (Benth.) Brenan) is one of the most invasive rangeland weeds in Australia, annually causing multi-million dollar losses to grazing industries. Recently, a dieback symptom has been reported from many sites in north Queensland. There are indications that this dieback phenomenon can be potentially incorporated into management strategies for this noxious weed. This investigation aimed to collect samples of dieback-affected prickly acacia plants, conduct laboratory isolations of putative causative fungal agents, identify the organisms to species level, conduct glasshouse pathogenicity tests to understand the disease symptoms and select the fungi with potential for further testing under field conditions. Different species of fungi were isolated from dieback affected stem samples of prickly acacia and *Botryosphaeria mamane* was supposed to be the causative one.

**Keywords** Dieback, prickly acacia, biological control, fungi.

### INTRODUCTION

Dieback of trees is a global phenomenon that is also observed on numerous species in many parts of Australia. Of the various causes of dieback, fungi are reported to be associated with a number of events (Taylor *et al.* 2009, Dakin *et al.* 2010, Sakalidis *et al.* 2011). Fungi were also found to be implicated in the dieback of several of Australia's worst weeds, such as mimosa (*Mimosa pigra*), parkinsonia (*Parkinsonia aculeata*), and European blackberry (*Rubus* spp.) (Wilson and Pitkethley 1992 and Diplock *et al.* 2006). At present dieback is the major limiting factor to the spread of parkinsonia (*Parkinsonia aculeata*) (van Klinken *et al.* 2009).

Prickly acacia (*Acacia nilotica* ssp. *indica*), another invasive weed of Australian rangelands, has been reported by landholders to display a naturally occurring dieback phenomenon in north Queensland (Galea 2010, unpublished data). We hypothesise that fungi are playing a key role in this process, based on the observed symptoms on prickly acacia and results from studies on other rangeland weeds (Wilson and

Pitkethley 1992, Diplock *et al.* 2006, Toh *et al.* 2008). If this is true, then it may offer novel ways of managing this species, and they could offer an exciting potential for biological control of many invasive weed species including prickly acacia.

In this paper, we present preliminary results on the identification of fungal isolates extracted from dieback-affected stem samples and on laboratory and glasshouse pathogenicity trials with selected isolates.

### MATERIALS AND METHODS

**Sample collection** Stem samples were collected from nine locations in North Queensland by selectively sampling dieback affected trees in July 2010 showing symptoms ranging from stem lesions, partial crown death through to widespread death of plant populations.

**Isolate acquisition** Wood shavings were sampled by drilling into surface sterilised stem pieces with a flame sterilised drill bit. These shavings were collected into sterile Petri dishes before being aseptically transferred to either ½ Potato Dextrose Agar (½PDA), Malt Extract Agar (MEA), V8 Juice Agar (V8A) or Oatmeal Agar (OA) plates amended with Penicillin (Sigma<sup>®</sup>, Penicillin G sodium salt) at 0.12 g 400 mL<sup>-1</sup> (300 ppm) and Streptomycin (Sigma<sup>®</sup>, Streptomycin sulfate salt) at 0.08 g 400 mL<sup>-1</sup> (200 ppm) (Waller 2002).

Plates were incubated at 25°C and sub-cultured 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 and then each variant was sub-cultured. All isolates were stored at 4°C.

**Identification** Isolates were grown in 1 mL clarified V8 Juice broth in wells of 24 well (Falcon<sup>®</sup> 3047) plates, rinsed with sterile water, transferred to sterile 1.5 mL Eppendorf tubes and lyophilised. Genomic DNA (gDNA) was extracted from small pieces of dry tissue using the UltraClean<sup>®</sup>-htp 96 well microbial DNA isolation kit (Mo Bio Laboratories Inc. viewed 2012).

Extracted gDNA was aliquoted to 96 well PCR plates and the ITS region amplified using primers ITS1 (tggtcatttaggaagtaa) and ITS4 (tctccgcttattgatgc) (White *et al.* 1990). ITS amplicons were purified from PCR mixtures using the Agencourt Ampure Xp system (Beckman Coulter Genomics viewed 2012) and were Sanger sequenced by Macrogen (Korea) in one direction using the ITS1 primer. Regions of good sequence were retrieved and compared to the non-redundant Gen-Bank sequence database using the BLAST algorithm.

**Experimental design and selection of isolates for glasshouse trials** In the first glasshouse trial, eight isolates of *Botryosphaeria mamane* originating from different dieback sites were tested for pathogenicity to prickly acacia juveniles using a randomised complete block design with six replicates.

In the second trial, two isolates of each of *Pseudofusicoccum vioaceum*, *Paecilomyces sinensis*, *Aureobasidium* sp. and *Phaeobotryosphaeria citrigena* were tested following a similar design with five replicates.

In both trials, an isolate of *Lasiodiplodia pseudotheobromae* NT039, previously isolated from parkinsonia and shown to be highly pathogenic to parkinsonia seedlings (Toh *et al.* 2008) and more recently to prickly acacia seedlings in another part of this study, was used as a positive control. The test isolates were selected based on a preliminary seedling assay (data not presented here), which was conducted following the technique outlined by Toh *et al.* (2008).

**Preparation of inoculum** Inocula were prepared following the technique outlined by Toh *et al.* (2008). French white millet seeds were processed by rinsing twice in water, followed by soaking for 24 hours in RO water and subsequent rinsing. Approximately 10 cc millet grain were transferred to 30 mL plastic McCartney tubes. Tubes were autoclaved twice (24 hours apart) at 121°C for 20 minutes. A 10 mm × 10 mm section of the fungal culture was cut from the growing colony margin and transferred aseptically into a McCartney tube containing previously autoclaved millet. The tubes were sealed with Parafilm® and incubated at 25°C in a dark incubator with gentle shaking at every 72 hours to achieve even colonisation. Once the substrate was fully colonised, the tubes were stored at 4°C before use. Un-inoculated autoclaved millet was used as the negative control.

Before inoculation of plants, the inocula were tested for their viability. Four colonised millet grains of each isolate were aseptically transferred to four corners of a ½PDA plate and incubated at 25°C. After required incubation the plates were critically observed

for inocula viability and purity. The negative controls were also checked in the same way.

**Inoculation** Glasshouse-grown juvenile plants were stem inoculated using an alcohol sterilised 3.5 mm drill bit mounted in a cordless drill to produce a 3–4 mm deep hole at 10 cm above soil level. Using sterilised forceps two colonised millet grains were placed into each hole. After inoculation the wound was sealed with Parafilm®. Six month old plants were used in the first trial, whereas seven month old plants were used in the second trial.

**Pathogenicity assessment** Pathogenicity was assessed by measuring the length (cm) of the visible stem lesion at 14 day intervals. After completion of the experiment the plants were harvested and the stems vertically dissected using a sharp knife to observe and photograph the internal lesions.

**Confirmation of infection** Confirmation of infection was achieved by isolating fungi from the harvested plants and comparing them to the original cultures used for their inoculation. The re-isolation was done by modifying the technique outlined by Diplock *et al.* (2006). Two plants from each treatment were randomly selected. A 5 mm piece of stem was excised from infected stem of each plant using a flame sterilised scalpel, and the bark was removed. Stem pieces were then soaked in 2% NaOCl for 2 minutes followed by rinsing in sterile water twice for 3 minutes. The segments were then dried using sterile blotting paper and aseptically transferred to ½ strength PDA media followed by incubation at 25°C. The plates were observed daily and after required incubation (3–4 days) the isolates were purified by subculturing on fresh ½PDA plates before comparing to original isolates.

**Data analysis** Collected data were analysed using Minitab version 16 and treatment means were compared using F-test.

## RESULTS

**Isolation and identification** A set of 149 fungal isolates were prepared from die-back affected stem samples of prickly acacia, of which 126 isolates were successfully sequenced and have been putatively identified by ITS comparisons.

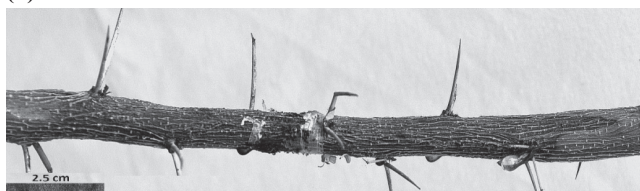
*Botryosphaeria* was found to be the most prevalent genus with 70% of the identified isolates belonging to this genus. Among these, 76 isolates were identified as *Botryosphaeria mamane* and 12 as *Botryosphaeria* sp. The remaining identified isolates belonged to 15 species: *Pseudofusicoccum vioaceum* (8 isolates),

*Scytalidium* sp. (6 isolates), *Paecilomyces sinensis* (5 isolates), *Phaeobotryosphaeria citrigena* (4 isolates), *Asteromella pistaciarium* (3 isolates), *Pleurostomomorpha ootheca* (2 isolates), *Rhytidhysterium rufulum* (2 isolates), *Cladosporium* sp. (1 isolate), *Cytospora* sp. (1 isolate), *Pyrenochaetopsis microspora* (1 isolate), *Exserohilum* sp. (1 isolate), *Alternaria alternata* (1 isolate), *Curvularia pseudorobusta* (1 isolate), *Curvularia lunata* (1 isolate), *Paecilomyces formosus* (1 isolate). An additional two isolates were unidentified ascomycete endophytes. Botryosphaeriaceae was the best represented family (*Botryosphaeria* spp., *Pseudofusicoccum viaaceum*, *Scytalidium* and *Phaeobotryosphaeria*).

(a)



(b)



**Figure 1.** Stem lesions observed on *Acacia nilotica* stems inoculated with one isolate of *Botryosphaeria mamane* (a) and *Lasiodiplodia pseudotheobromae* (b).

**Glasshouse trial** In the first trial, all the test isolates were found to produce black to brownish, sunken and necrotic stem lesions spreading in both directions from the inoculation point (Figure 1). Other dieback symptoms such as crown desiccation, defoliation, leaf necrosis, extensive internal staining, exudation of plant sap and plant death were also observed. Test fungi were successfully re-isolated from the inoculated plants. No symptom was recorded following inoculation with the negative control.

Lesions were evident for all isolates by week two (Table 1). Lesion elongation slowed after week two. At the same time plants started to exude sap through the inoculation point, which indicates activation of the plant's response to infection. Extension of lesion

length had almost ceased by the sixth week. At eight weeks after inoculation, stem lesion length was greatest for *Lasiodiplodia pseudotheobromae*. Significant differences in lesion lengths were also apparent among isolates of *Botryosphaeria mamane*. Negative controls did not produce lesions. In the second trial, no disease symptom was recorded in plants inoculated with isolates of *Pseudofusicoccum viaaceum*, *Paecilomyces sinensis*, *Aureobasidium* sp. and *Phaeobotryosphaeria citrigena*. However, the isolate of *Lasiodiplodia pseudotheobromae* (positive control) developed significant stem lesions and other dieback symptoms as observed in the first trial. At eight weeks after inoculation the average length of stem lesion with *Lasiodiplodia pseudotheobromae* was 10.68 cm.

**Table 1.** Length of stem lesion recorded following inoculation by various isolates of *Botryosphaeria mamane* collected across north Queensland. Means followed by the same letter are statistically identical at 5% level of probability by F-test.

Treatments (Isolates)	Lesion length (cm)			
	2 Weeks	4 Weeks	6 Weeks	8 Weeks
AN#4 ( <i>Botryosphaeria mamane</i> )	4.40 E	4.85 F	5.05 E	5.13 E
AN#17 ( <i>Botryosphaeria mamane</i> )	4.53 E	5.05 EF	5.16 E	5.20 E
AN#32 ( <i>Botryosphaeria mamane</i> )	6.41 BC	6.75 B	6.93 B	6.96 B
AN#49 ( <i>Botryosphaeria mamane</i> )	6.05 C	6.51 BC	6.70 BC	6.75 BC
AN#71 ( <i>Botryosphaeria mamane</i> )	6.75 B	7.23 B	7.37 B	7.41 B
AN#72 ( <i>Botryosphaeria mamane</i> )	6.28 BC	6.88 B	7.06 B	7.12 B
AN#108 ( <i>Botryosphaeria mamane</i> )	5.08 D	5.65 DE	5.73 DE	5.82 DE
AN#114 ( <i>Botryosphaeria mamane</i> )	5.30 D	5.83 CD	6.06 CD	6.08 CD
NT# 039 ( <i>Lasiodiplodia pseudotheobromae</i> )	8.23 A	9.83 A	11.20 A	11.70 A
Negative control (autoclaved millet)	0.00	0.00	0.00	0.00
Mean standard error	0.18	0.26	0.28	0.27

## DISCUSSION

From the above findings it is evident that several species of fungi could be implicated in prickly acacia dieback. However, our preliminary results suggest that *Botryosphaeria mamane* is the most likely to be the causative agent, as it was isolated from dieback affected trees in the field and could replicate similar symptoms in the glasshouse experiment. Based on sequence data, there may also be a second *Botryosphaeria* species involved, although its identification has yet to be resolved.

There was variation in stem lesion lengths induced by various *Botryosphaeria mamane* isolates. The presence of pathotypes would be of interest to the development of mycoherbicides. However, a genetic basis for these differences requires confirmation, as does differences in pathogenicity under field conditions.

*Lasiodiplodia pseudotheobromae* caused dieback symptoms in prickly acacia in our glasshouse trial. It has previously been shown to be highly pathogenic to *Parkinsonia aculeata* (unpublished data), but was not isolated from prickly acacia in our study. This is despite parkinsonia and prickly acacia co-occurring in north Queensland. The reason for this apparently restricted field host range requires further attention.

These initial results indicate that the test isolates *Botryosphaeria mamane* and *Lasiodiplodia pseudotheobromae* are pathogenic to prickly acacia plants. They may therefore be potentially utilised as a mycoherbicide for this noxious weed. The most pathogenic isolates are planned to be evaluated under field conditions.

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