Preliminary investigations of the *Mimosa pigra* dieback phenomenon

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Summary *Mimosa pigra* L. (Fabaceae), one of the most troublesome weeds in northern Australia, has been the target of biological control efforts for 30 years but still remains a serious problem. *M. pigra* is also host to a sporadic dieback phenomenon first reported in 1988 on the floodplains of the Adelaide River. This preliminary study aims to investigate the role of pathogens, insects and/or environmental conditions as possible contributing factors to *M. pigra* dieback in the Northern Territory. Understanding the underlying causes may help us design better management strategies. Laboratory isolations of fungi from diseased and healthy mimosa stems have resulted in 132 distinct isolates but have only tested 61 of them so far. Ten of these isolates were pathogenic towards mimosa seedlings in a laboratory trial. Five of these ten isolates were identified as *Lasiodiplodia theobromae* by DNA sequencing.

Keywords Dieback, *Mimosa pigra*, biological control, pathogens, *Lasiodiplodia theobromae.*

INTRODUCTION

Plants which appear to be suffering from ill health and death of growing points may be characterised as 'experiencing dieback'. Dieback is a condition often found in woody plants, and is associated with progressive death of stems, shoots, branches or roots often starting at the tips (Ostry *et al*. 2011). *Mimosa pigra* L. (Fabaceae) is a Weed of National Significance in Australia where it invades extensive wetland areas in the Top End of the Northern Territory (Thorpe and Lynch 2000). Widespread dieback of *M. pigra* has been reported in the Adelaide River floodplains (Northern Territory) since at least 1988 (Miller 1988). The fungus *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl. (Botryosphaeriaceae) has previously been isolated from typical dieback symptoms: reddish-brown lesions originating from leaf axils, yellowing of the leaves and subsequent stem death (Wilson and Pitkethley 1992). Significant patches of dieback in *M. pigra* continue to be observed in other parts of the Northern Territory including at sites in the Mary River and Daly River Regions (Natasha Burrows pers. comm.).

In this paper we present preliminary qualitative results of field studies and seedling pathogenicity trials of selected fungal isolates from diseased and healthy mimosa stems collected in the Northern Territory.

MATERIALS AND METHODS

Field data collection Fieldwork was conducted at the Melaleuca Station in the Mary River Region of the Northern Territory. Field data were gathered using three methods. First was an interview with the land manager to gather historical information on the presence of dieback on *M. pigra* across the region, second was the establishment of permanent plots to enable observations of plant demographic changes over time and to test whether pathogens, insects and/or environmental factors play a role in the dieback phenomenon, and lastly was the establishment of permanent transects to capture additional plots and to observe dieback through space.

Sixteen permanent plots were set-up throughout the *M. pigra* population. These were selected to represent different levels of dieback progression, from healthy populations that showed no symptoms, to populations where all juveniles and adults were dead but young seedlings were present. Within each plot, individual plants were rated for health using a ten-point plant scale. Soil samples collected in each quadrat were tested for pH and salinity. Two 100-metre long transects were set-up in parallel. Within each transect, a 5 × 5 metre plot was established at every 20 metre interval. The same measurements were recorded as the permanent plots.

Stem collection Stem samples were collected from two sites at the Melaleuca Station where *M. pigra* appeared healthy and free from dieback symptoms (12°38.49'S, 131°46.59'E) and one where *M. pigra* looked severely stressed and defoliated (12°26.21'S, 131°43.33E).

Fungal isolation Fifteen stem pieces (5 healthy and 10 dieback-affected stems) were used for the isolation process and cut, using a mitre saw, into 1 cm thick

sections. Under sterile conditions the stem pieces were surface sterilised in a 1.5% NaOCl solution for 2 minutes, rinsed twice in sterile water with stirring and dried between sheets of sterile paper towel. Three stem pieces were then aseptically placed onto either ½ strength potato dextrose agar (½ PDA; Difco) or acidified malt extract agar. Both were amended with penicillin G (200 µg mL^{-1}) and streptomycin (100 µg mL^{-1}). Plates were incubated at 25°C in the dark and observed regularly for growth. Subcultures were prepared from fungi growing out from the edge of the stem pieces.

Inoculum preparation French white millet (*Panicium milacium*) was rinsed twice and soaked in deionised water in a 1000 mL beaker for 24 hours at room temperature before being drained and rinsed again. The soaked millet was then transferred to McCartney tubes for autoclaving twice over 24 hours at 121°C for 25 minutes.

Agar blocks (10 mm × 10 mm) were cut from the growing margin of fresh subcultures and transferred to the prepared millet tubes. These were incubated at 25°C and shaken every 2 days to redistribute the grains and encourage even colonisation. The inoculum was dried once the fungus fully colonised the millet. The caps of the tubes were replaced with sterile tissue paper held in place with a rubber band. Drying was achieved by placing the tubes under vacuum in a desiccator containing silica gel for at least 48 hours. The caps were replaced and the samples were stored at 4°C until used.

Pathogenicity screening of isolates Preliminary screening using a 2-replicate trial was done to rapidly assess which of the 132 fungal isolates recovered from field-collected stems were pathogenic to mimosa seedlings and have the potential to be pathogenic to adult plants. Of the 61 isolates screened in the two-replicate seedling trials 26 showed potential for causing seedling mortality. Fourteen of these isolates were retested with 10 replicates and two negative controls.

For each isolate, one pre-germinated seed was planted into autoclaved vermiculite contained in a 30 mL plastic McCartney tube with a hole drilled in the base for drainage. Using sterile forceps, three grains of colonised millet were placed around the planted seed which was then covered with more sterile vermiculite. The same was done with the negative control except that sterile uncolonised grains were used. Tubes were then placed into plastic boxes (Décor) which were sealed and incubated in a constant temperature chamber at 25°C with a 14 h photoperiod. Seedlings were watered every 2 days and assessed qualitatively for disease symptoms every 4 days until 12 days after inoculation. A final assessment of seedling health was conducted at 14 days after inoculation.

Re-isolation of fungal isolates from diseased seedlings used in pathogenicity trials was performed to fulfill Koch's postulates. Approximately 5 mm sections were excised from diseased shoots. The procedure for surface sterilisation was the same as described in the stem isolation process. Shoot pieces were plated out on ½ PDA and incubated at 25°C for 7 days in the dark. Confirmation of pathogenicity was done by comparing the morphology of cultures of re-isolated fungi with that of the original isolates used in pathogenicity trials.

Identification of isolates Fungal isolates selected for DNA extraction were grown in 1 mL clarified V8 Juice broth in wells of 24 well plates (Falcon® 3047), rinsed with sterile water, transferred to sterile 1.5 mL eppendorf tubes and lyophilised. Genomic DNA was extracted using the UltraClean®-htp 96 well microbial DNA isolation kit. gDNA was aliquoted to 96 well PCR plates and the ITS region amplified using ITS1 and ITS4 (White *et al.* 1990). ITS amplicons were purified from PCR mixture using Agencourt Ampure Xp system (Beckman Coulter Genomics) and were Sanger sequenced by Macrogen (Korea) in one direction using the ITS primer. Sequences were examined, regions of good sequence retrieved and compared to the non-redundant GenBank sequence database using the BLAST algorithm.

RESULTS

Preliminary field observations The interview conducted with the land manager at Melaleuca Station indicated that dieback was first noticed there in 2007 (Tony Searle pers. comm.). It was specifically observed in an area called "the island", situated between Sampan Creek and Alligator Lagoon. It started off as patches but is now extensive. In 2008, the land manager stopped spraying *M. pigra* with herbicide because plants were sick and defoliated. Furthermore, he observed that the study area had an annual period of inundation of about 4 months to a depth of about 1 m.

All permanent plots were established on black cracking clay soil. The soil pH was lower in healthier *M. pigra* plots (4.14 ± 0.08 SE) than in the dieback-affected plots (4.93 ± 0.14 SE). Specific conductivity, as supported by ion analysis, indicated that there was only slight variation in the electrical conductivity (EC) values across all plots.

The phytophagous insects *Carmenta mimosa*, *Maroga* sp., *Platyomopsi*s sp. and *Neurostrota gunniella* were observed in all plots. Insect prevalence data

will be correlated with disease progression data to be collected in subsequent years to test whether herbivory is associated with the dieback phenomenon in anyway. *M. pigra* at healthy plots were predominantly rated as having a health class of 9; dieback plots have a health class of 7; recovering dieback plots have 3 and the dead plots have a health class of 0.

Pathogenicity screening of isolates A total of 284 fungal isolates was recovered from the diseased and healthy stems collected from Melaleuca Station. This number was reduced to 132 after grouping isolates with similar cultural morphologies. To date, a total of 61 isolates have been screened in the 2-replicate trials. Twenty-six (42%) of these isolates killed both inoculated seedlings within 14 days of inoculation. Of the 26 putatively pathogenic isolates, 14 have been re-screened in a 10 replicate assay. Of these, 10 isolates produced various proportions of seedling mortality and the remaining 4 did not kill any seedlings.

Disease symptoms observed on the inoculated seedlings were: presence of black necrotic spots and yellowing of the cotyledons, extensive necrotic tissues, and colonisation of the shoot and cotyledons by fungal mycelium. All controls in trials were healthy and symptomless.

Fifty percent of the fungal isolates used in the pathogenicity trials were from putatively healthy stems. Cultural morphology of the most aggressive fungal isolates on PDA plates was fluffy and whitish at the start of incubation, becoming brown to grey after 10 days. Molecular identification revealed that the five most aggressive isolates in the pathogenicity trials were *Lasiodiplodia theobromae*, including four isolates sourced from healthy stem pieces.

DISCUSSION

The results obtained from the seedling pathogenicity trials showed that *L. theobromae* can kill mimosa seedlings. *L. theobromae* commonly exists as an endophyte in healthy plant tissue (Punithalingam 1980), and was also previously identified as the likely causative agent of *M. pigra* dieback by Wilson and Pitkethley (1992). However, our isolation of this species from healthy *M. pigra* stems raises important questions regarding the factors that incite pathogenicity. We hope that our continuing quantitative studies will help determine the relative role of environmental conditions and feeding by phytophagous insects. In any case, this endophytic fungus may have the potential to be developed as a biocontrol agent. The use of endophytic fungi should be preferable to other biological agents as they are internal colonisers, and therefore compete within the vascular systems. As internal colonisers, their existence is buffered from environmental factors.

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