Control of *Mimosa pigra* by *Phloeospora mimosae-pigrae*: liquid culture production and application technique

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Abstract
Shake-flask liquid fermentation was investigated to mass-produce spores of the fungal pathogen, *Phloeospora mimosae-pigrae*, a potential biological control agent for the giant sensitive plant, *Mimosa pigra*. Twenty percent Campbell's V-8 juice in large shake flasks with a pH of 6.5 yielded 9.7 × 10^6 conidia mL^{-1} *in vitro* after ten days fermentation, producing ten times the required inoculum concentration of 1 × 10^6 for field applications. When sprayed using a backpack sprayer or from a helicopter, the inoculum successfully induced typical symptoms on plants under field conditions. The standardized production and application protocol for *P. mimosae-pigrae* inoculum can be used to treat large areas infested with this weed.

Introduction
Phloeospora mimosae-pigrae, an anamorph of *Sphaerulina mimosae-pigrae*, was the first fungal pathogen introduced to Australia as a potential biological control agent for *Mimosa pigra*, commonly known as mimosa or giant sensitive plant. In its centre of origin in Central and South America, this fungus causes canker on rachides and stems leading to premature leaf drop and dieback. *P. mimosae-pigrae* was introduced to Australia following evaluations of its effectiveness and host-specificity in the UK (Seier and Evans 1992, Seier 1998, Seier and Evans 1996, Evans *et al.* 1993).

Mimosa pigra, a woody perennial, reaches up to 6 m in height in northern Australia (Lonsdale 1992). In Central and South America, the native range of *M. pigra*, the plant rarely grows more than 2 m tall. The invasive shrub is believed to have been introduced to the Botanic Gardens in Darwin in the late 1800s but was not considered a weed of importance until the 1970s (Forno 1992, Miller and Lonsdale 1987). It now covers approximately 800 square kilometres of wetland and conservation areas in northern Australia. Chemical, physical and mechanical control play an important role in the management of this weed, but biological control is likely to be the long-term solution to the problem (Lonsdale 1988, Lonsdale *et al.* 1995, Braithwaite *et al.* 1989, Miller *et al.* 1981).

The search for biological control agents commenced in 1979 by the Commonwealth Scientific and Industrial Research Organization (CSIRO) Entomology and the Northern Territory Department of Primary Industry and Fisheries (NTDPI&F). To date, nine insects and two fungal pathogens have been introduced by CSIRO Entomology and NTDPI&F. Classical biological control and inundative or augmentative biological control (and in the case of fungal agents, mycoherbicides) are two strategies in biological control of weeds (Harley and Forno 1992). For a weed infesting a very large area of land of relatively low commercial value, the classical approach is more appropriate and cost-effective.

CSIRO and NTDPI&F introduced *P. mimosae-pigrae* into Australia in 1994. To ensure establishment in the field, investigations were conducted to achieve a sufficiently high volume of inoculum through liquid fermentation. Many filamentous fungi sporulate in submerged culture (Vezina *et al.* 1965), and liquid fermentation provides the simplest way to produce large number of spores (Auld 1993). Nutritional environment and oxygen transfer during culture growth are two major attributes that can influence the frequency of spore formation (Johnston and Booth 1983).

The objective of this study was to optimize the spore production of *P. mimosae-pigrae* in liquid culture. The effectiveness of conidia produced in liquid culture was tested in the field. In this paper, we describe optimization of spore production, standardized protocol for mass production of *P. mimosae-pigrae* conidia and its establishment on *M. pigra* in the introduced range of the host.

Materials and methods
Preparation of seed inoculum
The original single-conidium isolate of *P. mimosae-pigrae* was maintained on potato dextrose agar (PDA) (Becton Dickinson and Company, Coekeyville, MD 21030, USA) in 9 cm Petri dishes. Inoculated plates were incubated at 25°C for a minimum of ten days for subsequent seeding of liquid media.

Liquid fermentation
Bottled and canned Campbell's V-8 juice (Campbell's Soup Australia, Lemnos, Victoria 3631) was used as the nutrient source of the liquid medium. In preliminary experiments other vegetable juices were tried, but Campbell's V-8 gave the highest yield of conidia. One litre of sterile liquid medium (200 mL Campbell's V8 and 800 mL sterile distilled water (SDW)) was decanted into 2 L Erlenmeyer flasks. The nutritional analysis of Campbell's V-8 juice is given as 1.4 g protein, 0.2 g fat, 8.8 g total carbohydrate, 5.0 g sugars, 522 mg sodium, 862 mg potassium, 269 mg vitamin A, 42 mg vitamin C. The pH of the liquid medium was adjusted to 6.5 using 40% sodium hydroxide. Conidia were harvested from a single Petri dish culture by scraping the surface with a spatula, and one Petri dish was used to inoculate each flask. Inoculated flasks were incubated on a rotary shaker (190 rpm) for ten days at 21°C.

Comparison of conidia production methods
Liquid fermentation methods to produce conidia of *P. mimosae-pigrae* using bottled or canned Campbell's (V-8) liquid were compared. The two sources of Campbell's V-8 juice contain the same nutrients and are manufactured in the same way according to the manufacturer (Campbell's Soup Australia). They differed in exposure to light due to their storage containment. Bottled Campbell's V-8 juice is stored in a clear glass bottle and canned Campbell's V-8 juice is stored in an aluminium can. Erlenmeyer flasks were inoculated and incubated as described above. After ten days the concentration of conidia was determined with the aid of a haemocytometer. Viability of conidia was examined by germinating conidia on water agar.

Comparison of field applications
Ground-based application techniques to inoculate field plants were evaluated against aerial applications at the Finniss River in the Northern Territory. A motorized backpack sprayer (Oleo-Mac, AM 150) was used for ground-based applications. The spraying unit could hold 10 L of liquid. A mist of inoculum was applied amongst the plants for ground-based applications. The aerial applications were carried out by applying a fine mist from approximately 50 cm height above the canopy. A 15 metre wide boom was attached to a spraying unit under the helicopter using T Jet 8008E nozzles. The

spraying units were initially cleaned with activated charcoal and rinsed with water. Spraying volume by helicopter was adjusted to 100 L ha^{-1} with a concentration of 10^6 conidia mL^{-1}, and the delivery rate of the motorized backpack sprayer was 1 L 30–50 m^{-2} with a concentration of 10^6 conidia mL^{-1}. Methyl cellulose was added at a rate of 1 g L^{-1} as a sticking agent to prevent rain washing of inoculum. Field applications on mature plants were made early evening during the wet-season (November–April 1997/1998 and 1998/1999) as the pathogen requires relative humidity between 70–100% to germinate during a 24 hours incubation period (Seier 1998). Applications were carried out in blocks along edges of bulldozed transects of mimosa infestations (one block of 15 × 100 m for aerial application by helicopter and one block of 10 × 30 m for ground-based application by motorized backpack sprayer). The perimeters of the inoculated areas were marked. A buffer zone of 2 m was created inside the perimeters of an inoculated area. Excluding the 2 m buffer zone, 50 randomly chosen leaves were selected inside the inoculated area and assessed by rating all rachides (first and secondary rachides) of each leaf. Disease rating was carried out four weeks after inoculation and symptom expression varied from water soaked lesions to sporulation and necrotic cankers.

Germination tests

Petri dishes containing water agar were placed beside field plants prior to inoculation to provide an assessment of inoculum deposition on plants. The Petri dishes were incubated at 25°C for 24 hours. Two plates were used for each application technique. The plates were observed under a microscope and the number of germinated conidia was counted. Conidia were considered to have germinated when the length of the germ tube was as long as half the length of the spore. One hundred conidia sampled at random were assessed for germination.

Analysis

Analyses of variance were performed using Genstat 5 software package to assess the effect of the two different conidial production methods. A completely randomized design was used with eight subsamples taken from each of six replicates.

A two-sample *t*-test was carried out to test whether there was a difference between the germination rate of bottled and canned modified Campbell's V-8 juice on water agar.

Results

After 10 days on the rotary shaker a significant difference was detected between the two sources of Campbell's V-8 juice ($P<0.05$) based on an ANOVA. The average spore concentration was 1.9×10^6 conidia mL^{-1} (SE = 0.29) using bottled modified Campbell's V-8 juice and 9.7×10^6 conidia mL^{-1} (SE = 0.26) using canned modified Campbell's V-8 juice. This is greater than the 10^6 conidia mL^{-1} concentration recommended for inoculation (Seier 1998).

No significant differences were found between the germination percentages of conidia produced in bottled (94–98%) or canned (95–98%) modified Campbell's V-8 juice after ten days incubation on the shaker bench. The germination percentage on water agar after field application was between 95–99% which corresponded with the germination results from the conidia production.

Both the ground based and aerial application methods resulted in symptoms on sprayed plants. Ambient weather during the wet season provided optimum incubation conditions with a relative humidity between 70–100%. Differences in symptom expression and symptoms severity in relation to the delivery rate are presented in Table 1.

Discussion

Liquid fermentation using canned modified Campbell's V-8 juice provides a simple and economical way to produce large numbers of fungal spores of *P. mimosae-pigrae*. Commonly used spraying equipment in agriculture, such as a motorized backpack sprayer and helicopter, proved to be suitable for field application of *P. mimosae-pigrae*. Although field applications by motorized backpack sprayer induced higher symptom expression than aerial application by helicopter, increased delivery rate by helicopter may increase the infection rate.

The study demonstrated that the development of a culturing and application protocol for a classical biological control agent can be achieved by partly incorporating the mycoherbicide approach (Jackson 1997) into the development strategy.

A successful culturing and application protocol for *P. mimosae-pigrae* is an essential step for the initial establishment of this biological control agent.

In their native habits plant species and natural enemies coexist in a dynamic, but stable balance (Cullen and Hasan 1988, Harley and Forno 1992). The addition of this fungal pathogen to the existing suite of biological control agents (insects) for *M. pigra* in northern Australia may enhance the collective impact of the natural enemies and the target weed in its introduced range.

The ability of *P. mimosae-pigrae* to be produced easily increases the potential of the fungal pathogen as either a classical biological control agent or a mycoherbicide. Coelomycetes (*P. mimosae-pigrae*) that normally sporulate within the plant tissue are generally easily induced to sporulate in submerged fermentation. Therefore, greater production *in vitro* might be possible through further fermentation studies. However, as a classical biological control agent these further studies may not be required for the establishment of the fungus in the field.

The development of a culturing and application protocol through the utilization of the mycoherbicide approach needs to be followed by the development of a strategy to establish the fungal pathogen in the field.

Currently ground application by motorized backpack sprayer provides a reliable technique to initiate symptom development in the field. Aerial application by helicopter gives an opportunity to enhance the establishment of the fungal pathogen on a large scale and provides access to remote *M. pigra* infestations. However, studies on disease persistence and host-pathogen interactions need to be completed to refine the delivery rate of aerial application.

Table 1. Comparison of application methods to produce visible symptoms initiated by *Phloeospora mimosae-pigrae* on *Mimosa pigra* in the field.

Application equipment	Total number of diseased leaves with visible symptoms on rachide[A]	Number of leaves per severity rating scale[B]					Delivery rate
		0	1	2	3	4	
Motorized backpack sprayer	50	0	6	8	11	25	1 L 30–50 m^{-2}
Aerial application by helicopter	26	24	19	4	3	0	1 L 100 m^{-2}

[A] 50 leaves were assessed.

[B] Rating scale: 0= no symptoms, 1 = <25% covered with lesions, 2 = 25–50% covered with lesions, 3 = 50–75% covered with lesions, 4 = 75–100% covered with lesions.

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