In vitro assessment of *Stromatinia cepivora* as a potential biological control agent for angled onion (*Allium triquetrum*) in Victoria, Australia

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Summary Angled onion (*Allium triquetrum* L.) is a noxious weed in Australia and is difficult to control, especially in natural habitats. The genetic diversity of 11 provenances was assessed by RAPD-PCR and was relatively small, making it a suitable biological control target. Pathogenicity trials subsequently used five typical provenances in axenic conditions to test the potential of biological control by *Stromatinia cepivora* (Berk.) Whetzel (*Sclerotium cepivorum* Berk.), a fatal soil-borne pathogenic fungus of cultivated *Allium* species. Pathogenicity and virulence of the fungus were assessed using a *S. cepivora* isolate supplied by DPI Knoxfield. Micropropagation was necessary to free plants from endogenous infection with bacteria. Axenic plants were inoculated with the fungus in test-tubes. The fungus killed plants of all provenances except those from Wonthaggi, Victoria. This study suggests that *S. cepivora* has the potential to be developed as a mycoherbicide for *A. triquetrum* but that further provenances and strains need to be investigated.

Keywords *Allium triquetrum*, *Stromatinia cepivora*, *Sclerotium cepivorum*, mycoherbicide.

INTRODUCTION

Allium triquetrum (Liliaceae) is a noxious, invasive weed in Australia and South Africa (Parsons and Cuthbertson 1992, Walsh and Entwisle 1994, Department of Primary Industries 2008). It is classified as an environmental, pasture and urban weed in Australia and occurs in poorly drained soils along river lines and roadsides (Parsons and Cuthbertson 1992). The common name of *A. triquetrum* in Australia and New Zealand is angled onion, but it is also called three-cornered leek, triquetrous garlic or leek, and onion weed. It is native to the central Mediterranean region (Blood 2001, Parsons and Cuthbertson 1992) and is a herbaceous perennial that propagates by bulbs, bulblets and seeds (Parsons and Cuthbertson 1992, Walsh and Entwisle 1994, Hussey *et al.* 1997, Blood 2001). *A. triquetrum* reduces biodiversity and affects the regeneration of native flora. In addition, it can taint milk and meat in livestock. Its geophytic nature and high rate of reproduction means that it is difficult to manage by herbicides or manual removal, particularly in bushland, and there is no current selective control method.

Stromatinia cepivora causes a serious, fatal white rot of cultivated *Allium* species, identified easily by the small black sclerotia produced on white hyphae (Agrios 2005, Alexopoulos and Mims 1979). It is an imperfect fungus (Alexopoulos and Mims 1979) that produces many tiny sclerotia (0.25–0.6 mm diameter) (Kay and Stewart 1994). The sclerotia can survive dormant in soil for up to 40 years in the absence of host plants without losing their viability (Coley-Smith 1979, Coley-Smith *et al*. 1990) and are transferred by soil and water movement. Since the fungus attacks all known cultivated *Allium* species, it is possible that it will attack *Allium* weeds too. Since all *Allium* species are introduced into Australia, it is proposed that *S. cepivora* could be used as a biological control agent, particularly in bushland, where no native *Allium* species exist, provided that it is not detrimental to native species.

As a first step in investigating the potential of *S. cepivora* as a biological control agent for *A. triquetrum*, this study investigated the genetic diversity of *A. triquetrum* and the pathogenicity of *S. cepivora* under laboratory conditions.

MATERIALS AND METHODS

Biodiversity testing of *A. triquetrum* About 50 complete plants were collected from each of 11 provenances, mainly in Victoria (Dandenong Ranges, Kinglake, Wonthaggi, Yarra Bend Park, Reservoir, Yellow Gum Park, Gardiners Creek, Merri Creek, Bendigo Creek, Kangaroo Flat and White Hill) and maintained as a potted collection in a glasshouse. Three bulbs from each provenance were chosen randomly and DNA was extracted using a Qiagen DNeasy kit according to the manufacturer's protocol. The young foliage of seedlings was used for DNA extraction from seeds using the same protocol. RAPD-PCR amplification reactions were carried out using 56 10-mer primers (from Operon kits OPA, OPB and OPM, e.g. OPM-05 = GGGAACGTGT, OPB12 = CCTTGACGCA). Each 25 µL reaction contained 12.5 µL of PCR Mastermix (Fermentas), ~20 ng of genomic DNA, 2 µL

of primer and nuclease-free water. A thermal cycler was programmed for 35 cycles of: 30 s at 94°C, 60 s at 38°C, and 2 min 30 s at 72°C. Amplicons were separated on 1.5% agarose by gel electrophoresis, stained with ethidium bromide and images were viewed and recorded in a Bio-Rad Gel Doc system. Amplicon size was estimated by simultaneously electrophoresing 1 µL of Fermentas GeneRuler. Each PCR reaction was performed at least three times.

Plant regeneration by micropropagation Axenic test-tube grown plants were used to test the pathogenicity of the fungus. Meristems from within the bulbs were micropropagated in Murashige and Skoog (MS) medium to rid them of infection by endogenous deleterious bacteria (which were similar to *Ochrobactrum anthropi* (Holmes *et al*. 1988) based on DNA sequencing and physiological tests) (P. Tehranchian unpublished data). Bulbs were surface-sterilised with 1.5% NaOCl, rinsed in sterile water and cut vertically, avoiding the central meristem, using a sterile scalpel. Meristems were cultured upright in polycarbonate tubes (80 mm tall × 27 mm diameter) at 25°C in a 16 h photoperiod with 270 µmoles m^{-2} s^{-1} for 10–14 days and then transferred to water agar in glass test-tubes for pathogenicity tests. Pathogenicity was tested only on five provenances chosen for genetic diversity and availability of material not contaminated with endogenous bacteria.

Pathogenicity testing of *S. cepivora* There were 7–9 replicates of uniform size for each provenance. *S. cepivora* (VPRI12439a) culture was kindly supplied from the VPRI Herbarium National Collection of Fungi (DPI Knoxfield). For pathogenicity testing, sclerotia were harvested from cultures on PDA (potato dextrose agar) plates and stored in water agar plates to deplete any transferred carbon source. Sclerotial viability was assessed on V8 medium. Six sclerotia (4 × 4 mm) were placed on the collar region of each plant in its individual test-tube. Inoculated plants were maintained for 21 days in conditions as before. Pathogenicity was recorded and virulence scored as one of seven stages (7 = no infection to 1 = dead plant). Control plants were not inoculated. Experimental tubes were randomised during the experimental period and data were analysed by ANOVA using Minitab Version 15 (Minitab Inc.).

RESULTS

Biodiversity testing of *A. triquetrum* PCR amplification of DNA fragments extracted from bulbs and seeds demonstrated genetic diversity among provenances even within Victoria. Most primers did not produce polymorphic bands. OPB-12 (Figure 1) and OPM-05 produced 4–15 strong, consistent clear bands of 200–1500 bp that showed the greatest degree of polymorphism. There was some variation between provenances, e.g. White Hill (Bendigo) and Reservoir (Melbourne), and within a provenance, e.g. Wonthaggi. Geographically close provenances, e.g. Kangaroo Flat and Bendigo Creek, were no more similar than either was to more distant provenances, e.g. Dandenong Ranges.

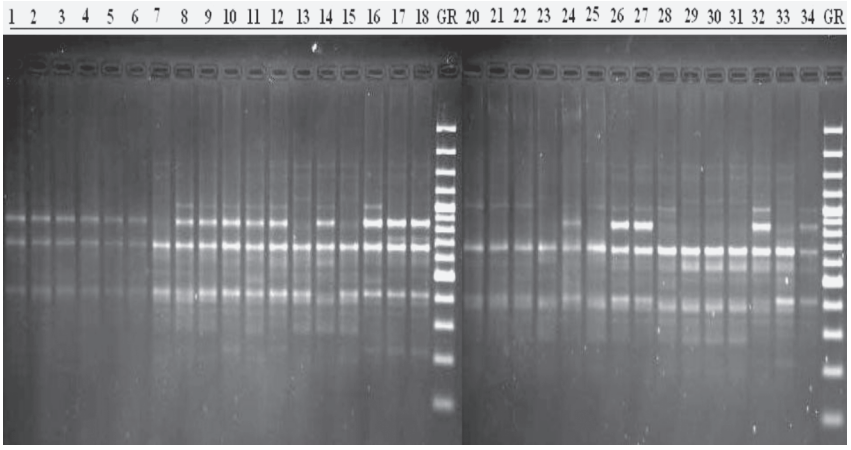

Figure 1. PCR amplification product (extracted DNA from bulbs) using primer OPB 12 on the 11 provenances in Victoria. Lanes are: GR: Gene Ruler, 1–3 Dandenong Ranges, 4–6 Kinglake, 7–9 Wonthaggi, 10–12 Yarra Bend Park, 13–15 Bendigo Creek, 16–18 Reservoir, 20–22 Kangaroo Flat, 23–25 Yellow Gum Park, 26–28 Merri Creek, 29–31 White Hill, 32–34 Gardiners Creek.

Pathogenicity testing of *S. cepivora* Sclerotial germination on V8 agar declined from 80–60% after storage on water agar but was judged adequate for pathogenicity testing. In most test-tubes, sclerotia germinated and fungal mycelium covered the surface of the water agar. After a few days, the fungal mycelium had covered the foliage and subsequently sclerotia were formed. The first disease symptoms became apparent 9–10 days after inoculation. Chlorosis, browning and necrosis increased (Figure 2) until 21 days, when infected plants died. Most (7/8) plants from Wonthaggi, remained green and healthy and sclerotia did not germinate.

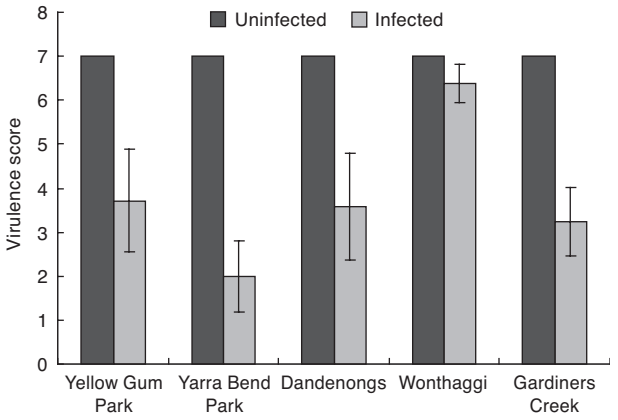

Figure 2. Pathogenicity and virulence levels of *S. cepivora* on test-tube grown *A. triquetrum* from five locations.

DISCUSSION

This is the first report of the genetic diversity of *A. triquetrum* in Australia, and shows that its relatively small genetic diversity makes it a suitable target for biological control. It is also the first report showing white rot infection of *A. triquetrum* by *S. cepivora.* Since all *Allium* species are exotic to Australia, *S. cepivora* thus has potential to control *A. triquetrum* in bushland, provided it is specific to *Allium*. It also may have limited applications in permanent pasture to control problems with livestock milk and meat taint caused by the weed.

The relatively small amount of diversity within and between the provenances is as expected from the *A. triquetrum* biology and breeding system. What was not expected, however, was as much genetic diversity within a provenance (one site of collection) as across all provenances. *A. triquetrum* reproduces clonally by bulbs and bulblets once established, and so no genetic diversity would be expected within a site. *A. triquetrum* is, however, chasmogamous and reproduces also by seeds (García *et al.* 2006) and so cross-pollination might be one of the reasons for biodiversity within each provenance (site). Genetic diversity of provenances in other states may alter their susceptibility to the fungus and so further provenances are being investigated.

Stromatinia cepivora strain VPRI12439a was fatal to four of the five provenances tested under axenic conditions inside test-tubes. The mycelium covered all areas of the plants, whereas in the field it appears only on roots and crowns (DPI 2008). This foliar invasion is probably caused by the axenic, high humidity conditions inside the test-tubes, since normally the fungus invades only roots and bulbs (Abd-El-Razik *et al.* 1973). While the results are promising, pot trials under more exacting conditions are needed to indicate more realistically what the effect of the fungus might be in infestations.

The exception to effectiveness of *S. cepivora* on *A. triquetrum* was 7/8 of the Wonthaggi plants, which showed no sign of infection, despite lack of genetic difference by RAPD-PCR. This suggests that this one strain would not be effective alone in biological control. Sclerotia did not germinate and no mycelium grew. In white rot disease in cultivated *Allium* species, sclerotial germination is stimulated by salkyl-L-cysteine sulphoxides in exudates from the host plant (Coley-Smith and Holt 1966, Coley-Smith *et al.* 1990). Plants from this provenance may not exude this chemical in sufficient quantities or they may exude a similar compound that is not recognised by the sclerotia. Another possibility is that the Wonthaggi plants still harboured the endogenous bacterium, despite being micropropagated and appearing uncontaminated on MS medium. The bacteria prevented sclerotia germinating in tests *in vitro* and so endogenous infection in the field may limit control by *S. cepivora.* On the other hand the bacteria could be useful in controlling sclerotial germination and hence white rot disease in cultivated *Allium* species. Experiments are currently under way to test both of these possibilities.

Testing of further provenances and strains is justified, not only to find the most effective combinations of plant provenance and fungal strain, but also to establish the host specificity (Muller-Stover and Kroschel 2005, Morin *et al.* 2006). Native Australian members of the Liliaceae must be tested as a priority to ensure that the fungus is specific to exotic *Allium* species. Releasing a virulent pathogen for cultivated *Allium* species into bushland or pasture is controversial

and any field release would require safeguards against spread to areas suitable for the production of cultivated *Allium* species, such as onions, leeks and garlic, before *S. cepivora* could be introduced as a potential biological control agent.

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