Bulb rot in live *Allium triquetrum* by *Pectobacterium carotovorum* subsp. *carotovorum*

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Summary *Allium triquetrum* L. (angled onion) is an invasive weed that threatens native ground flora such as orchids, lilies and grasses in natural habitats, especially in damp situations. A soft-rotting bacterium isolated from rotting *A. triquetrum* bulbs after 2 months of storage at 4°C (collected from Horsnell Gully, South Australia) was assessed for its potential as a biocontrol agent. The bacterium was identified as *Pectobacterium carotovorum* subsp. *carotovorum* Waldee by 16S r-DNA sequencing and physiological tests. In test-tube trials, the bacterium produced severe soft rot symptoms of bulbs 12 h post-inoculation and rotten young seedlings collapsed after 24 h incubation at 25°C. Identical symptoms were observed at 15°C and 4°C, but with a longer development period at 4°C. Histology of infected plants revealed that the bacterium invaded both the cortical and vascular tissue. In glasshouse tests, all *A. triquetrum* provenances inoculated with 10^8 CFU of the bacterium per plant showed soft rot symptoms 20 days post-inoculation, but cultivated *Allium* species and Australian native monocots were not affected 3 months post-inoculation. The soft-rotting bacterium was reisolated from infected *A. triquetrum* bulbs and leaves in the glasshouse, fulfilling Koch postulates. Although this bacterium is normally associated only with storage rots, it is potentially an effective biological control agent for *A. triquetrum* in the field, as it can attack live plants too. Field trials to demonstrate efficacy are currently in progress.

Keywords *Allium triquetrum*, *Pectobacterium carotovorum* subsp. *carotovorum*, bioherbicide, bacterial disease.

INTRODUCTION

Allium triquetrum L. (angled onion) is a native of African and European countries in the western and central Mediterranean region (Parsons and Cuthbertson 1992, Blood 2001). *A. triquetrum* is proclaimed as a noxious weed in parts of Australia (Walsh and Entwisle 1994). It is not known when this species was introduced to Australia, but it recorded as naturalised in temperate regions of South Australia by 1909 (Parsons and Cuthbertson 1992). It occurs in South Australia (Government of South Australia 2005), Western Australia (Walsh and Entwisle 1994), Tasmania and New South Wales (Weeds Australia 2008). As a result of its rapid growth in shaded areas (Blood 2001) and its allelopathic exudates (DPI 2008), it forms monocultures, especially in damp situations, and so is a threat to native herbaceous plants. Its strong onion odour can cause milk and meat taint if consumed by farm animals (Parsons and Cuthberston 1992, Weeds Australia 2008), which makes the products unfit for sale. Herbicides are ineffective because they fail to remove the underground bulbs and grubbing is labour-intensive and therefore costly. An alternative control is required.

Soft rot disease symptoms were noticed as rotted yellow smelly bulbs on a few *A. triquetrum* collected from Horsnell Gully (SA) after 2 months storage at 4°C. A bacterium was isolated and identified on the basis of physiological tests including API 20NE and sequencing as *Pectobacterium carotovorum* subsp. *carotovorum*.

Pectobacterium carotovorum Waldee 1945 (previously *Erwinia carotovora* (Jones 1901, Winslow *et al.* 1920)) is a member of the family Enterobacteriaceae. Dye (1983) described it as a Gram-negative motile rod (0.5–1 × 1–3 µm) that forms single or short chains of cells. It is a facultative anaerobe with an optimum growth temperature of 27–30°C. *P. carotovorum* is oxidative-negative and catalase-positive, produces acid by fermentation and assimilates a wide range of carbon sources such as glucose, sucrose, galactose, fructose and α–methyl glucoside (Dye 1983). *P. carotovorum* has three major important sub-species causing soft rots in plants: *P. carotovorum* subsp. *carotovorum*, which infects onions (Jones 1901, Bergey *et al.* 1923), *P. carotovorum* subsp. *atrosepticum* (Van Hall 1902) and *P. chrysanthemi* (Burkholder *et al.* 1953).

The aims of this study were to confirm the identity of the soft-rotting bacterium isolated from *A. triquetrum* using sequencing and to evaluate it as a biological control agent by testing its pathogenicity on test-tube and glasshouse-grown *A. triquetrum* plants, originating from various provenances from around Australia. A histological study of the infected bulbs was also performed to investigate the infection process.

MATERIALS AND METHODS

Isolation of soft-rotting bacterium and physiological tests Plants were collected from infestations in Victoria and South Australia, potted up and kept in a glasshouse until they died back. Bulbs were collected and washed with tap water, dried at room temperature and stored at 4°C for about 2 months. Rotted yellow smelly bulbs from Horsnell Gully, South Australia that emitted cellular debris on pressing were used for bacterial isolation. Loopfuls of bacterial mass were suspended in 0.9% sterile saline and streak-diluted on Difco Colombia blood agar base and modified trypticase soy agar (TSA) (Grant and Holt 1976).

Identification of bacterium with 16S r-DNA PCR and sequencing The bacterial DNA was extracted using a QIAGEN DNeasy Plant MiniKit modified protocol where bacterial cells were mixed with the AP1 buffer, boiled at 90°C for 4 min and subsequently cooled at 4°C instead of grinding in liquid nitrogen. 16S r-DNA was amplified using fD1 and rP2 primers (Weisburg *et al.* 1991). A PCR reaction was performed in 25 µL containing 12.5 µL of Go-Green Mastermix (Fermentas), ~16 ng of genomic DNA, 1µL of each 0.62 µM primer and sterile nuclease-free water. A thermocycler was programmed based on the Weisburg *et al.* (1991) protocol for: initial denaturation at 94°C for 10 min; followed by 35 cycles of: 95°C for 2 min, 51°C for 30 s, and 72°C for 4 min; with a final elongation at 72°C for 7 min. The PCR products were fractioned in 1.5% agarose gel electrophoresis and visualised in a Bio-Rad Gel Doc system. 16S r-DNA PCR products were purified using Qiagen PCR purification kit and sequencing reactions prepared using the Big Dye Terminator v3.1 protocol. Products were precipitated using the manufacturer's protocols, dried and sent to Micromon Sequencing Facilities located at Monash University for further analysis.

Pathogenicity of bacterium on test-tube-grown *A. Triquetrum* Micro-propagated test-tube-grown plants were used for *in vitro* pathogenicity testing of the isolated bacterium. *A. triquetrum* bulbs collected from provenances around Australia were surface-sterilised with 70% ethanol followed by 1.5% NaOCl and rinsed in sterile water. To avoid problems caused by an endogenous bacterium, the smallest shoot with a piece of inner disk attached to the basal stem was excised and cultured in Murashige and Skoog (MS) medium in polycarbonate tubes (80 mm tall × 27 mm diameter) at 25°C in a 16 h photoperiod provided by Fluora fluorescent tubes at 270 µmoles m^{-2} s^{-1} for 15 days. Young seedlings 10–15 cm high were transferred to water agar in glass test-tubes for pathogenicity tests.

For inoculation, a loopful of pure culture of the bacterium from -80°C stock was sub-cultured in Luria Broth (Difco) and incubated at 30°C in a Thermoline Scientific Shaker Incubator for 24 h at 140 rpm. The bacterial culture was centrifuged at 4500 × g for 10 min at 4°C in 50 mL Falcon tubes, the bacterial cells resuspended in sterile MilliQ water and adjusted to 10^8 bacterial cells mL^{-1}. Test-tube-grown plants were inoculated by injection of 1 mL (10^8 cells) of bacterial suspension into the water agar. There were three replicate tubes per provenance arranged in a randomised complete block design. Control tubes without bacterial inoculation were used for each provenance. Tubes were incubated at 25°C, 15°C and 4°C with a 16 h photoperiod (as above). Soft rot disease symptoms were scored with a combined bacterial growth/disease ranking scale of six stages (1: no infection to 6: dead plant).

Histology of infected tissues Infected collar region tissues were fixed for 24 h in 5% formaldehyde solution (Sigma) at room temperature and stored in 70% ethanol at 4°C. Fixed tissues were processed using a Leica ASP 200S tissue processor programmed for soft tissues as in the manufacturer's protocol and subsequently embedded in paraffin wax (Ruzin 1999) using a Shandon Histocentre 3 (Thermo Scientific). The embedded tissues were sectioned 3–4 µm thick, Gram-stained and fixed in DPX Mountant for examination with a Leica DM2500 compound microscope.

Pathogenicity of bacterium on glasshouse-grown *A. triquetrum*, cultivated *Allium* species and native monocots *A. triquetrum* plants from bulbs collected from provenances around Australia were grown in pots in a glasshouse using sand at 60–80% RH and 15–25°C. Cultivated *Allium* species or cultivars such as onion, leek, shallot etc. and some of the related Australian native monocots were also tested in the glasshouse simultaneously. The bacterial inoculum was prepared as above and inoculation was performed twice 30 days apart by adding 1 mL of a 10^8 cells mL^{-1} bacterial suspension to each pot placed in a separate saucer to catch runoff. The bacterial suspension culture was applied on the collar region of *A. triquetrum* using a *micropipette*. Plants were watered from the base every 2 days and fertilised every 2 weeks using modified Hoagland nutrient solution (Taiz and Zeiger 2002). There were four replicate pots for each provenance arranged in a randomised complete block design. Control pots without bacterial inoculation were used for each provenance. The soft rot disease symptoms on infected plants were scored with a six-point disease ranking scale as above.

RESULTS

Soft rotting bacterium identification The only type of colony observed on both media was a motile Gram negative rod that was oxidase-negative and catalase-positive. Physiological and biochemical tests showed the typical *P. carotovorum* characteristics such as fermentation of lactose and neither assimilation nor fermentation of maltose. The bacterium was resistant to erythromycin. On amplification of the 16S-rDNA of the bacterium, a band of 1500 bp was observed on 1.5% agarose gel. Sequence quality was checked by alignment of forward and reverse sequences using BioEdit software. This produced a 16S region sequence of 1100 bp and 100 bp of the 5´ beginning of the sequences was deleted due to low quality sequence. A Blast search of GenBank through NCBI showed that the closest match was *Pectobacterium carotovorum* subsp. *carotovorum* (Maximum identity: 98%, E value: 0.0).

Pathogenicity on test-tube-grown *A. Triquetrum* The bacterium was pathogenic and highly virulent on plants from all provenances tested (Figure 1). Wilting of leaves was the first disease symptom observed, after only 24 h at 25°C. Disease symptoms also developed at 15°C and 4°C after 7 days and 2 months, respectively. The water agar colour in the test-tubes changed to pale yellow and bacterial clumps were observed. Infected plants collapsed from the top and the rotted leaves became attached to the test tube. After 48 h incubation at 25°C, green or yellow masses of leaves were collapsed on the agar surface. The collar region of the test-tube-grown *A. triquetrum* dissolved in all almost samples while the control plants without bacterial inoculation remained healthy. The bacterium killed most of the plants (rank 6) in the experiment (Figure 1). One replicate from Kangaroo Flat (Bendigo, Victoria) was given a disease ranking of 5, because the top leaf was healthy although the plant had collapsed from the collar region. Only *P. carotovorum* subsp. *carotova* was re-isolated from the infected plants on nutrient agar plates. Statistical analysis based on randomised complete block design indicated that inoculated plants had significantly greater disease ratings but there was no significant difference in disease rating among provenances.

Histology of infected tissues Bacterial cells were observed in infected tissues collected after 12 h and 24 h post-inoculation. The plant samples were much rotted after 24 h inoculation and were difficult to process to show sections through recognisable tissues. There was no bacterial infection in control treatments and plant tissues were intact. In infected *A. triquetrum* collar regions, bacteria were observed intercellularly between the cortical parenchyma cells and in the vascular tissues (Figure 2). The epidermal cells remained intact in some sections. Largely intact cortical parenchyma cells were observed in samples collected after 12 h, but masses of bacteria were visible adjacent to the cell walls. Cortical parenchyma cells invaded by the bacterium were collapsed in samples collected after 24 h and vascular tissues were also destroyed.

Pathogenicity on glasshouse-grown plants The bacterium was pathogenic and virulent on almost all

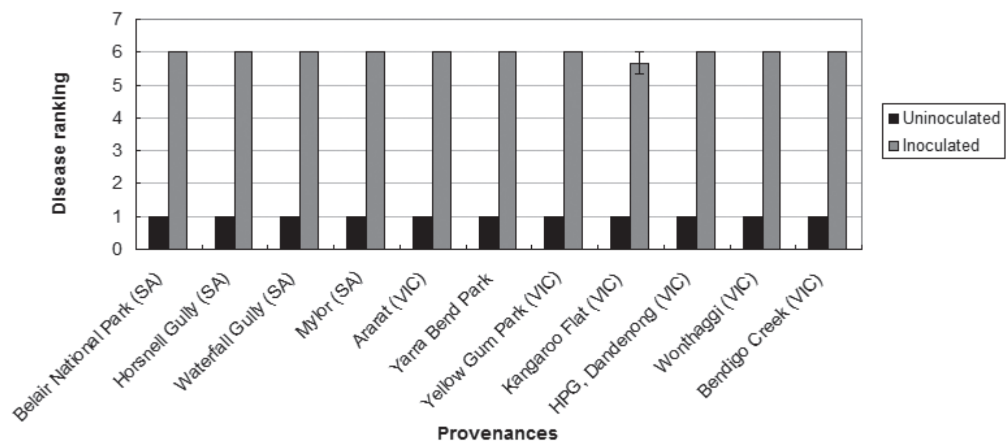

Figure 1. Disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* on test-tube-grown *Allium triquetrum* plants from provenances around Australia. HPG: Hardy's Picnic Ground, Dandenongs. (1: no infection to 6: dead plant) (Bars = 2 × standard error).

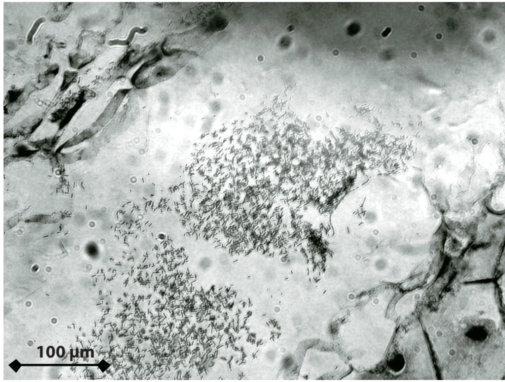

Figure 2. *Pectobacterium carotovorum* subsp. *carotovorum* in dissolved cortical parenchyma of *Allium triquetum* bulb.

A. triquetrum provenances from Australia in the RMIT glasshouse but not on cultivated *Allium* species and native monocots tested (data not shown here). The disease ratings for most plants were 4–6 (Figure 3). The first disease symptoms were observed 2 weeks after inoculation, as wilted, collapsed older leaves. Infected plants collapsed from the collar region and rotted from the base of the stem. Death was observed after 20 days as dried collapsed leaves and rotted bulbs in the sand. Control plants without bacterial inoculation remained healthy. Various disease rankings were given to plants from Waterfall Gully (SA), Mylor (SA), Ararat (VIC), Yarra Bend Park (VIC), Wonthaggi (VIC), Bendigo Creek (VIC) and Yellow Gum Park (VIC). Among the various provenances, Mylor (SA) replicates developed the least disease symptoms. Plants from Belair National Park (SA), Horsnell Gully (SA), Gardiner's Creek (VIC), Kangaroo Flat (VIC) and Hardy's Picnic Ground, Dandenongs (VIC) were ranked the maximum of 6 for all replicates. Statistical analysis based on a randomised complete block design indicated significant differences among replicates but not among provenances. This was because plants either became infected and collapsed (score of 6) or did not become infected and were healthy (score of 1), as shown with the four inoculated replicates from Mylor.

DISCUSSION

Pectobacterium carotovorum subsp. *carotovorum* has not previously been considered as a potential biological control agent for *A. triquetrum* in Australia. The pathogenicity test results for both test-tube and glasshouse-grown *A. triquetrum* plants obtained in this study showed that this strain of the bacterium was highly virulent on *A. triquetrum*. Similar disease symptoms were observed in the field where some of the provenances were collected in Victoria and in retrospect it would have been good to collect these and attempt to isolate the causal organism from them.

The isolation of pure cultures from the infected plants in the glasshouse, 16S-rDNA sequencing and positive 600 bp amplicon with *P. carotovorum* subsp. *carotovorum*-specific primers (Kanf *et al.* 2003) (data not shown) suggests that the symptoms in the bulbs

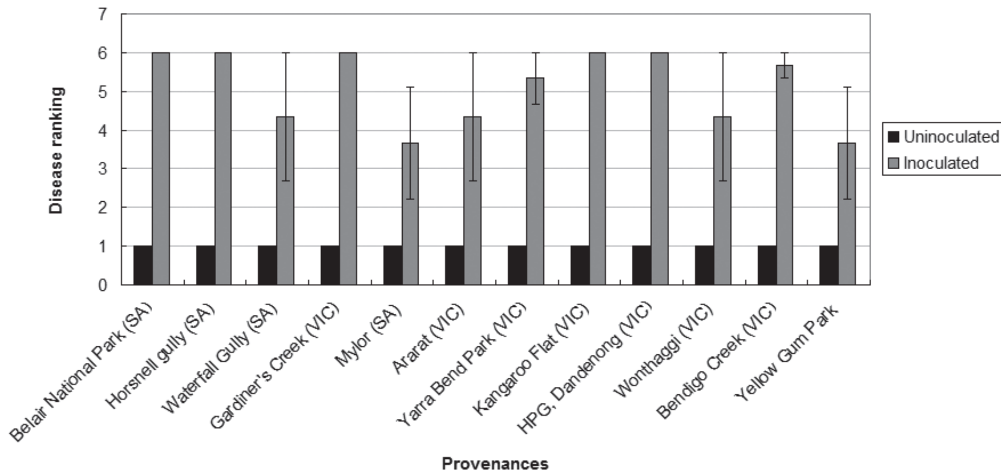

Figure 3. Disease caused by *Pectobacterium carotovorum* subsp. *carotovorum* on glasshouse-grown *Allium triquetrum* plants from provenances around Australia. HPG: Hardy's Picnic Ground (1: no infection to 6: dead plant) (Bars = 2 × standard error).

were caused by *P. carotovorum* subsp. *carotovorum*. This was confirmed by the physiological and biochemical characterisation based on the API 20NE and other tests (Pérombelon 1973, Lelliott 1974, Dickey 1979, Dye 1983) (data not shown). This bacterium is well known as a storage rot, including of onions (Palacio-Bielsa *et al.* 2006), but has not previously been considered as a possible biological control agent. Soft rot is a widespread storage disease caused by fungi and bacteria. It has been recorded on a wide range of plant species (crops and ornamentals) worldwide, including cultivated onion (Liao *et al.* 1993, Barras *et al.* 1994). Soft rotting agents infect parenchyma tissues of the host, producing macerating enzymes (Dye 1983, Cother and Sivasithamparam 1983).

The disease symptoms caused by *P. carotovorum* subsp. *carotovorum* in both pathogenicity tests developed rapidly and caused foliage collapse—after only 24 h incubation at 25°C for test-tube grown plants and 2 weeks at 15–25°C for glasshouse-grown plants. Such rapid disease development would be ideal for biocontrol of this weed if it can be repeated in a field situation. A further point in favour of the use of this bacterium for biocontrol is that there seemed to be no inherent resistance to infection, as there was no variation in mortality of test-tube plants among provenances, except in plants from Kangaroo Flat (Bendigo, VIC). Only one replicate of *A. triquetrum* from this provenance exhibited healthy green leaves after 24 h incubation, but a highly infected collar region was observed at 24 h and the plant collapsed after 48 h incubation.

According to Dye (1983), the optimum temperature for *P. carotovorum* subsp. *carotovorum* growth is 28–30°C. The longer times taken until disease symptoms were observed on test-tube-grown plants at 15°C and 4°C (7 days and 2 months) incubation respectively, suggest that temperature affected how the host plant defence system reacts against the bacterium, directly affecting the pathogenicity and virulence of the bacterium (Colhoun 1964, Bell 1981). However, temperature affects the development rate of the bacterium. In some other weedy species, plant resistance to pathogens is reported to vary as temperature changed (Mayama *et al.* 1975, Wang *et al.* 2009). Pathogenicity at temperatures less than the optimum suggests that the bacterium can be as effective in cool environments similar to those experienced during winter growth of *A. triquetrum* in the field and so is a good attribute for a biological control agent.

Pathogenicity of the bacterium varied between *A. triquetrum* provenances in the glasshouse, unlike the uniformity seen in test-tubes, where conditions greatly favoured the pathogen. Glasshouse-grown plants of the Mylor (SA) provenance had the least infection among all provenances and two replicates out of three test-tube-gorwn plants remained healthy. This could either be because of innate genetically acquired resistance in the plants, ,or disease escape. Genetic diversity of *A. triquetrum* between and within provenances has been demonstrated in previous studies (Tehranchian *et al.* 2010) and one of the Mylor replicates clustered separately from other South Australian and Victorian provenances in RAPD tests (data not shown). Therefore genetic variation within this provenance could be the reason for the lack of susceptibility to the bacterium in some plants. Disease escape is also a possibility, in that bacteria may have been washed below the rooting zone in the pots and so not been present at a sufficiently high dose to cause disease. This is unlikely, as 2×10^8 cells were inoculated per pot and this was sufficient to kill all replicates of most other provenances. It may also be a function of the numbers of replicates used; only four replicates were used in the glasshouse trials. This number of replicates may have been too low to cover for occasional disease escape and still show the pathogenicity of the bacterium in statistical analysis. However; small numbers of replicates are typical in weed management studies (Auld *et al.* 1988, Auld 1993).

The lack of soft rot disease symptoms in cultivated *Allium* species in particular and native Australian monocots (data not shown) under the same conditions as those that produced soft rot in *A. triquetrum* in the glasshouse is a good feature for a biological control agent. However, *P. carotovorum* supsp. *carotovorum* is known to be pathogenic on a wide range of vegetables and crops with fleshy tissues, bulbs and tubers (Babadoost 1990). Centrifugal phylogenetic testing is the most important factor for assessing biological control agent and release the pathogen into a new environment (Muller-Stover and Kroschel 2005, Morin *et al.* 2006). The lack of infection in other *Allium* species in the glasshouse, in particular, raises the question of this strain of *P. carotovorum* subsp. *carotovorum* being specifically pathogenic to *A. triquetrum*. *P. carotovorum* has many subspecies with different ranges of hosts, e.g. *P. carotovorum* subsp. *atrosepticum* on potato, and it is possible that this is the case, though more *Allium* species would need to be tested to find out.

Pectobacterium carotovorum subsp. *carotovorum* colonises intercellularly *A. triquetrum* as shown by the presence of bacteria between the cortical parenchyma cells and cells of the vascular bundles. The cortical parenchyma cell walls showed evidence of early rupture and vascular bundles showed evidence of damage, probably due to pectolytic activity. Delahaut

and Stevenson (2004) stated that pectolytic bacteria digest the middle lamella of host plant cell walls, which causes cells to be pulled apart. It is likely that *P. carotovorum* subsp. *carotovorum* enters the cortex through the epidermis and multiplies between and then in the infected cells. Its enzyme ruptures the cell wall and destroys cells. The bacterium was even seen in the vascular-bundles, an indication that it could move up through vascular tissues and this may be why the upper leaves of test-tube-grown plants collapsed 12 h after inoculation. As the bacterium travels through the vascular tissues, it is distributed quickly to the mesophyll of young leaves and causes collapse. In this study only the infected collar region of test-tube-grown *A. triquetrum* were used for light microscopy as it was not possible to section infected leaves due to highly softened tissues and low leaf thickness. A further study of the infected leaf tissue at different times after inoculation would test this hypothesis of the bacterial movement through the vascular tissues.

The pathogenicity of *P. carotovorum* subsp. *carotovorum* and its high virulence on *A. triquetrum*, coupled with its apparent lack of pathogenicity towards other plants tested, suggests that it could potentially be developed as biological control agent for this noxious weed in Australia.

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