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A new leaf blight disease of *Trifolium dasyurum* caused by *Botrytis fabae*

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Abstract A new disease was observed on *Trifolium dasyurum*, with symptoms beginning as a halo spot and developing into a leaf blight. The causal organism was identified by microscopy and DNA sequence studies as *Botrytis fabae*. This strain of *B. fabae* was also demonstrated to cause disease on foliage of a range of pulse crops, including *Vicia faba*, *Pisum sativum*, and *Lens culinaris*. This study demonstrates the potential of this strain of *B. fabae* to not only pose a significant threat to *T. dasyurum* but also to pulses grown in rotation with *T. dasyurum* that are susceptible to this strain of *B. fabae*.

Keywords *Trifolium dasyurum* · *Botrytis fabae* · *Botrytis cinerea* · Leaf blight

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New pasture legume types are being evaluated in Australia to accommodate new farming systems that include phase pastures (i.e., a period of perennial based pasture followed by a period of cropping), green and brown manures, and specialist fodder production across the matrix of soil types and rainfall zones. There are a wide range of alternative pasture legume species, mostly *Trifolium* species, being evaluated, and a number of species new to the cropping zones have already been or are being released to replace the most widely grown species of *Trifolium subterraneum* and annual *Medicago* spp., especially for ley-farming systems. Farmer surveys have shown widespread adoption of these new pasture legumes in Australia, with this trend expected to increase (Nichols et al. 2007), and they are becoming an increasingly important component of sustainable agricultural systems (Graham and Vance 2003). One of the species of *Trifolium* that has been particularly promising as an alternative pasture legume in southern Australia is *T. dasyurum* (eastern star clover). *Trifolium dasyurum* is native to north Africa, particularly Egypt and Libya, to the temperate regions of western Asia such as Cyprus, Iran, Iraq, Israel, Syria, and Turkey, and to the Mediterranean areas of Europe (Greece, Aegean islands, Cyprus; USDA, ARS, National Genetic Resources Programme 2008; Loi et al. 2007).

A new symptom on *T. dasyurum* was first observed in 2004 in an experimental site at Baker's Hill, Western Australia. The plants displayed symptoms

of small spot lesions with chlorotic margins at the early phases of symptom development. However, as the disease progresses, the small spot lesions merge, and the centres of the lesions become light grey, eventually resulting in a severe leaf blight. For younger plants, severe disease could even lead to death of the entire plant. Preliminary investigations suggested potential involvement of a *Botrytis* strain resembling *Botrytis fabae*.

Botrytis spp., including *B. fabae*, have long been recorded as the cause of severe disease on legume crops such as field peas, faba beans, horse beans, lentils, vetch, and chickpeas and are generally widely distributed around the world wherever these species are grown. The typical symptom on faba bean was described as chocolate spot (Davidson et al. 2004), and this commonly occurs worldwide, including Australia (Tivoli et al. 2006). This paper reports the identity of the *Botrytis* strain causing a new leaf blight disease of *T. dasyurum*, occurring in Australia and outlines the implications of this new disease for non-pasture legume crops grown in rotation.

The symptoms were first observed in the field on leaves of *T. dasyurum* plants growing in an experimental area 90 km northeast of Perth at the Yalanbee Research Station, Baker's Hill, Western Australia (latitude, 31.76°S; longitude, 116.48°E). The most common early phases of the symptom included the development of small spot lesions with chlorotic margins (Fig. 1). As the disease progressed, typical symptoms of a necrotic leaf blight were observed (Fig. 2). When plants were attacked when still at the seedling stage, symptoms of severe blight leading to death of plants were observed, frequently with obvious sporulation of the *Botrytis* on the affected tissues (Fig. 3).

Fig. 1 Halo spots with chlorotic margins caused by *B. fabae* in *T. dasyurum* during early disease development

Fig. 2 Symptoms of leaf blight caused by *B. fabae* particularly on young leaves of *T. dasyurum*

Koch's postulates were followed to confirm the cause of the symptoms observed. The *Botrytis* strain used was isolated from diseased *T. dasyurum* leaves collected from Baker's Hill. Diseased leaves were placed in a covered tray containing moist blotting paper and incubated at 22±2°C for 2 days. Single conidia were taken from a *Botrytis* isolate found sporulating on the leaf tissues showing typical disease symptoms and subsequently transferred onto potato dextrose agar plates. After 7 days, mycelium plugs were taken and transferred onto V-8 juice agar plates and incubated at 22±2°C until mycelium nearly covered the whole plate (about 7 days). The agar plate cultures were then seeded with sterilised barley seeds. Sterilised barley seeds were prepared by soaking seeds in deionized water for 12 h, then

Fig. 3 Symptoms of severe blight leading to death of young plants caused by *B. fabae* on young leaves of *T. dasyurum*. Note sporulation of *B. fabae* on infected tissues

draining the water from the seeds, and autoclaving at 120°C for 30 min each day over three consecutive days. The plates containing the barley seeds were incubated at 22±2°C for 7 days until the mycelium covered the barley seeds and then placed in a freezer (−4°C) for 48 h to induce sporulation of the *Botrytis* isolate and subsequently thawed in a laminar flow cabinet. The barley seeds colonized by the *Botrytis* isolate were then placed in a Petri dish containing sterilised moist filer paper and left at room temperature (22±2°C) until sporulation occurred (approximately 7 days later). Conidia of the *Botrytis* isolate were then collected by washing the colonised barley seed in 10 ml of sterilised water/plate, and a sterilised metal tea strainer was used to separate barley seeds from the conidial suspension. Conidial concentration was estimated using a hemocytometer and adjusted to 10^5 conidia ml^{-1}.

The conidial suspension was sprayed with a hand-operated nebuliser onto 28 day-old *T. dasyurum* seedlings until runoff. The inoculated plants were grown in 10 cm pots filled with a pasteurised commercial potting mix and maintained in a controlled environment chamber at >95% humidity for 4 days in total darkness, conditions conducive for *Botrytis* conidial germination and infection (Holz et al. 2004). Subsequently, plants were maintained in a controlled environment chamber with 12/12 h light/dark, a light intensity of 150 μE m^{-2} s^{-1} at 13/18°C (night/day) until the appearance of the symptoms. Conidia were re-isolated from experimental plants showing the same symptoms as observed in the field.

Conidia from naturally diseased and experimental plant leaves of *T. dasyurum* were mounted onto glass slides, and the size of 100 mature conidia and 100 sclerotia was determined using a light microscope at 200× magnification for conidia and 100× magnification for sclerotia.

A single conidial isolate of the *Botrytis* isolate (WAC13179) from *T. dasyurum* that morphologically matched *B. fabae* was grown on potato dextrose broth for 14 days at 22±2°C. The mycelium was harvested, lyophilised, submerged in liquid nitrogen, and ground into powder. Genomic DNA was extracted from 10 to 20 mg mycelium using a phenol/chloroform extraction method (Raeder and Broda 1985). DNA pellets were dissolved in 100 ml of TE buffer and stored until required at −20°C.

The primer combinations CAACAATTGA GATTTGCCCACAAG and GATGGATCCAGTGG TACCGAGCAT were used to amplify one of the three nuclear DNA genes as used by Staats et al. (2005), viz., the heat-shock protein 60 (HSP60) to define the molecular phylogeny of *Botrytis* spp. isolates. The polymerase chain reaction products obtained were cloned for sequencing. DNA sequences then were compared using 'Blast' of the GenBank database to identify the best match with *Botrytis* spp. The sSequence has been submitted to the NCBI Genbank, and the assigned number is EU 365876.

Six species of various pasture and crop legumes from across six genera (see Table 1) were tested for their reaction to a single isolate each of two different *Botrytis* spp., viz. (1), *B. cinerea* (WAC9965) isolated from *Cicer arietinum* and (2) the newly isolated *Botrytis* isolate (WAC13179) from *T. dasyurum*. There were four replicate pots for each *Botrytis* sp. × genotype combination and appropriate water-only inoculated control comparison treatments of each genotype organised in a randomised block design.

Table 1 Severity of disease in six legume species 28 d after inoculation with *Botrytis cinerea* or *B. fabae*

Host genotype	Disease severity[a]	
	Botrytis cinerea	*Botrytis fabae*
Vicia faba cv. Cairo (faba bean)	9.1	2.7
Lathyrus sativus cv. Ceora (grass pea)	5.9	0.5
Lens culinaris cv. Digger (lentil)	9.9	3.3
Pisum sativum cv. Dunwa (field pea)	8.6	1.4
Trifolium dasyurum cv. AGWEST Sothis (Eastern star clover)	10	9.2
Cicer arietinum cv. Norwin (chickpea)	9.1	0.5

[a] 0 to 10 scale, where 0=nil disease, and 10≥90% of leaf area lesion from infection by *Botrytis cinerea* or *B. fabae*. In every case, a score of 10 represented total collapse of all leaves and the plant dead. Sporulation level corresponded with disease severity level in all tested species, viz., the higher the score the more sporulation observed.

Seeds of each genotype were sown into 10 cm diam pots containing 750 g of pasteurised commercial potting mix and maintained in an artificially lit controlled environment chamber with a 12-h photoperiod, a light intensity of 150 μE m^{-2} s^{-1}, and a temperature regime of 13/18°C (night/day). Twenty-eight days after sowing, a conidial suspension of concentration 2×10^5 ml^{-1} of either *Botrytis* isolate (WAC9965 or WAC13179) was spray-inoculated on to each plant host until run-off. After the inoculation, plants were maintained at >95% humidity in the dark for the first 4 days and then returned to the 12-h photoperiod as above.

Twenty-eight days after inoculation, plants were scored for their disease severity using a 0 to 10 scale (Barbetti 1987; Barbetti and Nichols 2005) where 0=nil disease and 10≥90% of leaf area lesioned from *Botrytis* infection. In every case, a score of 10 represented total collapse of all leaves and plant death.

Trifolium dasyurum inoculated with *Botrytis* isolate WAC13179 developed symptoms identical to those observed in the field; initially, a halo spot which subsequently developed into a leaf blight symptom as the disease progressed (Figs. 1 and 2). The same *Botrytis* strain was consistently re-isolated from diseased tissues showing these symptoms. The size of conidia produced by the *Botrytis* strain isolated ranged from 15 to 21 μm in width and from 21 to 24 μm in length, and sclerotial size ranged between 1 and 2 mm in diameter. Both conidial and sclerotial descriptions and sizes matched those given for *B. fabae* by Ellis and Waller (1974).

The DNA partial sequence of the nuclear protein-coding gene HSP60 of the *Botrytis* strain isolated from *T. dasyurum* confirmed that this isolate was *B. fabae*. The sequence consisted of 983 characters and showed 99% identity with each of four recorded *B. fabae* strains (viz., EU563098.1, EU563095.1, AJ716075.1, and AJ716074.1) in GenBank. The total query coverage was 98% for these four recorded *B. fabae* strains ranking at the top in terms of identity, coverage and maximum and total scores.

Botrytis fabae WAC13179 caused significant disease on four of the six species inoculated (Table 1). While *T. dasyurum* showed severe disease symptoms and the greatest susceptibility to *B. fabae* isolated from *T. dasyurum*, this isolate also caused disease on foliage of the pulse crops tested, in particular on *Vicia faba*, *Pisum sativum*, and *Lens culinaris*. In contrast, on *C. arietinum* and *Lathyrus sativus*, while this same *B. fabae* isolate was still readily able to infect both of these genotypes, it resulted only in limited disease. Sporulation level of *B. fabae* WAC13179 corresponded with disease severity level in all tested species, viz., the higher the score the more sporulation observed.

Botrytis cinerea caused severe disease on all six of the tested pasture and crop legume genotypes. The diseased leaves and stems showed dark brown discolouration and a tan colored fungal growth (mixture of mycelium and conidia) growing on the surface of both leaves and stems (Fig. 3). No disease occurred in any of the 'water only'-treated controls.

Botrytis fabae has been recorded as a cause of disease on *Vicia* spp., *Pisum* spp., *Lens* spp., and *Phaseolus* spp. (Staats et al. 2005; Tivoli et al. 2006). To our knowledge, this is the first report of *B. fabae* as a pathogen of *T. dasyurum* or any other *Trifolium* species. Although the typical *B. fabae* disease symptoms on faba bean are reported as a 'chocolate spot' symptom (Davidson et al. 2004), *B. fabae* infection on *T. dasyurum* leaves resulted in a distinct halo spot-type symptom at the early stage, which later developed into a severe leaf blight particularly on younger leaves. It even resulted in plant death when seedlings were attacked. While pathogenicity variations between isolates of *B. fabae* are known (Deverall and Wood 1968; Hutson and Mansfield 1980; Mansfield and Hutson 1980; Hanounik and Maliha 1986), the fact that *T. dasyurum* was the most severely diseased among the six host genotypes we tested suggests host specialization in these strains of *B. fabae* from *T. dasyurum*.

Since this initial report in 2004 at Baker's Hill, similar severe disease has been observed on *T. dasyurum* in the Many Peaks region on the south coast of Western Australia, approximately 480 km south of Baker's Hill.

Although the biology and pathogenicity of *Botrytis* spp. have been studied on a variety of plant species (Jarvis 1977; Coley-Smith et al. 1980; Verhoeff et al. 1992; Holz et al. 2004), *B. fabae* has not previously been reported to cause disease on *Trifolium* species. Our study demonstrates the potential of *B. fabae* strain (WAC13179) to not only pose a significant threat to susceptible genotypes of *T. dasyurum* but also to susceptible genotypes of pulses grown in rotation with *T. dasyurum*. In particular, the cross-pathogenicity of this strain could result in the continuous buildup of inoculum of *B. fabae* such that

it may eventually adversely affect the productivity of legumes in all rotations in Western Australia where one or more species each of *Pisum*, *Cicer*, *Vicia*, and *Lens* are grown in rotations with pastures.

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