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Long term viability of the northern anthracnose pathogen, *Kabatiella caulivora,* facilitates its transportation and spread

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Abstract

A range of materials, including metals, fabrics, woods and plastics, were all effective carriers that maintain the long term viability of conidia and resting hyphae of the northern anthracnose pathogen of *Trifolium* species, *Kabatiella caulivora.* Conidia and hyphae became thick walled and melanised with

time. There were significant (P<0.001) differences between carrier materials and between temperature regimes in terms of conidia/resting hyphae survival. At 23°C/4°C day/night, conidia and resting hyphae remained viable on steel, corrugated steel, galvanised steel and all tested fabrics, wood and random mixed materials for up to 8 months. At 36ºC/14ºC day/night, conidia and resting hyphae remained viable for up to 8 months, but only on cotton, denim, fleece, silk, leather, paper, plastic and all the wood materials. At 45ºC/15ºC day/night, conidia and resting hyphae remained viable up to 8 months, but only on fleece wool, *Eucalyptus marginata* (jarrah wood) and on paper. There were significant differences between carrier materials in their abilities to retain conidia and resting hyphae after washing (P<0.001). Metabolic activity was confirmed for conidia and resting hyphae recovered after 8 months and *K. caulivora* colonies successfully re-established on potato dextrose agar. Findings confirmed the critical importance of materials as long-term carriers of viable *K. caulivora* conidia and resting hyphae. Findings not only highlight the potential for spread of a highly virulent *K. caulivora* race inside and outside of Australia via farming equipment, clothing and other associated materials, but the wider biosecurity implications from the transportation of fungal-infested carrier materials previously considered as low-risk.

Introduction

In the era of increased personal and business travel, military deployments and exercises, sporting events and trade in commerce, the risk of inadvertent introduction of exotic fungal pathogens particularly by spores via introduction of contaminated equipment and commercial goods into a country or an area is increasing (McKirdy *et al.*, 2012, Hovmøller *et al.*, 2008). While pathogens may get introduced through flower, seed and plant product imports, and also by machinery movement and natural phenomena, their entry is not limited to these pathways only (McKirdy *et al.*, 2012). Further, while fungal spores may be primarily dispersed by aerial or rain splash, dispersal can also occur

through human activities allowing pathogens to spread locally or to new geographic areas (Fitt *et al.*, 1989, Savage *et al.*, 2012, Roper *et al.*, 2010) which plays a critical role in the disease spread and development.

There is a wide range of potential 'carrier materials' that could enable entry and spread of exotic fungal spores with materials contaminated by fungal spores widely used in many different situations, such as fabrics, metals, paper, rubber tyre, leather, wood and building materials (Whinam *et al.*, 2005, Sterflinger, 2010). For example, Osyczka *et al.* (2012) isolated four species of perithecia-forming Ascomycota, five species of corticioid and polyporoid Basidiomycota, and four unidentified sterile mycelia from basidiomycetes taxa from timber with some ascocarps containing vital spores. Further, Hill *et al.* (1995) isolated 38 species of fungi from timber flooring of freight containers including species such as *Alternaria*, *Aspergillus*, *Phoma*, *Mucor, Eurotium, Penicillium,* and *Trichoderma*. Fungal contamination is also a major problem on library books, paper sacks, and fibreboard cartons and also in food packing materials (Zyska, 1997, Tindale *et al.*, 1989, Rakotonirainy *et al.*, 2003). Although there has been research investigations on carrier materials, most of these investigations were related to live materials as plant germplasm, fruits, seeds, pollens (Vanneste *et al.*, 2011) rather than inert materials. Current data regarding materials such as metal, plastics, fabrics and paper etc. as spore carriers is extremely limited. The relative risk of incursion of pathogenic fungal spores with different materials needs urgent assessment as resulting data is needed to guide the biosecurity and quarantine sectors to take appropriate precautions and action.

Northern anthracnose, also known as 'clover scorch' in Australia, caused by the fungus *Kabatiella caulivora*, is of major economic importance in the temperate regions of the world particularly in Asia, North America, Europe and Australia (Beale, 1972; Johnsson, 1975; Barbetti & Sivasithamparam, 1986; Barbetti 1996). The disease remains a major limitation to *Trifolium* species forages (Johnstone & Barbetti, 1987; Barbetti, 1989), particularly reducing herbage yield, hay and seed production of annual subterranean clover (*Trifolium subterraneum*) (Johnsson, 1975; Bokor *et al*., 1978;

Anderson *et al*., 1982). In highly susceptible varieties such as Woogenellup and Yarloop, seed yield reductions of 90% or more have been measured (Barbetti, 1989; You *et al*., 2007). Reductions of about 30% in grazing capacity and up to 50% reduction in whole-farm hay production have also been attributed to clover scorch during seasons favourable for disease development (Barbetti, 1989; You *et al*., 2007). High rainfall areas of south western and southern Australia remain severely affected despite the widespread uptake by farmers of varieties with improved resistance (Nichols *et al*., 2014).

The disease progresses rapidly at temperatures of 18°C to 25°C, with low light intensity and wet conditions (Leach, 1962; Beale, 1972). *K. caulivora* produces conidia by budding and forms yeast-like colonies (Berkenkamp, 1969) with the conidia primarily spread by wind-driven rain-splash, livestock and also by attachment to hay cutting equipment (Beale, 1972; You *et al*., 2007, Barbetti & You, 2014) and in infested hay or seed (Beale, 1972; Bokor et al., 1978; You *et al*., 2007). Conidia can change form and colour over time producing several unusual features such as thick melanised conidia and thick walled hyphae (Colotelo & Grincmenko, 1962). Past investigations have demonstrated that the conidia of *K. caulivora* can survive a wide range of temperature conditions from 3-24°C for at least for 150 days and that they can be stored for more than three months at -20°C (You *et al*., 2005) and for 20 months at -10°C (Cole, 1957). *K. caulivora* also forms thick walled resting hyphae, presumably for long term survival (Bayliss *et al*., 2003), allowing it to survive in stored infested seed and in dead infested plant residues for at least for 18 months (Kellock, 1971; Beale, 1972), and enabling it to persist from one annual growing season to the next (Johnstone & Barbetti, 1987).

The pathogen invades, predominantly through the petiolules causing 'turning' the leaflets to expose the under surface (Bokor, 1972; Bayliss *et al*., 2001; Barbetti & You, 2014), the characteristic symptom of *K. caulivora* infection on subterranean clover (Barbetti, 1989) and red clover (Sampson, 1928). Brown to black lesions become visible on the petiolules, petioles, stems, runners and peduncles, destroying the vascular tissues and causing leaves to wilt and collapse (Barbetti & You, 2014).

The disease was initially controlled in Australia by sowing resistant subterranean clover varieties (Bayliss *et al.,* 2002; Nichols *et al*., 2014). While a single race, Race 1, was predominant in the clover growing regions of Australia for decades (Bayliss *et al.,* 2001), a new race, Race 2, was identified in 1990 in Western Australia (Barbetti, 1995) that has devastated fields of subterranean clover varieties such as Junee and Esperance, previously resistant to Race 1 (Barbetti, 1995; Barbetti & You, 2014). Race 2 continues to spread within Western Australia and likely will in future spread across southern Australia, and potentially outside of Australia (Barbetti & You, 2014). However, although *K. caulivora* is known to survive in infested seed and dead infested plant residues, there is no information about its survival and/or dispersal via inert carrier materials and under different environmental conditions, despite such information being particularly relevant for curtailing the spread of Race 2 both inside and outside of Australia. Hence, we investigated the viability of *K. caulivora* conidia and resting hyphae on a range of different carrier materials and under different temperature conditions to determine their potential to foster or impede future spread of new races of *K. caulivora* inside and outside of Australia.

Materials and Methods

Kabatiella caulivora inoculum

A single isolate of *K. caulivora* Race 2, WAC5757 (Barbetti, 1995), was used. Lyophilised cultures in glass ampoules were rehydrated using sterile deionised water and plated onto potato dextrose agar containing 100 mg l^{-1} of aureomycin hydrochloride. Cultures were maintained at 15°C and subcultured every 5 - 8 d to maintain the budding, yeast-type form required for inoculation (Cole & Couch, 1959; Barbetti, 1995). A platinum wire loop of conidia was scraped from each culture and used to inoculate 100 ml of sterile malt extract broth in 250 ml Erlenmeyer flasks that were incubated on a rotary shaker at 20°C at 150 rpm for 72 h. The conidial suspensions were filtered through four layers of muslin cloth

to remove any hyphae. The concentration of conidia was determined using a haemocytometer counting chamber (Superior® Marienfeld, Germany) and the suspensions stored at -20°C until needed. Prior to inoculation, conidia were resuspended in 0.001% Tween 20™ and adjusted to a concentration of 10^6 conidia ml^{-1} using a haemocytometer counting chamber.

Selection of carrier materials

A total of 20 different carrier materials were selected to determine both their effectiveness as potential conidia carriers per se and also any direct effects of the materials upon conidial survival. These were: *Metals* - aluminium, brass, corrugated iron sheet, galvanised steel, steel, zinc; *Fabrics* - cotton, denim, fleece, silk, fibre polyester *Woods* - *Eucalyptus marginata* (jarrah), *Pinus radiata* (pine), *Eucalyptus regnans* (Tasmanian oak); and *Miscellaneous* - glass, leather, jute, paper, plastic and rubber tyre. These were selected as test materials as they are commonly used materials found around farms, and/or associated in one way or another in commercial and farm transport, and/or used by travellers.

Inoculations of carrier materials and measurement of survival of *K. caulivora* over time at different temperatures

The materials were cut into 0.5cm squares and randomly placed into rows in a sterile 48-well cell culture plate (Greiner® sterile) along with an equal number of the non-inoculated control treatments.The material in each well was inoculated individually with 10µl (10^6 conidia ml^{-1}) of inoculum and allowed to dry in a laminar flow for 2-3 h. All sides of the inoculated culture plates were sealed with Parafilm™ and placed under one of the three controlled environmental conditions: 23 ± 1ºC day/8 ± 1ºC night, 36 ± 1ºC day/14 ± 1ºC night, or 45 ± 1ºC day/15 ± 1ºC night, with a photoperiod of 14 h from a light source consisting of LED cool white along with incandescent light bulbs with an overall intensity of 250 µmol.$m^{-2}s^{-1}$, 260 µmol.$m^{-2}s^{-1}$, 320 µmol.$m^{-2}s^{-1}$, respectively (Quantum Flux MQ100, Apogee). There were six replicates for each carrier material, for every sampling time and for each temperature

treatment. A fully randomized design was used. The experiment was run over an eight month period, with sampling to assess the effect of test carrier materials and temperature on viability of conidia, on thick walled melanised conidia, and on thick walled hyphae that developed over time. The ability of the test carrier materials to retain or release conidia, thick walled melanised conidia and hyphae was assessed daily from day 1 until day 7, then weekly till day 30 (month 1), after which sampling was undertaken at regular 30 d intervals concluding at day 240 (month 8). The entire experiment was fully repeated twice.

Recovery of *K. caulivora* conidia and hyphae from inoculated carrier materials and determination of viability of conidia

The viability of conidia and hyphae was assessed using an Alamar Blue resazurin dye (7-hydroxy-3H-phenoxazin-3-one 10-oxide) (Barua *et al*., 2017). Briefly, conidia and resting hyphae were recovered from the carrier materials by adding 800 µl of 0.001% Tween 20™ directly to the treatment plates and placing plates on a rotary shaker for 40 min at 700 rpm. After washing, the carrier materials were removed from the residual conidia suspension and prepared for microscopy studies. Initially, the Alamar Blue bioassay was optimized as a conidia viability indicator for *K. caulivora* and optimum time for maximum metabolic activity was determined as 2 h. *K. caulivora* conidia suspensions ranging in concentration from 10^7 ml^{-1} to 10 ml^{-1} by tenfold serial dilution were set up in 96-well assay plates and used as comparison standards. Three replicate wells were used for each concentration and were set up using 100 µl of fresh conidia for the standards, with 20 µl of Alamar Blue reagent. In the same way residual conidia solutions after washing were set up with 100 µl of residual solution/wash suspension containing conidia with 20 µl of Alamar Blue reagent. Negative controls were also similarly set up with no conidia but only deionised water to determine the extent of any background absorbance. The absorbance at 600 nm and 570 nm measured using the spectrophotometer (Thermo Scientific Multiskan® Spectrum). The average of the background absorbance value at 600 nm for control wells

was subtracted from all absorbance values of experimental wells at 570 nm. A standard curve of 570-600nm absorbance versus conidia and hyphae concentration was plotted to calculate the viability percentage of *K. caulivora* conidia and hyphae in residual solutions after washing. The percentage of viable conidia was determined for each replicate using the regression equation from the standard curve, and the mean calculated from the six replicates.

Observation of conidia germination, melanised conidia and hyphae

The carrier materials removed from the residual conidia suspensions after washing at each time interval were prepared, first, for scanning electron microscopy studies to observe the attachment of conidia and the hyphae on different carrier materials and, second, for light microscopic studies carried out to observe germination of conidia and formation of thick walled melanised conidia and resting hyphae. A 50 µl of each residual solution with conidia and hyphae after washing the carrier materials was placed on a microscope glass side and covered with a glass cover slip. The percentage of hyaline conidia, melanised conidia and thick walled melanised hyphae was determined using an Olympus (BX51) microscope. There were three replications for each sample. Time period of maximum percentage of conidia germination was also assessed. The hyphae derived from the carrier materials were rehydrated in 500 µl of sterile deionised water for 6 h and plated on potato dextrose agar media, maintained at 20°C, and observed for 7 d to monitor viability and growth of the hyphae.

Experimental design and data analyses

All the test carrier material treatments in the experiments were arranged in a completely randomized design with numbers of replications as indicated above. All experiments were fully repeated twice and the relationship between the initial and repeat experiments assessed using a paired *t*-test using GenStat (15th edition, GenStat Procedure Library Release PL23.2). Where there were no significant differences between experiments ($P>0.05$), the data sets from the two most-similar experiments were

pooled, re-analysed and presented as a single data set. Single and multiple factor ANOVA were conducted using GenStat to determine the effects of time and temperature on the viability of the *K. caulivora* conidia across an 8 month period and to determine the rate of melanisation of the conidia and hyphae over a 7 d period.

Results

Effect of temperature and time on recovery of viable *K. caulivora* conidia and hyphae from inert materials

The average percentage of viable conidia recovered from each carrier material varied with the temperature and the type of carrier material (P<0.001). Viable conidia and hyphae were recovered for up to 8 months from all materials incubated at 23ºC/8ºC day/night except aluminium, zinc and brass which remained viable only for 6 months, 5 months and 21 days, respectively (Table 1). At 36ºC day/14ºC, conidia and hyphae viability was more variable, with conidia on brass and galvanised steel remaining viable for only 14 days, but conidia on other materials remaining viable for up to 8 months (Table 1). At 45ºC/15ºC day/night, the maximum time period of viability of conidia and hyphae was markedly reduced for most materials, but viability persisted, for 8 months on fleece (Table 1).

Viability of conidia and hyphae of *K. caulivora* recovered from carrier materials over time and temperatures

The different groups of materials varied in their capacity to retain viable conidia and hyphae and this also varied according to the temperature at which the materials were maintained. The percentage of viable conidia recovered from materials from a particular group varied with the temperature. There were significant (*P*<0.001) differences between carrier materials, over time and between temperatures, in terms of viability of conidia and hyphae (Fig. 1).

Metals: At 23ºC/8ºC day/night, the maximum percentage of viable conidia and hyphae recovered at 8 months was 0.3% from corrugated iron and also from steel (0.3%); at 1 month it was 9.8% from steel and at one week it was 19.1% from steel (Fig. 1). At 36ºC/14ºC day/night, the maximum percentage of viable conidia and hyphae recovered at 6 months was 0.2% from steel, at 1 month it was 3.4% from aluminium and at one week it was 5.4% from aluminium. The maximum percentage of viable conidia and hyphae recovered at 5 months at 45ºC/15ºC day/night was 0.2% from zinc, at 1 month it was 1.1% from steel and at one week it was 6.1% from corrugated iron (Fig. 1)

Fabrics: At 23ºC/8ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 10.9% from cotton, at 1 month it was 29.2% for cotton, while at one week it was 39% from fibre polyester. At 36ºC/14ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.3% from cotton, while at 1 month it was 18.4% from denim and at one week it was 19.1% from cotton. At 45ºC/15ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.2% from denim, while at 1 month it was 0.5% from silk and at one week it was 7.7% from fleece. (Fig. 1).

Woods: At 23ºC/8ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 8.3% from jarrah wood, at 1 month it was 12.1% from Tasmanian oak and at one week it was 15% from Tasmanian oak. At 36ºC/14ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 5.1% from pine wood, at 1 month it was 9.6% from jarrah wood and at one week was 10.7% from Tasmanian oak. At 45ºC/15ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.3% from jarrah wood, at 1 month it was 4.8% from Tasmanian oak and at one week it was 8.9% from Tasmanian oak. (Fig. 1).

Miscellaneous: At 23ºC/8ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.5% from plastic and rubber tyre, at 1 month it was 19.1% from jute and at one week it was 26.1% from jute. At 36ºC/14ºC day/night, the maximum viable conidia and hyphae recovered at 8 months was 0.2% from plastic, at 1 month it was 3.6% from plastic and at one week it was 8.9% from

rubber tyre. At 45ºC/15ºC day/night, the maximum viable conidia and hyphae recovered at 7 months was 0.2% from leather and paper, at 1 month it was 2.3% from plastic and at one week it was 7.7% from rubber (Fig. 1).

Observation of melanised conidia and resting hyphae: Light microscopy studies revealed that the number of hyaline conidia observed at the time of inoculation decreased from day 1 (25%) to day 7 (0%) and some (12%) of conidia degenerated (Table 2). Within 2 days of inoculation, geminated and non-germinated conidia (>70%) changed their colour and shape, then elongated and formed thick cell walls that became melanised and septate in the majority (65%) of cases (Fig. 3 A). Many of the conidia germinated and formed thick walled resting hyphae over time (Fig. 2, 3). Maximum average percentage (up to 55%) of conidia germination was observed within 1 - 5 days after inoculation (dai) across all carrier materials. All conidia that germinated were melanised, but approximately 15% of the melanised conidia did not germinate. Thick walled melanised hyphae were observed from day 1 and the percentage of hyphae increased with the increase in germination (Table 2). At day 7, >50% of germinated conidia, 15% of non-germinated conidia and 35% of resting hyphae developed clear melanisation (Table 2). Conidia produced germ tubes and then formed mycelial colonies on the surface of the various materials (Fig. 2A-F). The colour of these mycelial colonies varied and changed over time (Fig. 3A-C). The mycelial colonies were pale white to light olive coloured or light brown when beginning to form, but changed to darker brown or black from 15 dai onwards (Fig. 3A-C). The mycelia eventually formed thick walled resting hyphae within 30 dai (Fig. 3). After 5 months, while hyphae did not show any metabolic activity immediately, when soaked in water over a 4 – 6 hour period, the subsequent swollen hyphae demonstrated viability by changing the colour of the resazurin dye. Resting hyphae then transferred to potato dextrose agar germinated and produced several germ tubes and then branching mycelia, further confirming their viability. Scanning electron microscopy studies revealed that *K. caulivora* conidia and resting hyphae remained attached to each of the carrier materials even after washing (Fig. 2A-F).

Discussion

This is the first study to demonstrate that *K. caulivora* can remain viable on inert carrier materials at temperatures up to 45°C for up to 8 months. In the absence of a plant host, *K. caulivora* survived on tested carrier materials by forming thick walled melanised conidia and thick walled melanised resting hyphae. While most conidia changed size and shape, developed thick walls and became melanised within 1 to 2 days of inoculation on the carrier materials, the 12% of the conidia that remained hyaline all degenerated. Where conidia had become elongated, melanised and septate, they produced multiple germ tubes arising randomly from germinating conidia to and resting hyphae form mycelial colonies. These melanised thick walled conidia and resting hyphae had a clear survival advantage, especially under higher temperatures and for a much longer period than did conidia remaining hyaline and degenerating. The colour of the mycelial colonies varied initially from pale white, light green to light brown and finally to darker brown, and were heavily melanised and black from 15 dai onwards. Cole and Couch (1959) reported colour variation of mycelial colonies of *K. caulivora* as varying among and within colonies, from white to pink to olive-green, brown and black in many shades, especially across different growth media. While such studies detailed changes in colour and shape of conidia on different growth media and the formation of melanised, thick-walled, dark green conidia and even anastomosis of mycelium under limited nutrient availability, the current study is the first report of such occurring on inert carrier materials. Such cell wall thickening and melanisation changes have been assumed to assist with the survival of the pathogen under adverse environmental conditions (Sampson, 1928; Cole, 1957; Colotelo & Grincmenko, 1962). The current study also demonstrated that melanised conidia and mycelia can form a hyphal mass on carrier materials, with mycelia becoming anastomosed and forming round thick resting hyphal coils. Formation of such hyphal coils may be a thigmotropic response of the pathogen to the surface of the carrier materials, as adverse environmental conditions have been suggested as a cause of such hyphal coils (Bayliss *et al*., 2001; Piérard *et al*., 2007). These fungal coils

were also proposed as a possible mechanism to overcome host resistance (Bayliss *et al*., 2003). Further, and perhaps more importantly, the current study demonstrated for the first time that these hyphal coils not only remain viable for up to 8 months, but that they can, without producing any conidia, 'germinate' to produce multiple germ tubes and branching mycelial on potato dextrose agar.

There were significant differences between temperature regimes in terms of survival of conidia and resting hyphae, and upon which carrier materials best prolonged survival. For example, at 23°C/4°C day/night, conidia and resting hyphae remained viable on many test materials for up to 8 months, including steel, corrugated steel, galvanised steel and all tested fabrics, wood and random mixed materials. However, at 36ºC/14ºC day/night, conidia and resting hyphae remained viable for up to 8 months only on cotton, denim, fleece, silk, leather, paper, plastic and all the wood materials. In contrast, at 45ºC/15ºC day/night, conidia and resting hyphae remained viable up to 8 months only on fleece wool, *E. marginata* (jarrah wood) and on paper. Although disease epidemics develop best at lower temperatures and in wet weather, conidia and hyphae of the pathogen are known to survive long periods at high temperatures in infested residues and with ability to subsequently initiate disease when conditions are suitable (Sigrianski & Minyaeva, 1937; Leach, 1962; Helms, 1977). Berkenkamp (1969) reported that production of conidia and disease development was best at cool temperatures between 12-20°C. Anderson *et al.* (1982) highlighted a strong relationship between environment and disease development in subterranean clover forages, with mean temperatures of 11-17°C and frequent rainfall favouring disease development. In contrast, others have reported that more severe disease symptoms occur at temperatures 20°C and 22.5°C (Guerret *et al*., 2016) or at temperatures 20°C and 24°C (Cole & Couch, 1958; Darunday & Hanson, 1967). Prolonged warm to hot sunny weather stops disease development (Anderson *et al.*, 1982) and *K. caulivora* is inactivated when the temperature reaches 28°C (Berkenkamp, 1969; Johnsson, 1975). However, that conidia and resting hyphae of *K. caulivora* could survive at temperatures up to 45°C on fleece wool, *E. marginata* (jarrah wood) and on paper for a period of at least 8 months in the current study, highlights the significant potential for *K. caulivora*

conidia and resting hyphae to survive for long periods at much higher temperatures than previously considered possible from other epidemiological studies with this pathogen. These findings have major implications both for longer effective *K. caulivora* carryover across multiple forage growing seasons and for domestic and international movement of people and commodities that can effectively carry and retain conidia and resting hyphae. This poses an environmental and economic biosecurity threat in regions where northern anthracnose, or specific race(s) of it, do not yet occur.

While there were significant differences between carrier materials, that *K. caulivora* thick walled melanised conidia and hyphae remained attached to and viable on a range of carrier materials for up to 8 months is of concern. Importantly, there were also significant differences between carrier materials in their abilities to retain conidia and resting hyphae after washing. That metabolic activity was confirmed for conidia and resting hyphae recovered after 8 months and that *K. caulivora* colonies could be successfully re-established on potato dextrose agar from such spores is of even greater concern. That even after two to three washes, not all conidia or resting hyphae could be removed from the carrier materials in the current study, highlights the challenges in eliminating fungal spore and hyphae contamination from farm machinery, clothes of regional, national and international travellers, etc. Such contamination allows transportation and spread of *K. caulivora* into areas where it, or a specific race of it, is not yet present. For example, *K. caulivora* Race 2 is predominantly found in the Esperance region of Western Australian (Barbetti, 1996; Barbetti, 2007) but could quickly spread much further afield on insert materials such as farm equipment and clothing. Many of the varieties of subterranean clover with resistance against *K. caulivora* Race 1 are very susceptible to Race 2 (Barbetti, 1995,1996; Bayliss *et al*., 2002), and the threat of spread of Race 2 more widely across southern Australia threatens >1 m ha of varieties currently with effective resistance against Race 1 (MJ Barbetti, unpubl.). Further, the increasingly variable and changing climatic conditions will likely encourage development of further new races of *K. caulivora* (Guerret *et al*., 2016), and as is already happening with other fungal pathogens in Western Australia such as *Sclerotinia sclerotiorum* on oilseed rape (Uloth *et al*., 2015). Any such *K.*

caulivora isolates that were better adapted to warmer conditions would pose a potential threat not only to currently affected regions per se but also to regions where the disease is currently not important due to current unfavourable environmental conditions. As already noted, attachment of any new highly virulent races to one or more of these carrier materials opens the way for their spread into new regions internal and external to Australia.

Current studies confirm the critical importance of materials as long-term carriers of viable *K. caulivora* conidia and resting hyphae. They highlight the threat posed by the long-term maintenance of viability of *K. caulivora* conidia and hyphae on inert materials associated with movement of humans, farming equipment, clothing and commodities across regions inside and outside of Australia where Race 2 is not yet present. Finally, the current studies have wider biosecurity implications in relation to the transportation of fungal-infested carrier materials in general, as they highlight an urgent need to re-evaluate potential carrier materials that have historically long been considered of low biosecurity risk in regards to the movement of fungal plant pathogens.

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TABLE CAPTIONS

Table 1 Maximum time period of viability of *Kabatiella caulivora* conidia observed on different carrier materials over an 8 month time period under three different controlled temperature regimes.

Table 2 Percentage of conidia melanised and germinated, unmelanised, degenerated and the percentage of melanised resting hyphae of *Kabatiella caulivora* over a time period of seven days.

FIGURE CAPTIONS

Fig. 1 Box plot of viability of *Kabatiella caulivora* conidia observed on different carrier materials over an 8 month time period under three different controlled temperature regimes. Each box shows the viability of the conidia on the respective material over time period from day 1 to 8 months where T1 = 23 ± 1°C day/8 ± 1°C night; T2 = 36 ± 1°C day/14 ± 1°C night and T3 = 45 ± 1°C day/15 ± 1°C night. l.s.d values at $P < 0.05$ are 0.23 (carrier material), 0.22 (time), 0.09 (temperature), 1.68 (carrier material x time), 0.41 (carrier material x temperature), 0.37 (time x temperature) and 1.68 (carrier material x time x temperature).

Fig. 2 Scanning electron micrographs showing germination of conidia of *Kabatiella caulivora* 5 days after inoculation on **A**, steel and on **B**, plastic; and showing formation thick walled hyphae at 3 months after inoculation on **C**, silk, **D**, cotton, **E**, fleece, and **F**, denim.

Fig. 3 Light micrographs showing the formation of thick walled resting hyphae and melanisation (change in the colour) of hyphae of *Kabatiella caulivora* on glass slides over an 8 month period; **A**, at 3 days after inoculation, **B**, at 3 months after day of inoculation, **C**, **D**, **E** and **F** at 8 months after inoculation.

Table 1 Maximum time period of viability of *Kabatiella caulivora* conidia observed on different carrier materials over an 8 month time period under three different controlled temperature regimes.

Material	**Temperature**		
	23°C/4°C day/night	**36ºC/14ºC day/night**	**45ºC/15ºC day/night**
Metals			
Aluminium	6 months	3 months	3 months
Brass	21 days	14 days	2 days
Corrugated	> 8 months	2 months	21 days
Galvanised	> 8 months	14 days	6 days
steel	> 8 months	2 months	2 months
Zinc	5 months	2 months	2 months
Fabrics			
Cotton	> 8 months	> 8 months	5 months
Denim	> 8 months	> 8 months	6 months
Fleece	> 8 months	> 8 months	> 8 months
Silk	> 8 months	> 8 months	6 months
Fibre polyester	> 8 months	7 months	4 months
Wood			
Jarrah	> 8 months	> 8 months	> 8 months
Pine	> 8 months	> 8 months	7 months
Tasmanian Oak	> 8 months	> 8 months	7 weeks
Miscellaneous			
Glass	> 8 months	7 months	6 months
Leather	> 8 months	> 8 months	7 months
Paper	> 8 months	> 8 months	> 8 months
Plastic	> 8 months	> 8 months	6 months
Rubber	> 8 months	6 months	6 months
Jute	> 8 months	7 months	7 months

Table 2 Percentage of conidia melanised and germinated, unmelanised, degenerated and the percentage of melanised resting hyphae of *Kabatiella caulivora* over a time period of seven days.

Time (days)	Melanised conidia (germinated)	Unmelanised conidia	Degenerated conidia	Melanised resting hyphae
1	40	25	10	2
2	43	15	12	10
3	50	2	12	28
4	53	1	12	29
5	55	0	12	30
6	54	0	12	31
7	50	0	12	35

	Melanisation	*Time*	*Melanisation x Time*
Significance	<0.001	*n.s.*	<0.001
l.s.d (P<0.05 =)	0.69	*n.s.*	1.82

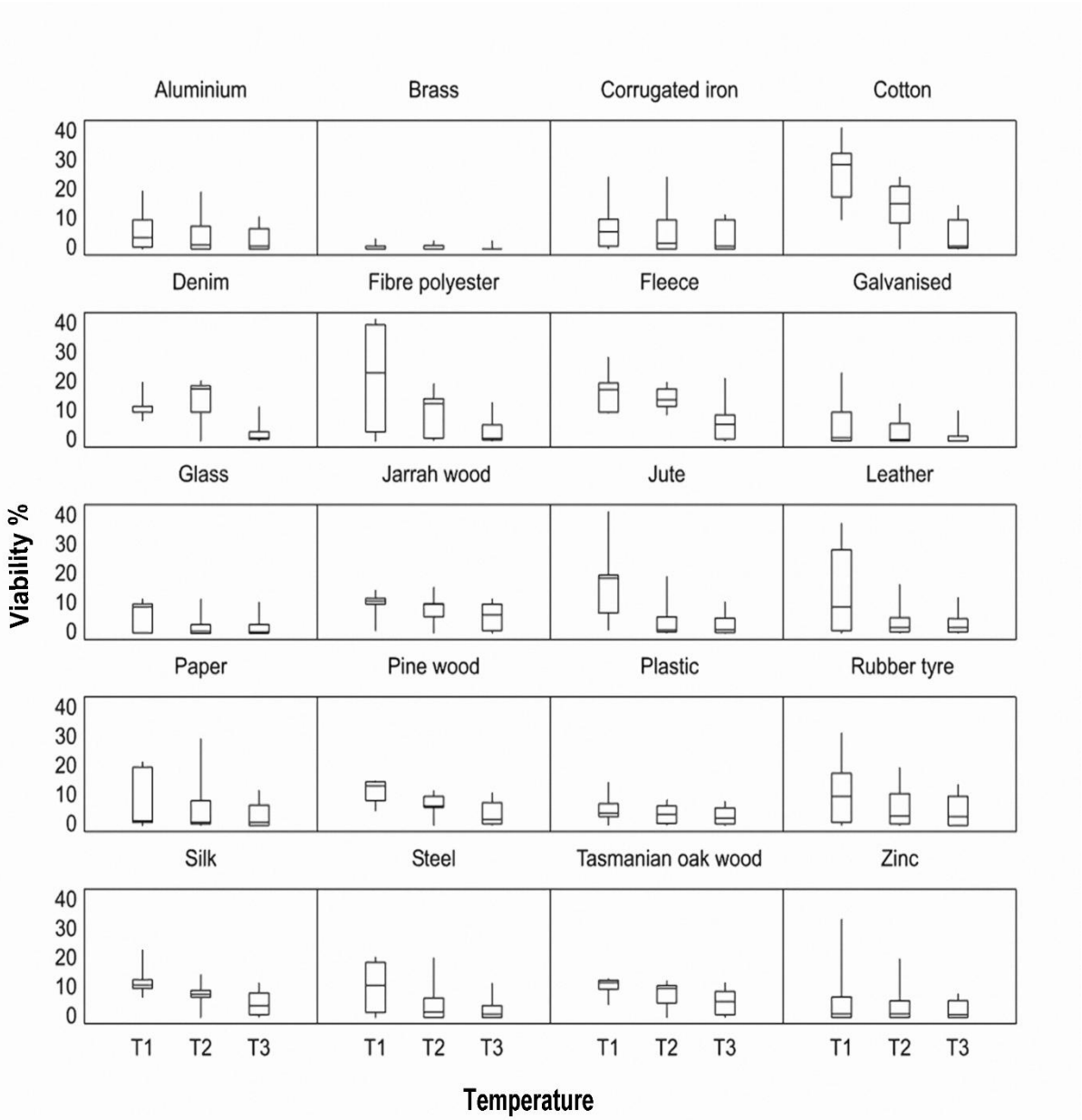

Fig. 1

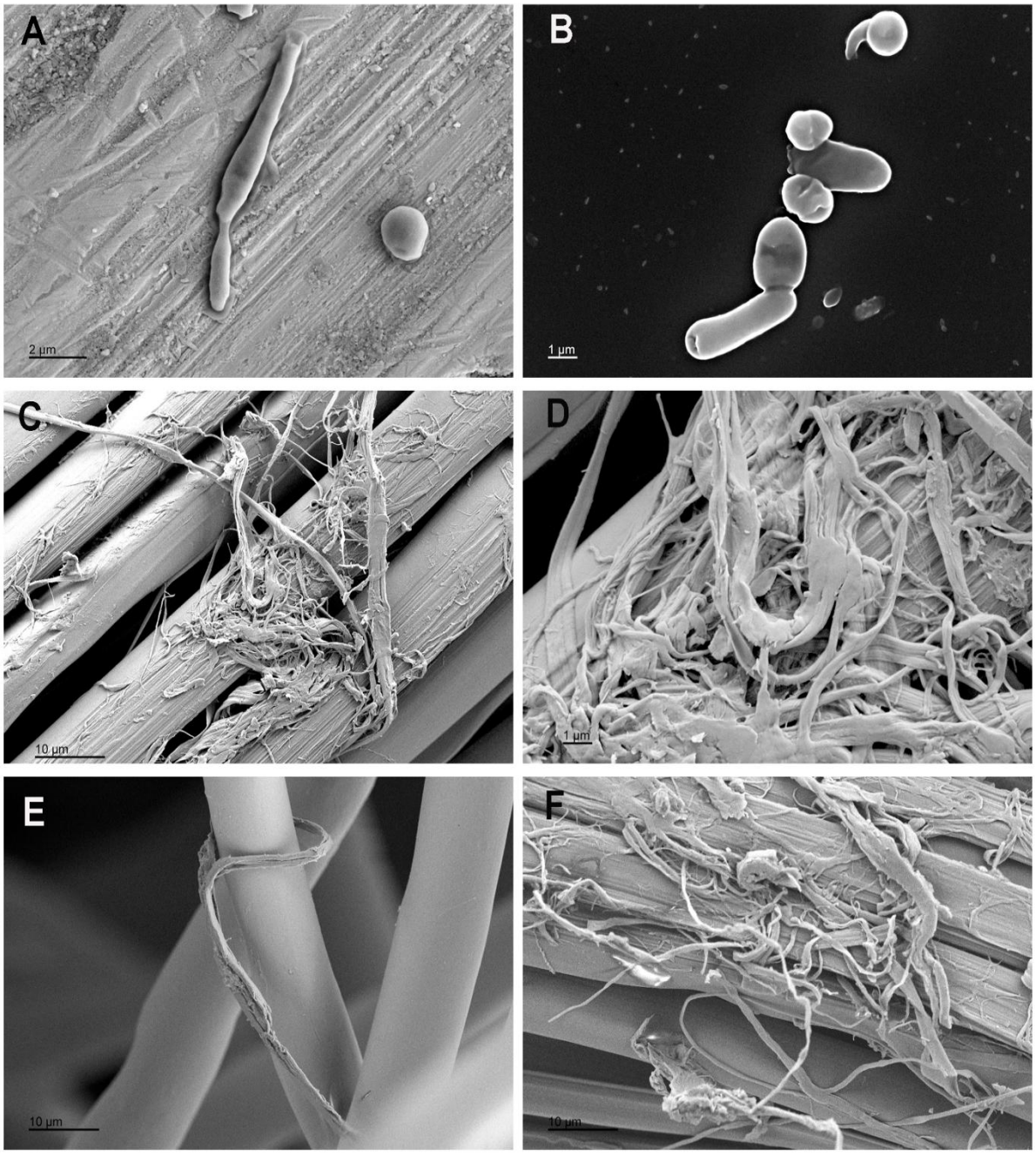

Fig. 2

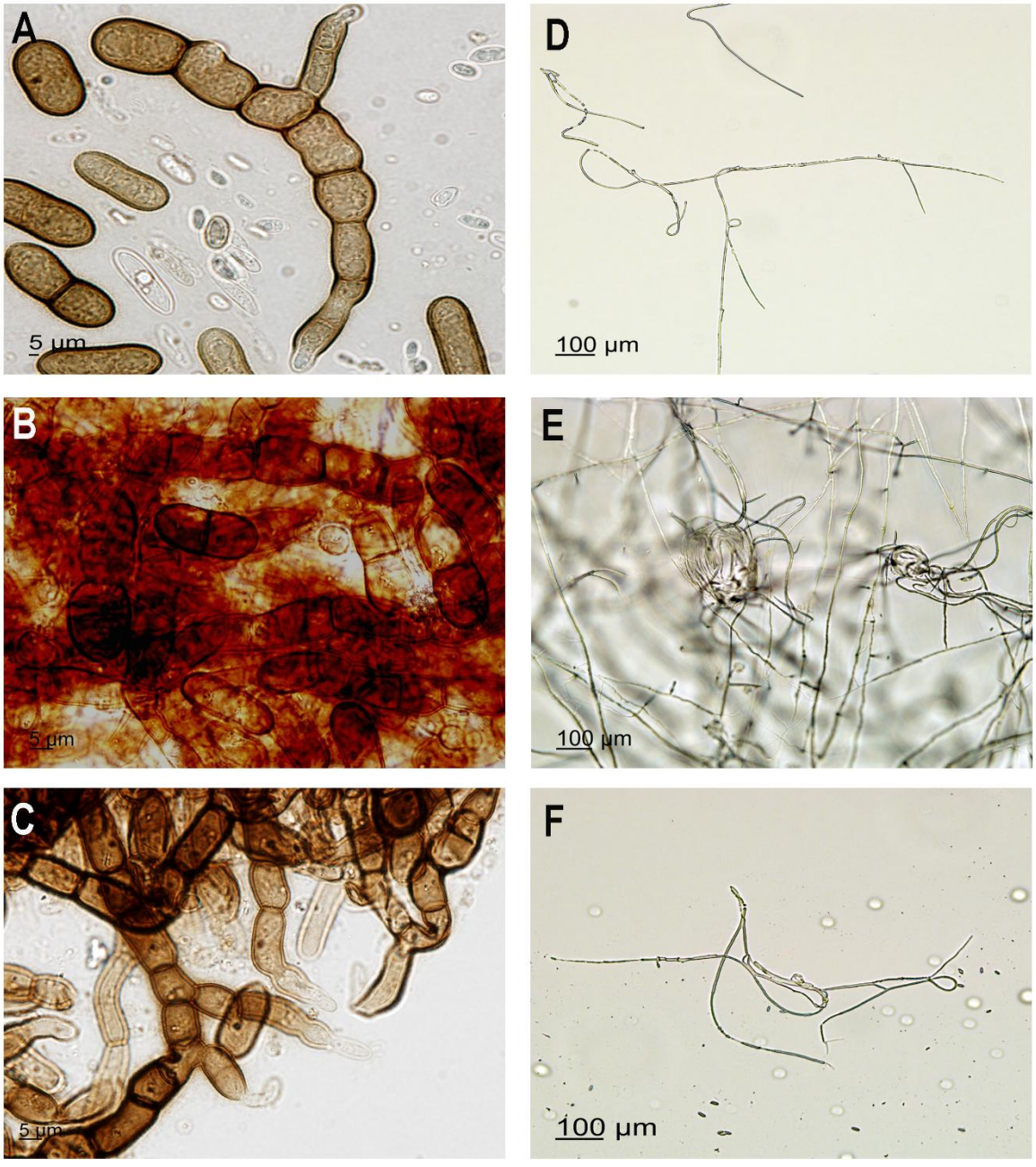

Fig. 3