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Contrasting carbon metabolism in saprotrophic and pathogenic microascalean fungi from *Protea* trees

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ABSTRACT

Protea-associated *Knoxdaviesia* species grow on decaying inflorescences, yet are closely related to plant pathogens such as *Ceratocystis albifundus*. *C. albifundus* also infects *Protea*, but occupies a distinct niche. We investigated substrate utilization in two *Knoxdaviesia* saprotrophs, a generalist and a specialist, and the pathogen *C. albifundus* by integrating phenome and whole-genome data. On shared substrates, the generalist grew slightly better than its specialist counterpart, alluding to how it has maintained its *Protea* host range. *C. albifundus* grew on few substrates and had limited cell wall-degrading enzymes. It did not utilize sucrose, but may prefer soluble oligosaccharides. Nectar monosaccharides are likely important carbon sources for early colonizing *Knoxdaviesia* species. Once the inflorescence ages, they could switch to degrading cell wall components. *C. albifundus* likely uses its limited cell wall-degrading arsenal to gain access to plant cells and exploit internal resources. Overall, carbon metabolism and gene content in three related fungi reflected their ecological adaptations.

1. Introduction

Arthropod-vectored fungi in the Microascales have been the source of great economic losses world-wide. These include various pathogens of food crops (e.g. *Ceratocystis fimbriata s.s.*, *Ceratocystis cacaofunesta* and *Ceratocystis manginecans*; Engelbrecht et al., 2007; Van Wyk et al., 2007) and of plantation trees (e.g. *Ceratocystis albifundus*; Wingfield et al., 1996; Roux and Wingfield, 2013), as well as saprotrophic or weakly pathogenic species (e.g. *Ceratocystis adiposa*) that cause sap stain of plantation trees (Uzunovic et al., 1999). Arthropod-vectored fungi in this order have also been reported to colonize the flower heads (infructescences) of South African *Protea* species without showing any obvious signs or symptoms of disease (Wingfield et al., 1988; Wingfield and Van Wyk, 1993). The fungi from this unusual niche were identified as species of *Knoxdaviesia* (Wingfield et al., 1988; Wingfield and Van Wyk, 1993; De Beer et al., 2013).

In addition to *Protea*, *Knoxdaviesia* species have been reported from a range of dead, decaying or diseased wood and plant tissues (Morgan-Jones and Sinclair, 1980; Pinnoi et al., 2003; Van der Linde et al., 2012; De Beer et al., 2013), as well as weevil galleries (Kolařík and Hulcr, 2009). The genus includes nine species, of which only three are associated with *Protea*. One of these is *Knoxdaviesia wingfieldii* that was discovered on *Protea caffra* in KwaZulu-Natal (Crous et al., 2012). The remaining two species are known only from the Cape Floristic Region (CFR) biodiversity hotspot in the Western Cape Province of South Africa (Mittermeier et al., 1998; Bergh et al., 2014). Here, *Knoxdaviesia proteae* occurs exclusively on *Protea repens* (Wingfield et al., 1988), while the closely related *Knoxdaviesia capensis* occurs on several *Protea* species (Wingfield and Van Wyk, 1993; Roets et al., 2009b; Aylward et al., 2015).

Protea-associated *Knoxdaviesia* species are primarily vectored by mites (Roets et al., 2011b), and these mites, in turn, are phoretic on the beetle, and possibly bird, pollinators of *Protea* species (Roets

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et al., 2009a; N. Theron pers. comm.). The fungi enter the *Protea* inflorescences early in the flowering stage, when flowers are pollen receptive (Roets et al., 2009a). During this time, the inflorescences produce copious amounts of nectar (Cowling and Mitchell, 1981; Wiens et al., 1983) that likely support the growth of the fungi. Once the inflorescences of serotinous *Protea* plants have completed flowering, they are enclosed by involucral bracts to form fire-safe, seed storage structures known as infructescences (Rebelo, 2001). Within these enclosed structures, *Knoxdaviesia* species flourish and can occur on the styles, pollen presenters and perianth of the dead flowers as well as on the inner surface of the involucral bracts (Lee et al., 2005). Their sporulating ascomata are some of the most dominant fungal features observed within infructescences (Marais and Wingfield, 1994; Lee et al., 2005) and can proliferate for more than a year after pollination (Roets et al., 2005). At this stage, nectar sugars are likely depleted and it is expected that the *Protea*-associated fungi rely on the decaying floral parts as a food source.

The decaying infructescence habitat occupied by *Knoxdaviesia* species varies greatly from that of many other microascalean fungi that typically invade living vascular plant tissue (Morris et al., 1993; Van Wyk et al., 2007; Roux and Wingfield, 2009). For example, the black wattle (*Acacia mearnsii*) pathogen, *C. albifundus*, infects vascular tissue through wounds caused by other biotic or abiotic factors (Roux et al., 2007). Easy re-isolation of the pathogen from discoloured sapwood suggests that it has direct contact with both xylem and phloem vessels, although the rapid upward infection (Roux et al., 1999) could implicate xylem as the primary mode of spread. Phloem sap is rich in carbohydrates, of which sucrose is frequently the dominant component (Pritchard, 2007). Xylem does not contain sugars, but transports mineral nutrients to which *C. albifundus* would have direct access (Lucas et al., 2013). Although the eventual death of the plant results from wilting, the observed lesions and gummosis caused by this pathogen suggest that *C. albifundus* also attacks plant cells (Morris et al., 1993; Roux et al., 1999).

In the Western Cape Province, tree hosts of the pathogenic microascalean fungi are widespread across commercial plantations that often border areas of natural fynbos vegetation and several plantation species have invaded fynbos (Van Wilgen, 2009). Despite this, the *Protea*-associated species have not been found on the hosts of their pathogenic relatives. The converse is also true, where both apparently native and introduced microascalean pathogens have been found on plantation and even indigenous forest trees (Roux et al., 2007; Kamgan et al., 2008; Roux and Wingfield, 2009), yet these pathogens have never been found in *Protea* infructescences. *C. albifundus* has, however, been reported from stem cankers of several indigenous trees in South Africa, including *Protea* species (Crous et al., 2004; Roux et al., 2007; Lee et al., 2016). Even though the *Protea* host is shared in this case, a distinct difference in niche occupation is apparent, with the pathogenic species occupying the vascular tissue associated with wounds (Morris et al., 1993; Roux et al., 1999) and the saprotrophic species decaying floral parts in seed cones.

Considering that the different responses of fungal taxa to carbon resources may facilitate niche partitioning (Hanson et al., 2008), we integrate phenome and whole-genome data to consider the substrate utilization of the generalist *K. capensis*, the specialist *K. proteae* and the pathogen *C. albifundus*. Our first objective was to investigate the difference in host range between the two *Knoxdaviesia* species. Roets et al. (2011a) found that although the chemistry of *Protea* is not the only factor influencing the occurrence of *Protea*-associated ophiostomatoid fungi, it is the dominant factor in *Protea* species that host *Knoxdaviesia*. These authors also observed that both *K. capensis* and *K. proteae* grow optimally in culture when flowers of their natural *Protea* host are added to the medium. We hypothesize that *K. capensis* is capable of using a wider range of substrates than *K. proteae*, enabling it to associate with different *Protea* hosts with variation in host chemistries. Our second objective was to characterize metabolic pathways that facilitate life in *Protea* infructescences in contrast to a lifestyle involving pathogenic wound association. We hypothesize that the vascular tissue habitat of *C. albifundus* enables it to persistently exploit the availability of sugars, whereas *Knoxdaviesia* species may only be exposed to nectar sugars for a short period after which they must switch to breaking down complex polysaccharides from decaying plant cells.

2. Materials and methods

2.1. Fungal isolates

One isolate of each of *K. capensis* (CBS139037), *K. proteae* (CBS140089) and *C. albifundus* (CMW17620) were used. These isolates were selected because their genome sequences are in the public domain (Van der Nest et al., 2014; Aylward et al., 2016). For the analysis of glucose, fructose and sucrose usage, we included four additional isolates of *C. albifundus* (CMW4068, CMW13980, CMW17274, CMW24685). For the duration of the study, all isolates were grown on malt extract agar (MEA; Merck, Wadeville, South Africa) at 25 °C and have been conserved in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2. Phenotype microarrays

Biolog FF MicroPlates PM1 and PM2 (Biolog Inc., Hayward, CA) were used to investigate carbon metabolism in *K. capensis*, *K. proteae* and *C. albifundus* CMW17620. Each microplate contained 95 distinct carbon sources and one water control (Table S1, Supplementary File 1). Fungal cultures were grown on MEA plates overlaid with sterile cellophane (Sigma-Aldrich, Steinham, Germany) until the fungal growth covered two thirds of the cellophane (approximately 10 d). Fungal material was scraped from the cellophane, placed in 1.5 ml sterile water and homogenized with three 3 mm glass beads in an MM301 TissueLyser (Retsch, Inc., Germany). The mycelial debris was pelleted by centrifugation at 1.1*g* for 1 min and the suspension transferred to a new Eppendorf tube. A dilution series of the suspension was prepared and the transmittance (T) measured at 600 nm using a PowerWave™ HT Scanning Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Dilutions were adjusted to obtain a suspension with a T of 62% (absorbance = 0.21). The 62% T suspension was used to prepare the fungal inoculum as described in the Biolog protocol (PM Procedures for Filamentous Fungi, 25-Aug-07) and 100 µl of the inoculum was pipetted into each well of the microplates. Three technical replicates were performed several weeks apart. A new inoculum was prepared for every replicate, whereas the same inoculum was used on both PM1 and PM2 for each individual replicate of a species. The plates were incubated at 25 °C and the amount of mycelial biomass produced in each well was measured at regular intervals (*ca*. five times daily) for seven days at 750 nm (Atanasova and Druzhinina, 2010; Mchunu et al., 2013), using the microplate spectrophotometer.

Glucose, fructose and sucrose utilization was subsequently verified using the two *Knoxdaviesia* isolates and all five of the *C. albifundus* isolates included in this study. We aimed to replicate the conditions in the Biolog microplate by using agar (15 gl^{-1}) medium supplemented with the 20% (weight:volume) of the relevant sugar and inoculating 15 µl of a spore suspension prepared according to the Biolog protocol. Additionally, 11 mgl^{-1} phenol red sodium salt (Sigma-Aldrich, Steinham, Germany) was added to act