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

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Highlights

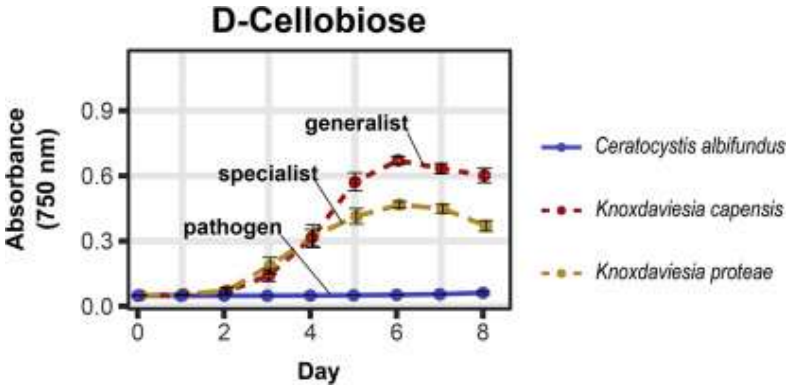
- Saprotrophic *Knoxdaviesia* species can utilize the simple sugars in nectar.
- Cellulose and hemicellulose-degrading enzymes are abundant in *Knoxdaviesia*.
- Cell wall-degrading enzymes are limited in the pathogen, *Ceratocystis albifundus*.
- *C. albifundus* lacks key enzymes and transporters to utilize exogenous simple sugars.
- Carbon metabolism and gene content in related fungi reflect their ecologies.

Abstract

Protea-associated *Knoxdaviesia* species grow on decaying inflorescences, yet are closely related to plant pathogens such as *Ceratocystis albifundus*. *Ceratocystis albifundus* also infects *Protea*, but occupies a distinct niche. We investigated substrate utilization in two *Knoxdaviesia* saprotrophs, a generalist and 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. *Ceratocystis 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. *Ceratocystis 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.

Keywords: *Ceratocystis*, carbon utilization, *Knoxdaviesia*, Microascales, proteome, growth substrate

Graphical abstract



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.*, *C. cacaofunesta* and *C. manginecans*; Engelbrecht et al., 2007; Van Wyk et al., 2007) and of plantation trees (e.g. *C. albifundus*; Roux and Wingfield, 2013; Wingfield et al., 1996), as well as saprotrophic or weakly pathogenic species (e.g. *C. 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 and Van Wyk, 1993; Wingfield et al., 1988). The fungi from this unusual niche were identified as species of *Knoxdaviesia* (De Beer et al., 2013; Wingfield and Van Wyk, 1993; Wingfield et al., 1988).

In addition to *Protea*, *Knoxdaviesia* species have been reported from a range of dead, decaying or diseased wood and plant tissues (De Beer et al., 2013; Morgan-Jones and Sinclair, 1980; Pinnoi et al., 2003; Van der Linde et al., 2012), 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 *K. wingfieldii* that was discovered on *P. 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 (Bergh et al., 2014; Mittermeier et al., 1998). Here, *K. proteae* occurs exclusively on *P. repens* (Wingfield et al., 1988), while the closely related *K. capensis* occurs on several *Protea* species (Aylward et al., 2015; Roets et al., 2009b; Wingfield and Van Wyk, 1993).

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 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 (Lee et al., 2005; Marais and Wingfield, 1994) 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; Roux and Wingfield, 2009; Van Wyk et al., 2007). For example, the black wattle (*Acacia mearnsii*) pathogen, *Ceratocystis 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 (Kamgan et al., 2008; Roux et al., 2007; Roux and Wingfield, 2009), yet these pathogens have never been found in *Protea* infructescences. *Ceratocystis albifundus* has, however, been reported from stem cankers of several indigenous trees in South Africa, including *Protea* species (Crous et al., 2004; Lee et al., 2016; Roux et al., 2007). 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 *Knoxdaviesia capensis* (CBS139037), *Knoxdaviesia proteae* (CBS140089) and *Ceratocystis albifundus* (CMW17620) were used. These isolates were selected because their genome sequences are in the public domain (Aylward et al., 2016; Van der Nest et al., 2014). 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 days). 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^{TM} 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 as a visual indicator. Potato Dextrose Agar (Merck, Wadeville, South Africa) with phenol red sodium salt was used as a positive control and medium containing agar only (15 gL^{-1}), also with phenol red

sodium salt, served as negative control. The experiment was performed in triplicate. Phenol red is a pH indicator that is yellow at pH < 6.5 and red at pH > 8, and can, therefore, indicate the accumulation of acidic metabolic by-products. The initial pH of the culture media was increased by adding 1-2 µl of 5M NaOH per millilitre agar until a pink colour was achieved. Cultures were grown for two weeks at 25°C after which growth was investigated with the aid of a Leica ES2 dissection microscope.

2.3. Phenome analysis

Growth curves from the absorbance values recorded from the inoculated PM1 and PM2 BioLog plates were drawn in R 3.2.5 (R Core Team, 2016) using a custom script (Shaffer, 2017). With this script, the time points at which measurements were taken were collapsed into days and the average absorbance per day across the three replicates were plotted with the standard error of the mean. Welch t-tests, implemented in R, were used to test whether the absorbance values of each substrate were significantly higher ($P < 0.05$) from the negative control across the entire experiment. The false discovery rate was controlled for by considering the Benjamini and Hochberg (1995) corrected P-values.

2.4. Genome analysis

The genomes of *K. capensis* (LNGK00000000.1), *K. proteae* (LNGL00000000.1) and *C. albifundus* (JSSU00000000.1) are available on GenBank® (Benson et al., 2013). We annotated the *Knoxdaviesia* genomes with MAKER (Cantarel et al., 2008; Holt and Yandell, 2011) in a previous study (Aylward et al., 2016), whereas the MAKER annotations of the *C. albifundus* genome (Van der Nest et al., 2014) were provided by D. Roodt. To characterize

metabolic pathways, the predicted proteomes of these species were annotated with KEGG (Kyoto Encyclopedia of Genes and Genomes) Orthology (KO) identifiers using the online BlastKOALA version 2.0 server (Kanehisa et al., 2016; www.kegg.jp/blastkoala/). Via KEGG Mapper analysis, each protein with a KO annotation was mapped to its KEGG pathway and BRITE functional hierarchy (Kanehisa et al., 2011). KO numbers associated with carbon and carbohydrate metabolism were investigated to identify functions unique to each species. Additionally, carbohydrate-active enzymes (CAZymes) in the predicted proteomes of all three species were annotated with dbCAN (Yin et al., 2012), a program that uses hidden Markov models (HMMs) to search for the signature domain of each CAZyme family. Hits with e-values above 10^{-6} and covering <50% of the HMM were discarded. Local tBLASTn and BLASTp searches were conducted against the genomes and predicted proteomes, respectively, of the other species to ensure that each unique protein was truly absent in the other two and not the result of incomplete or incorrect annotation. Based on sequence similarity only, protein homology can be inferred confidently above 40% amino acid identity (Rost, 1999). In doing BLAST searches, however, we were also interested in functional conservation as described by the Enzyme Commission (EC) numbers associated with KO annotations (Bairoch, 2000; Kanehisa et al., 2004). As suggested previously (Addou et al., 2009), proteins were considered EC functional homologs when they shared an amino acid sequence similarity of at least 60% over ≥ 60% of the query sequence length. Proteins predicted from the full length gene models of the identified polysaccharide degrading enzymes and CAZymes were interrogated with SignalP 4.1 (Petersen et al., 2011) to predict whether they are secreted or intracellularly localized.

3. Results

3.1. Carbohydrate utilization of *Knoxdaviesia* and *C. albifundus*

Knoxdaviesia capensis showed biomass production on 62 of the 190 substrates tested, while *K. proteae* grew on 59 substrates. Of the nine substrates on which *K. proteae* grew, but not *K. capensis*, only one (the di-peptide Gly-Pro) stimulated substantial biomass production, indicated by high absorbance values, while the remainder had very low, although statistically significant, absorbance values (Table 1, Fig. S1 and S2, Supplementary File 4). In contrast to the *Knoxdaviesia* species, we observed positive growth for *C. albifundus* on only 19 of the 190 substrates tested. *Ceratocystis albifundus* showed very weak growth relative to *Knoxdaviesia*, a phenomenon that may partially be explained by the light colour and often aerial nature of *C. albifundus* mycelium (Wingfield et al., 1996). Although only two of the substrates had absorbance values significantly different from the negative control, *C. albifundus* biomass production could be inferred by close inspection of the growth curves (Fig. 1, Supplementary File 4).

For *Knoxdaviesia*, substrates that resulted in the most biomass production were primarily simple carbon sources (i.e., monosaccharides and disaccharides) (Fig 2A, B). Few differences in carbohydrate utilization were observed between the two *Knoxdaviesia* species. *Knoxdaviesia proteae* showed weak growth on the monosaccharide D-ribose and the disaccharide sucrose, whereas *K. capensis* had notable growth on the sugar alcohol D-dulcitol and two polysaccharides, glycogen and pectin (Tables 1 and 2, Fig. 2C, D).

Table 1. Results of the phenome analysis in *Knoxdaviesia capensis* (*Kc*), *K. proteae* (*Kp*) and *Ceratocystis albifundus* (*Ca*).

Substrate	Compound category	Growth[a]		
		Ca	***Kc***	***Kp***
D-Salicin	alcoholic glucoside		x	x
L-Lactic Acid	alpha hydroxy acid		x	x
D-Alanine	amino acid		x	
D-Threonine	amino acid	x		
L-Alanine	amino acid		x	x
L-Asparagine	amino acid		x	
L-Aspartic Acid	amino acid		x	x
L-Glutamic Acid	amino acid		x	x
L-Glutamine	amino acid		x	x
L-Histidine	amino acid	x		
L-Isoleucine	amino acid		x	
L-Leucine	amino acid	x	x	
L-Ornithine	amino acid		x	x
L-Proline	amino acid		x	x
L-Serine	amino acid			w
L-Threonine	amino acid			w
L-Pyroglutamic Acid	amino acid	x		
γ-Amino-n-Butyric Acid	amino acid		x	
Ethanolamine	amino alcohol		x	x*
Acetic Acid	carboxylic acid			w
Butyric Acid	carboxylic acid		x	x
D-Galacturonic Acid	carboxylic acid		w	w
Glyoxylic Acid	carboxylic acid	x		
m-Hydroxy-Phenylacetic Acid	carboxylic acid		x	
Propionic Acid	carboxylic acid		x	x
4-Hydroxy-Benzoic Acid	carboxylic acid		x*	x
Bromo-Succinic Acid	dicarboxylic acid	x		

Fumaric Acid	dicarboxylic acid		w	w
L-Malic Acid	dicarboxylic acid		x	x
Succinamic Acid	dicarboxylic acid	x		
Succinic Acid	dicarboxylic acid		x	x
Gly-Pro	dipeptide			x
D-Cellobiose	disaccharide		x	x
D-Trehalose	disaccharide		x	x
Lactulose	disaccharide		x	x
Sucrose	disaccharide			?
α-D-Lactose	disaccharide		x	x
β-Gentiobiose	disaccharide		x	x
L-Galactonic Acid-γ-Lactone	ester	x	x	x
Methyl Pyruvate	ester	x	w	w
Mono-Methyl Succinate	ester			w
Arbutin	glycosylated hydroquinone		x*	x
Pyruvic Acid	keto acid		x	x
α-Keto-Butyric Acid	keto acid		x	x
α-Keto-Glutaric Acid	keto acid		x	x
α-Keto-Valeric Acid	keto acid	x		
3-Hydroxy-2-Butanone	ketone		x	
Dihydroxy-Acetone	ketone	x		
D-Fructose	monoshaccaride		x	x
D-Fucose	monosaccharide	x		
D-Galactose	monosaccharide	x	x	x
D-Glucose	monosaccharide		x	x
D-Mannose	monosaccharide		x	x
D-Ribose	monosaccharide	x		w
D-Xylose	monosaccharide		x*	x
L-Arabinose	monosaccharide		x	x
L-Lyxose	monosaccharide		x	x
N-Acetyl-D-Glucosamine	monosaccharide		x	x

β-Methyl-D-Galactoside	monosaccharide		x	x
β-Methyl-D-Glucoside	monosaccharide		x	x
Adenosine	nucleoside		x	
Thymidine	nucleoside		x*	
Uridine	nucleoside		x	x
Gelatin	peptide			w
Dextrin	polysaccharide	x		
Glycogen	polysaccharide		x	
Inulin	polysaccharide	x		
Laminarin	polysaccharide		x	x
Pectin	polysaccharide		x	
Tween 20	polysorbate surfactant		w	w
Tween 40	polysorbate surfactant		w	w
Tween 80	polysorbate surfactant		x	x
Adonitol	sugar alcohol		x	x
D-Arabitol	sugar alcohol		w	w
D-Mannitol	sugar alcohol	x	x	x
D-Sorbitol	sugar alcohol		x	x
Dulcitol	sugar alcohol		x	
Glycerol	sugar alcohol		x	x
L-Arabitol	sugar alcohol	x		
myo-Inositol	sugar alcohol		x	x
Maltotriose	trisaccharide	x	x	x
D-Gluconic Acid	uronic acid		x	x
D-Glucuronic Acid	uronic acid			w

[a] Phenome assays were conducted with Biolog FF MicroPlates PM1 and PM2 (Biolog Inc., Hayward, CA). Growth is indicated with ‘x’; very weak growth is indicated with ‘w’; growth inferred from the growth curves, but not supported by significance tests is indicated with an asterisk (*); empty cells indicate no growth.

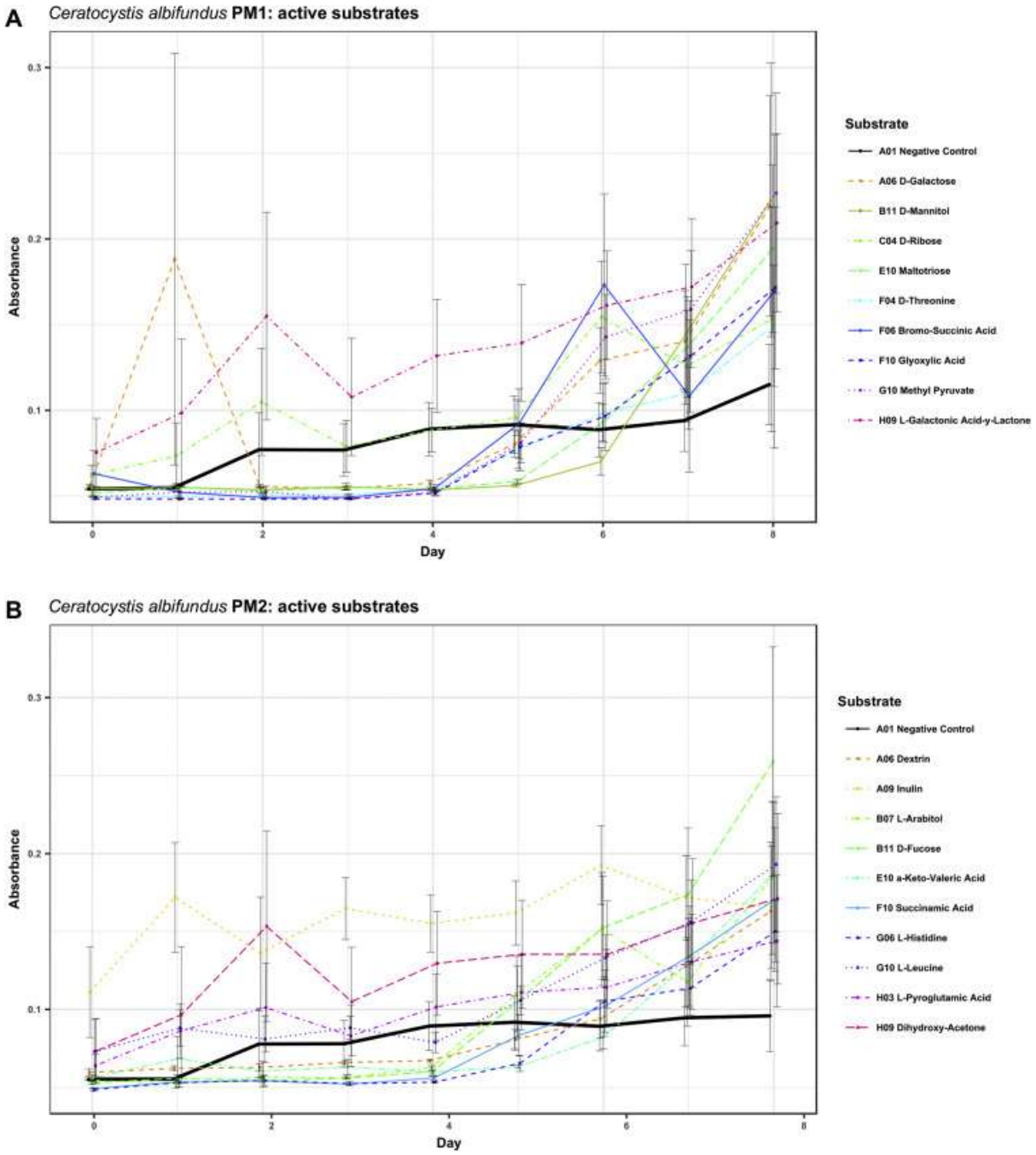
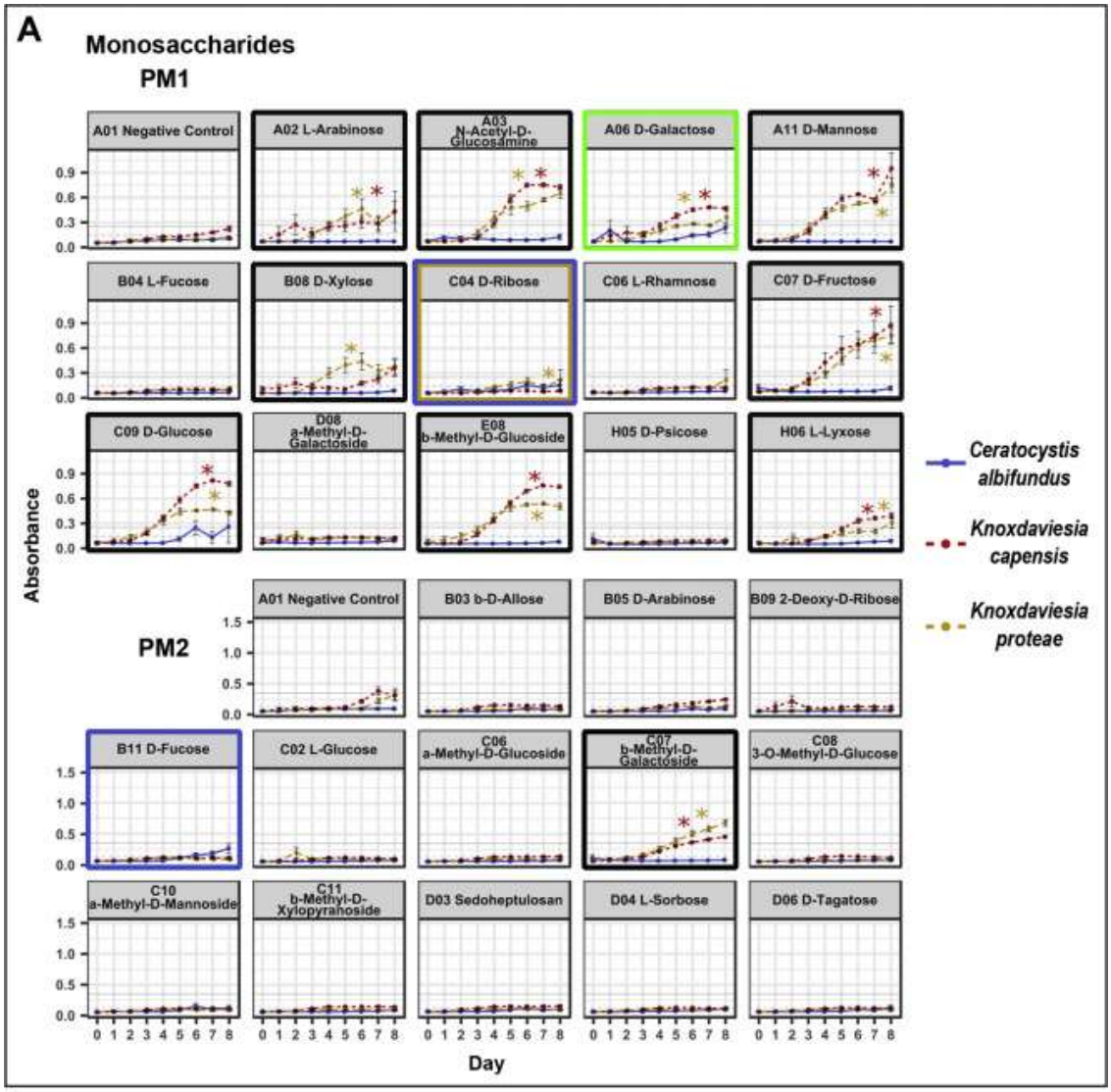


Fig. 1. Growth curves for the 19 (A) PM1 and (B) PM2 substrates that stimulated growth in *Ceratocystis albifundus*. The thick black curve is the negative control. Error bars represent the standard error of the mean (for three replicates on each day of the experiment). For the growth curves of all substrates, see Supplementary File 4.


A
Monosaccharides
PM1
A01 Negative Control
A02 L-Arabinose
A03 N-Acetyl-D-Glucosamine
A06 D-Galactose
A11 D-Mannose
B04 L-Fucose
B08 D-Xylose
C04 D-Ribose
C06 L-Rhamnose
C07 D-Fructose
C09 D-Glucose
D08 a-Methyl-D-Galactoside
E08 b-Methyl-D-Glucoside
H05 D-Psicose
H06 L-Lyxose
PM2
A01 Negative Control
B03 b-D-Allose
B05 D-Arabinose
B09 2-Deoxy-D-Ribose
B11 D-Fucose
C02 L-Glucose
C06 a-Methyl-D-Glucoside
C07 b-Methyl-D-Galactoside
C08 3-O-Methyl-D-Glucose
C10 a-Methyl-D-Mannoside
C11 b-Methyl-D-Xylopyranoside
D03 Sedoheptulosan
D04 L-Sorbose
D06 D-Tagatose
Absorbance
Day
Ceratocystis albifundus
Knoxdaviesia capensis
Knoxdaviesia proteae
B
Disaccharides
PM1
A01 Negative Control
A10 D-Trehalose
C10 D-Maltose
C11 D-Melibiose
C12 Thymidine
D09 a-D-Lactose
D10 Lactulose
D11 Sucrose
F11 D-Cellobiose
PM2
A01 Negative Control
B12 b-D-Galactopyranosyl -D-Arabinose
C01 b-Gentiobiose
C12 Palatinose
D07 Turanose
Absorbance
Day
Ceratocystis albifundus
Knoxdaviesia capensis
Knoxdaviesia proteae

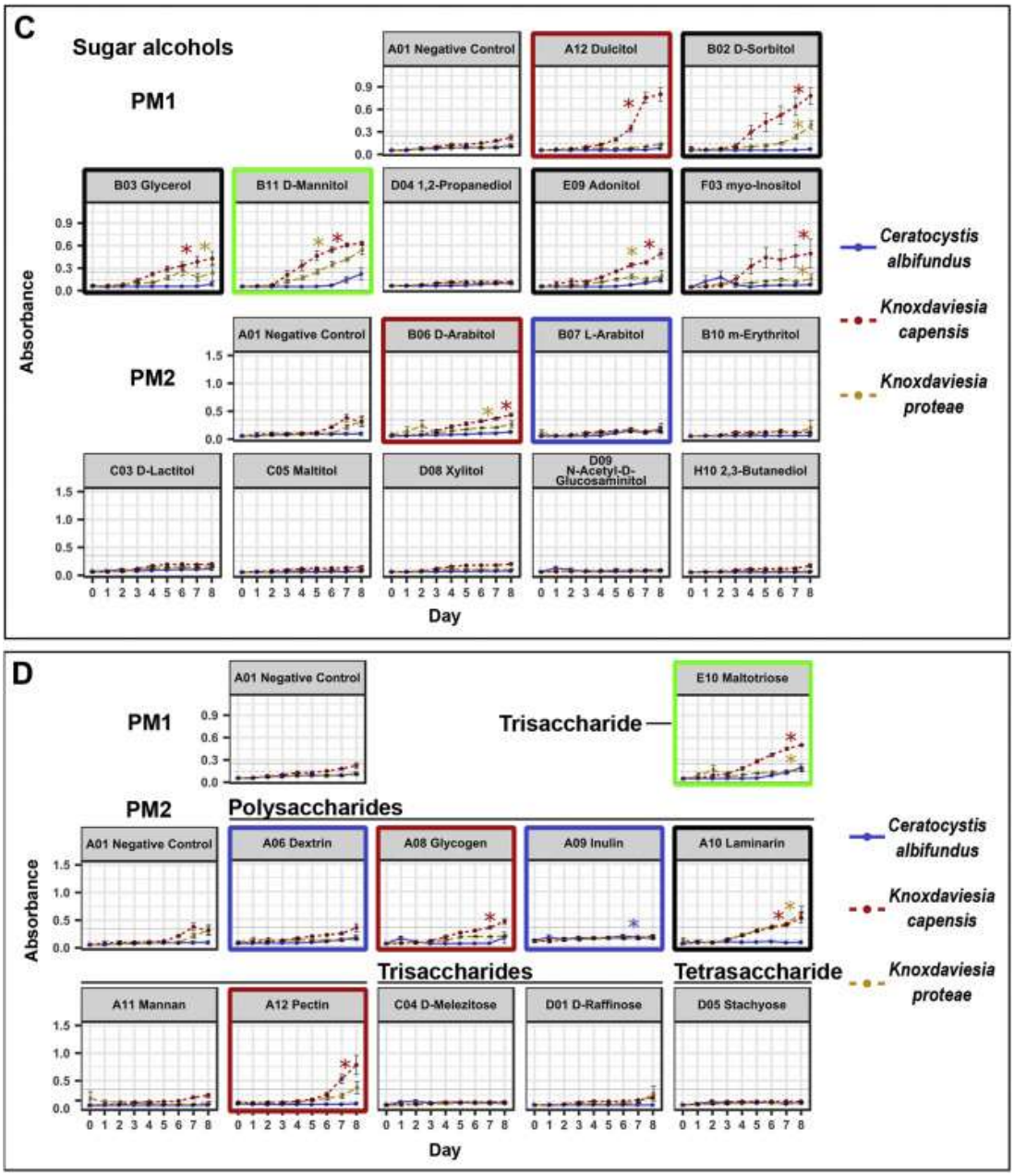


Fig. 2. Growth curves on the (A) monosaccharide, (B) disaccharide, (C) sugar alcohol, and (D) polysaccharide substrates tested during this study. Graphs outlined with a thick border indicate those substrates in which a particular substrate stimulated growth ($P < 0.05$ for *Knoxdaviesia*): red = *Knoxdaviesia capensis*, gold = *K. proteae*, blue = *Ceratocystis albifundus*, black = both *Knoxdaviesia* species, green = all three species. Dashed borders indicate probable growth that is not statistically supported ($P > 0.05$). Error bars represent the standard error of the mean (for three replicates on each day of the experiment). *Knoxdaviesia* curves that are significantly different from the negative control are indicated with an asterisk. The negative control for each plate (PM1 and PM2) is shown for comparative purposes. For the growth curves of all substrates, see Supplementary File 4.

Table 2. Categories of substrates used by *Knoxdaviesia capensis* (*Kc*), *K. proteae* (*Kp*) and *Ceratocystis albifundus* (*Ca*).

Compound category	*Ca*	*Kc*	*Kp*
Amino acids	**4**	**12**	**11**
Single amino acids	*4*	*12*	*9*
Peptides	*0*	*0*	*2*
Sugars	**8**	**26**	**25**
Monosaccharides	*3*	*10*	*11*
Disaccharides	*0*	*5*	*6*
Polysaccharides	*3*	*4*	*2*
Sugar alcohols	*2*	*7*	*6*
Others	**7**	**24**	**23**
Carboxylic acids	*3*	*8*	*8*
Ketones	*2*	*4*	*3*
Nucleosides	*0*	*3*	*1*
Polysorbates	*0*	*3*	*3*
Other	*2*	*6*	*8*
Total substrates	***19***	***62***	***59***

[a] Category as detailed in Table S1 (Supplementary File 1).

[b] Values represent the total number of PM1 and PM2 substrates in each category considered to have stimulated growth in each species.

Ceratocystis albifundus grew on very few carbohydrates (Table 2). Surprisingly, irregular and ambiguous growth was observed on glucose, whereas fructose and sucrose did not stimulate any growth. It grew on three other monosaccharides, D-fucose, D-galactose and D-ribose, as well as two oligosaccharides with α-1,4 and α-1,6, glycosidic bonds, respectively,

maltotriose and dextrin (Fig. 1). Although no fructose utilization was apparent, *C. albifundus* showed weak growth on inulin, a β-2,1 fructan.

The apparent inability of *C. albifundus* to grow on fructose and sucrose and its inconclusive growth pattern on glucose, prompted verification of the utilization of these substrates. For *K. capensis* and *K. proteae*, the agar only and sucrose plates retained their pink colour. Some spore germination and sparse hyphal growth was apparent on the agar only control, but this growth pattern is known in *Knoxdaviesia* species when they are cultivated on water agar and this behaviour is exploited to store cultures for extended periods of time. *Knoxdaviesia* hyphal growth occurred on the glucose and fructose plates and sporulation was observed at the points of inoculation. The positive control displayed typical vegetative growth and sporulation. All *C. albifundus* strains demonstrated the same phenotypes. The only *C. albifundus* treatment that retained its initial pink colour was the agar control, although the growth form on this control could not be distinguished from the glucose, sucrose and fructose treatments. In each of these treatments, limited spore germination, similar to that of the negative control, was observed and growth did not seem to occur beyond initial germination. A small number of ascomata developed on the fructose, glucose and sucrose treatments in isolate CMW17274. The yellow colour development on the glucose, fructose and sucrose plates indicated metabolic activity, but biomass production was distinct from the positive control on which growth was apparent without the aid of a microscope. Even if these sugars facilitate initial spore germination and metabolic activity, the lack of growth on the treatment plates suggested limited exploitation of these substrates after germination and indicated that *C. albifundus* could not utilize them as a sole carbon source. Ultimately, *C. albifundus* does not use glucose, fructose or sucrose as sole carbon source, whereas the *Knoxdaviesia* species use glucose and fructose.

3.2. Predicted proteome-based analysis of carbon and carbohydrate metabolism

Less than 37% of the *Knoxdaviesia* and 42% of the *C. albifundus* predicted proteins could be mapped to KEGG (Table S2, Supplementary File 2). Although a large portion of all three genomes remains unclassified, the KEGG database is sufficient to classify well-characterized proteins involved in metabolism (Kanehisa et al., 2011; 2016). All three species had similar proportions of their annotated proteins devoted to carbohydrate metabolism (7.6 – 7.9%), although the actual number (216 proteins) is the lowest in *K. proteae*. The primary carbohydrate utilization pathways predicted for these fungi are summarized in Fig. S4 (Supplementary File 4).

A repertoire of enzymes for degrading plant cell wall components, storage polysaccharides and cell surface carbohydrates were predicted from both *Knoxdaviesia* and *C. albifundus* (Tables 3 and 4). Although the KEGG and CAZyme annotations showed the same pattern of degradation, the CAZyme annotation (Table 3) identified more polysaccharide degrading proteins than KEGG (Table 4). The discrepancy in the number of proteins identified between the three species was primarily due to the number of different cell wall-degrading enzymes they encode. According to KEGG, *K. capensis* and *K. proteae*, respectively, have 37 and 36 different proteins predicted to act on plant cell wall components, whereas *C. albifundus* has 23. More than 100 cell wall-degrading CAZymes were identified in each *Knoxdaviesia* species, compared to only 69 in *C. albifundus*. Both datasets indicated that *C. albifundus* encodes several polysaccharide lyases and glycosidases associated with degrading pectic polysaccharides that were not identified in *Knoxdaviesia*. In contrast, the *Knoxdaviesia* species have a higher copy number of enzymes that degrade cellulose and hemicellulose compared to *C. albifundus*. In addition to the cell wall-degrading enzymes shared with *C.*

Table 3. Number of carbohydrate-active enzyme (CAZyme) families and proteins identified from the *Knoxdaviesia capensis, K. proteae* and *Ceratocystis albifundus* proteomes.

	K. capensis		***K. proteae***		***C. albifundus***	
CAZy class[a]	**Families**	**Proteins[b]**	**Families**	**Proteins[b]**	**Families**	**Proteins[b]**
Auxiliary Activity (AA)	8	37 (26)	8	34 (23)	7	18 (11)
Carbohydrate-Binding Module (CBM)	12	24 (16)	12	24 (15)	9	15 (12)
Carbohydrate Esterase (CE)	11	40 (16)	11	39 (16)	8	29 (4)
Glycoside Hydrolase (GH)	38	122 (66)	38	119 (61)	38	90 (51)
Glycosyl Transferase (GT)	30	71 (4)	29	67 (6)	25	58 (58)
Polysaccharide Lyase (PL)	0	0	0	0	3	5 (5)
TOTAL[c]	***99***	***285 (124)***	***98***	***273 (114)***	***90***	***205 (82)***
Likely substrates						
Cellulose & hemicellulose	24	104 (63)	24	102 (64)	19	60 (31)
Pectin & pectin sidechains	1	1 (0)	1	1 (0)	7	9 (8)
Storage carbohydrates	7	14 (6)	7	14 (4)	7	14 (8)
Other	19	57 (25)	19	54 (21)	18	49 (19)

[a] See Tables S4 and S5 (Supplementary File 3) for a full list of CAZyme families in each class.

[b] The number of proteins per family with the number of potentially secreted proteins indicated in brackets.

[c] The total proteins is not a sum of the number in each individual family, since some CBM domains were identified in the same protein as a GH or CE domain.

Table 4. Identified glycosidase, carbohydrate esterase and polysaccharide lyase enzymes associated with carbohydrate degradation.

EC number[a]	Enzyme name[b]	Substrate	Number of proteins (Proteins with secretion signal)[c]		
			Kc	***Kp***	***Ca***
Cellulose degradation					
3.2.1.4	cellulase	cellulose	5(3)	6(6)	2(2)
3.2.1.91	cellulose 1,4-β-cellobiosidase	cellulose	5(5)	4(4)	1(1)
3.2.1.21	β-glucosidase	glucans, e.g. cellobiose	7(2)	6(3)	5(0)
Hemicellulose degradation					
3.2.1.8	endo-1,4-β-xylanase	xylans	6(5)	6(4)	2(2)
3.2.1.37	β-xylosidase	xylans	1(1)	1(0)	0
3.2.1.177	α-xylosidase	xyloglucans	2(0)	2(0)	0
3.2.1.155	xyloglucan-specific exo-β-1,4-glucanase	xyloglucans	0	0	1(1)
3.1.1.72	acetylxylan esterase	acetylated xylan	4(3)	4(3)	1(1)
3.2.1.25	β-mannosidase	mannosyl-oligosaccharides	1(0)	1(0)	1(0)
3.2.1.78	β-mannanase	mannan	1(1)	1(1)	0
3.2.1.55	α-L-arabinofuranosidase	arabinan in xylan/pectin	1(0)	1(0)	2(1)
3.2.1.22	α-galactosidase (melibiase)	galactans	1(0)	1(0)	0

3.2.1.23	β-galactosidase	galactans	3(1)	3(1)	1(0)
Pectin degradation					
3.2.1.67	galacturan 1,4-α-galacturonidase	pectin backbone	0	0	1(1)
3.2.1.89	arabinogalactan endo-β-1,4-galactanase	arabinogalactans	0	0	1(1)
3.2.1.99	arabinan endo-1,5-α-L-arabinanase	arabinans	0	0	1(1)
4.2.2.2	pectate lyase	homogalacturonan	0	0	1(1)
4.2.2.10	pectin lyase	homogalacturonan	0	0	2(2)
4.2.2.23	rhamnogalacturonan endolyase	rhamnogalacturonan	0	0	1(1)
Storage polysaccharide degradation					
3.2.1.1	α-amylase	starch	0	0	1(1)
3.2.1.3	glucoamylase	glucans	1(0)	1(0)	1(1)
3.2.1.20	α-glucosidase	e.g. sucrose, starch	1(0)	1(0)	0
3.2.1.33	amylo-α-1,6-glucosidase	glycogen	1(0)	1(0)	1(0)
3.2.1.26	β-fructofuranosidase (invertase)	e.g. sucrose & maltose	0	0	2(1)
3.2.1.28	α,α-trehalase	trehalose	2(1)	2(0)	2(1)
3.2.1.39	glucan endo-1,3-β-D-glucosidase	glycans	1(1)	1(1)	1(0)
3.2.1.58	glucan 1,3-β-glucosidase	β-1,3-D-glucans	1(1)	1(1)	2(1)
3.2.1.6	endo-1,3(4)-β-glucanase (laminarinase)	glucans	1(0)	1(0)	1(0)

Cell surface carbohydrates (glycoproteins / glycolipids)					
3.2.1.24	α-mannosidase	mannan	1(0)	1(0)	1(0)
3.2.1.52	β-N-acetylhexosaminidase	N-acetylglucosides / N-acetylgalactosides	2(1)	2(1)	2(1)
3.2.1.84	glucan 1,3-α-glucosidase	α-1,3-D-glucans	1(1)	1(0)	1(1)
3.2.1.106	mannosyl-oligosaccharide glucosidase	mannosyl-oligosaccharides	1(1)	1(1)	1(1)
3.2.1.113	mannosyl-oligosaccharide 1,2-α-mannosidase	mannosyl-oligosaccharides	5(2)	5(2)	4(1)
3.2.1.164	galactan endo-1,6-β-galactosidase	arabinogalactans	0	0	1(1)
Other					
3.2.1.14	chitinase	chitin	4(2)	5(0)	4(3)
3.2.1.-	SUN family β-glucosidase		1(1)	1(1)	1(1)
3.2.1.101	mannan endo-1,6-α-mannosidase	α-1,6-mannan	4(4)	4(4)	3(0)
	Total number of different polysaccharide-degrading enzymes				
	Glycosidases (EC 3.2.1.)*		*60(33)*	*60(30)*	*47(24)*
	Carbohydrate esterases (EC 3.1.1.)*		*4(3)*	*4(3)*	*1(1)*
	Polysaccharide lyases (EC 4.2.2.)*		*0*	*0*	*4(4)*
	Total number of enzymes that degrade specific plant structures				
	Cell wall		*37(21)*	*36(22)*	*23(15)*

Storage polysaccharides	*8(3)*	*8(2)*	*11(5)*
Cell surface carbohydrates	*10(5)*	*10(4)*	*10(5)*

[a] EC, Enzyme Commission (Bairoch, 2000).

[b] Enzyme names are according to the KEGG ENZYME database (Kanehisa et al., 2004). The total number of glycosidases, carbohydrate esterases and polysaccharide lyases as well as the total number of enzymes associated with degrading specific plant structures are indicated.

[c] The number of proteins with specific EC functionalities predicted with BlastKOALA are shown (Kanehisa et al., 2016). The number of these proteins that are putatively secreted, as determined by SignalP (Petersen et al., 2011), is indicated in brackets. *Kc* = *Knoxdaviesia capensis*, *Kp* = *K. proteae*, *Ca* = *Ceratocystis albifundus*.

albifundus, they may also be able to degrade xylans, xyloglucans, galactans and mannans in hemicellulose.

Although the copy numbers of enzymes associated with degrading plant storage polysaccharides were similar between the species, the GH32 family of glycoside hydrolases and a secreted α-amylase (EC 3.2.1.1; GH13) enzyme were identified in *C. albifundus* and not in *Knoxdaviesia*. Enzymes to degrade the carbohydrate component of glycolipids and glycoproteins were also identified in both species, although some classes (e.g. CE9, GH38, GH109 and GH127) had no proteins with secretion signals, suggesting that these are rather involved in cellular maintenance. Our analysis also suggested that *C. albifundus* had a unique secreted enzyme (EC 3.2.1.164) to degrade arabinogalactans attached to lipids or proteins.

3.3. Comparison of the phenome assays and predicted carbohydrate metabolism of *Knoxdaviesia* and *C. albifundus*

Considering both the phenome and predicted proteome results of this study, genotype is not necessarily an accurate predictor of phenotype. We show here that the slight differences in substrate use between *K. capensis* and *K. proteae* are not explained by the genes predicted for carbon metabolism. Additionally, some substrates that would be expected to induce growth, such as sucrose for *C. albifundus*, failed to do so. It is possible that silenced genes were identified as functional or that certain enzymes have evolved a specific substrate affinity. In the context of these phenome assays, we note that experimental conditions are far removed from natural conditions and that complex regulatory mechanisms may have prevented growth on certain substrates.

3.3.1. Galactose metabolism

Galactose was the only monosaccharide used by all three species studied (Table 1, Fig. 2A). The KEGG proteome analysis indicated that this sugar enters glycolysis through the Leloir pathway, encoded by *GAL* genes in yeast (Sellick et al., 2008). Initial KO annotations did not identify the second enzyme in this pathway, galactokinase (EC 2.7.1.6), in *K. proteae*. However, the BLASTp and tBLASTn searches with the *K. capensis* galactokinase protein had significant BLAST hits in the *K. proteae* proteome and genome, respectively, and all three species thus contain this enzyme. All species are also theoretically able to source galactose from galactan-containing oligo- and polysaccharides, such as lactose, via their β-galactosidase enzymes, although only the *Knoxdaviesia* species grew on lactose.

3.3.2. Xylan and D-xylose metabolism

Both *Knoxdaviesia* species grew on D-xylose as the sole carbon source (Table 1, Fig. 2A). This is particularly intriguing since the nectar of some of their *Protea* hosts contain small amounts (0.1 – 5%) of D-xylose (Nicolson and Van Wyk, 1998). Our proteome analyses (Table 4), indicated that this ability is likely linked to the D-xylose reductase enzyme (Fig. S4; Verduyn et al., 1985) encoded by *Knoxdaviesia*, but not identified in *C. albifundus*. The use of xylose as an energy source appears essential, because the *Knoxdaviesia* species also encode α- and β-xylosidases that release D-xylose from xyloglucans and xylan in the plant cell wall. A unique, secreted xyloglucan-specific exo-β-1,4-glucanase (GH74; EC 3.2.1.155) enzyme was identified in *C. albifundus*, yet it did not grow on D-xylose.

3.3.3. Fructose and mannose metabolism

One difference between *Knoxdaviesia* and *C. albifundus* was noted in the fructose metabolism pathway: the absence of ketohexokinase (fructokinase; EC 2.7.1.3) in *C. albifundus* (Table S3, Supplementary File 2) and its inability to grow on D-fructose. In

bacteria and some eukaryotes such as mammals, fructose metabolism is a branched pathway (Fraenkel and Vinopal, 1973; Tappy and Lê, 2010). Hexokinase (EC 2.7.1.1) may phosphorylate D-fructose to fructose-6P that is subsequently phosphorylated to form the glycolytic intermediate fructose-1,6-diP. Alternatively, the ketohexokinase enzyme that is missing from *C. albifundus* may produce fructose-1P that is broken down to glyceraldehyde-3P to enter glycolysis. In some bacteria, the fructose-1P pathway is the preferred method of utilizing exogenous fructose (Kelker et al., 1970) and it is tempting to speculate that this preference, along with the missing ketohexokinase enzyme, may be the reason why *C. albifundus* cannot utilize exogenous fructose. However, baker's yeast, *Saccharomyces cerevisiae*, requires only hexokinase to grow on fructose (Gancedo et al., 1977), indicating that the first pathway is sufficient for the metabolism of exogenous fructose.

The branched pathway of fructose utilization has not been described in yeast or filamentous fungi and fungal references of the ketohexokinase enzyme are not available on UniProt (http://www.uniprot.org). A tBLASTn search of the *K. capensis* ketohexokinase enzyme yielded multiple good hits (bit score > 200, e-value < 10^{-6}, query coverage > 90%) to fungal proteins, indicating that this enzyme is not absent from the Fungi and may play a role in fungal fructose metabolism. The best hit was to a hypothetical protein in the Microascalean species *Scedosporium apiospermum*, followed by hits to species in the genera *Colletotrichum*, *Aspergillus* and *Fusarium*, amongst others.

Utilization of D-mannose as a carbon source presumably proceeds by phosphorylation and subsequent isomerization of mannose-6P to fructose-6P (Valentine and Bainbridge, 1978). Although all three species appeared to encode the necessary enzymatic machinery to accomplish these reactions, only the *Knoxdaviesia* species grew on D-mannose. The ability of these species to utilize mannose may be linked to the presence of a putatively secreted GH5 endoglucanase enzyme in *Knoxdaviesia* (β-mannase, EC 3.2.1.78; not identified in *C.*

albifundus) that could degrade the β-1,4 linked polysaccharides of D-mannose that form the primary chain of this sugar in hemicellulose (Moreira and Filho, 2008).

3.3.4. Starch and cellulose metabolism

Glucoamylase and amylo-α-1,6-glucosidase, that cleave α-1,4 and α-1,6 linked glucose residues from the terminal ends of starch and glycogen molecules (Divakar, 2013), were identified in the proteomes of *Knoxdaviesia* and *C. albifundus* (Table 3). Only *K. capensis*, however, grew on glycogen (Fig. 2D). Additionally, the endoglucanase, α-amylase, that cleaves internal α-1,4 linkages (Divakar, 2013) was predicted from *C. albifundus* and may explain its growth on dextrin (Fig. 1B), the hydrolysed form of starch, and maltotriose. Since α-amylases are widespread in the fungal kingdom, their absence in *Knoxdaviesia* is likely a derived state (Guimarães et al., 2006; Sundarram and Murthy, 2014). All three species also lack lytic polysaccharide mono-oxygenases (LPMOs) that act on starch (AA13).

Secreted cellulolytic enzymes with endoglucanase, exoglucanase and oxidative (AA9) activity were predicted from all three proteomes (Table 3). These may act synergistically on the plant cellulose fibres, because CAZyme family AA9 LPMOs are able to oxidatively cleave the crystalline cellulose structure, making the polysaccharide available to glucanases (Beeson et al., 2012). Endoglucanases expose substrates for the exoglucanases (Teeri, 1997), which in turn, cleave the disaccharide cellobiose from cellulose terminals, enabling β-glucosidase to complete the hydrolysis to β-D-glucose. Although all three species possessed several copies of the β-glucosidase enzyme, secretion signals were identified in only the *Knoxdaviesia* β-glucosidase enzymes, suggesting that *C. albifundus* cannot release glucose from cellobiose. Regarding the shared enzyme classes, the *Knoxdaviesia* species encoded approximately double the number of cellulolytic and hemicellulolytic enzymes predicted from the *C. albifundus* proteome (Tables 3 and 4). This disparity most likely reflects the

importance of cellulose and hemicellulose as energy sources for *Knoxdaviesia*. In contrast, *C. albifundus* does not appear to use cellulose for energy and may only weaken these structures.

3.3.5. Sucrose metabolism

Although not able to grow on sucrose, the *Knoxdaviesia* species grew on its monomers, glucose and fructose (Table 1, Fig. 2A). The α-glucosidase enzymes in the predicted proteomes of these species could hydrolyse sucrose, but did not contain secretion signals and have also previously been described as intracellularly localized (Batista et al., 2005; Sutton and Lampen, 1962). The two *Knoxdaviesia* species would, therefore, not be able to degrade sucrose outside the cell. These enzymes would also be responsible for cleaving the α-1,4 bond in maltose and their absence explains the lack of growth on this substrate. The lack of *C. albifundus* growth on sucrose was surprising since two GH32 β-fructofuranosidases (invertases) that hydrolyse the sucrose β-1,2 bond were identified in this species (Table 3). Additionally, β-1,2 bonds are also found in other fructans, such as inulin, on which *C. albifundus* growth was observed.

3.3.6. Pectin degradation

The enzymes involved in the first half of the pectin degradation pathway (pectin to D-galacturonic acid) appears to be present only in *C. albifundus*, while those involved in the second half (D-galacturonic acid to L-glyceraldehyde) were present only in the *Knoxdaviesia* species (Table 3; Fig. S5, Supplementary File 4). The latter, coupled with their ability to grow on pyruvate (pyruvic acid) explains the weak growth of *Knoxdaviesia* on D-galacturonic acid (Fig. S1D, Supplementary File 4). The final enzyme to convert L-glyceraldehyde to glycerol could not be identified in any of the three species, implying that the downstream reactions of pectin degradation are non-functional.

The only polysaccharide lyases (EC 4.2.2.-) that could be identified in the three proteomes examined belong to *C. albifundus* and are implicated in pectin degradation (Table 3 and S5, Supplementary File 3). This species also encoded three pectin-degrading glycosidases and a pectin methylesterase (CE8) that were not identified in *Knoxdaviesia* (Table 3 and S4, Supplementary File 3). Although *C. albifundus* was apparently incapable of utilizing pectin (Table 1**,** Fig S3E, Supplementary File 4), the presence of these putatively secreted enzymes in its predicted proteome suggests that degradation of pectin may be an important process during *C. albifundus* infection.

Both *Knoxdaviesia* species had a unique CE12 acetylesterase protein that could act on pectin components or xylan (Table 4), although there is no indication that this protein is secreted. Based on the proteome data and the weak growth of the *Knoxdaviesia* species on D-galacturonic acid, neither was expected to degrade pectin, yet *K. capensis* used it as a sole carbon source (Table 1, Fig. 2D). The pectin used in the phenome assay was purified from apple (P8471, Sigma Aldrich) that contains xylogalacturonans in its pectic fraction (Schols et al., 1995). Although the major pectin-degrading enzymes were absent from *K. capensis*, its growth could be explained by metabolism of xylogalacturonan, since the *Knoxdaviesia* species are potentially capable of releasing 1,4 linked D-xylose residues via β-xylosidase (Van den Brink and de Vries, 2011). *Knoxdaviesia capensis* is also the only species with a predicted secretion signal in its β-xylosidase enzyme (Table 3), potentially explaining why *K. proteae* did not grow on pectin. Likewise, the inability of *C. albifundus* to utilize pectin sidechains may be due to the specific type of pectin in the phenome assay.

3.3.7. Sugar transporters

Although the glycolytic intermediates D-glucose-6P and fructose-6P are ubiquitous in living organisms, they did not stimulate growth in *Knoxdaviesia* or *C. albifundus*. Similarly, several

Table 5. Carbohydrate transport proteins identified in *Knoxdaviesia capensis* (*Kc*), *K. proteae* (*Kp*) and *Ceratocystis albifundus* (*Ca*).

KO identifier[a]	Transporter family[b]	Description	Potential substrate(s)	Number of proteins		
				Kc	*Kp*	*Ca*
K08150	SLC2: Facilitative GLUT transporter	myo-inositol member 13	myo-inositol	1	1	1
K15378	SLC45: Putative sugar transporter	member 1/2/4	glucose, galactose	2	2	2
K08141 (TC:2.A.1.1)	Sugar porter (SP) family	general alpha glucoside:H+ symporter	trehalose, maltose, turanose, isomaltose, α-methyl-glucoside, maltotriose, palatinose, melezitose	4	4	2
K02429 (TC:2.A.1.7)	Fucose:H+ symporter (FHS) family	L-fucose permease	fucose	1	1	0
K06902 (TC:2.A.1.24)	Unknown major facilitator-1	UMF1 family	uknown	1	1	1

[a] KO, KEGG Orthology identifiers predicted by BlastKOALA (Kanehisa et al., 2016). TC numbers refer to the Transporter Classification Database (www.tcdb.org).

[b] Transporter families are listed as described in the BRITE functional hierarchy (Kanehisa et al., 2011). SLC, Solute Carrier Family; GLUT, Glucose Transporter

other substrates were not used, despite identification of the necessary enzymes (e.g. sucrose in *C. albifundus*). However, substrate utilization depends on the physical presence of the metabolic enzyme, enzyme expression, biological activity, and transport machinery to translocate the substrate into the cell (Saier, 2000). We, therefore, investigated the proteins of each species that have KO identifiers associated with membrane transporters. Only five families associated with sugar transport were identified by KEGG (Table 5), although the single identified member of the GLUT family (K08150) transports myo-inositol rather than hexose sugars (Nikawa et al., 1991). The remaining four families are potentially able to transport glucose, galactose, trehalose, maltose and fucose. Additionally, each species encodes a major facilitator transporter with unknown substrate affinity.

4. Discussion

4.1. Cell-wall degrading enzymes: key to the saprotrophic lifestyle of *Knoxdaviesia*

The phenome assays have shown that *Protea*-associated *Knoxdaviesia* species are able to feed on exogenous glucose, fructose and xylose, three of the four sugars often present in *Protea* nectar (Nicolson and Van Wyk, 1998). As such, these would likely be the dominant source of energy during the early colonization of young *Protea* flowers (Roets et al., 2009a). We hypothesize that *Knoxdaviesia* species do not metabolize sucrose, because they are unable to translocate it into the cytoplasm. Consequently, they cannot utilize it, since their only sucrolytic enzyme (α-glucosidase) is not secreted. Colonization of living *Protea* flower heads would be the only period when sucrose is available and, during this time, plant invertases cleave sucrose into glucose and fructose, causing sucrose levels to decrease with

flower age in *Protea* nectar (Nicolson and Van Wyk, 1998). Due to their direct access to glucose and fructose in inflorescences and the presumed absence of sucrose in infructescences, sucrose transport and utilization would, therefore, be redundant for *Knoxdaviesia* species.

The phenome results suggests that *Knoxdaviesia* species predominantly exploit arabinose, glucose, galactose, mannose and xylose, monomers abundant in cellulose and hemicellulose (Brown, 2004; Scheller and Ulvskov, 2010). Once *Protea* infructescences have formed, nectar is depleted and the cell walls of flowers, the involucral bracts and any storage polysaccharides that may remain will likely be the only sources of carbon for these fungi. At this stage, the *Knoxdaviesia* species would need to switch from the uptake and metabolism of simple nectar sugars to the utilization of complex carbohydrates.

Available data suggests that the number of CAZymes identified in *Knoxdaviesia* (Zhao et al., 2014), including plant cell-wall degrading enzymes (Martinez et al., 2008; Van den Brink and de Vries, 2011), is far below average for saprotrophic Sordariomycete fungi. Their reduction in polysaccharide degrading enzymes was particularly evident with regards to pectin. It is typical for fungi to contain less enzymes dedicated to pectin degradation compared to cellulose and hemicellulose (Espagne et al., 2008), e.g. only six pectinolytic enzymes were predicted from *Neurospora crassa*, but the absence of these genes in plant-associated fungi such as *Knoxdaviesia* is enigmatic However, *Protea* infructescences represent the only niche from which these fungi are known and complete plant cell wall degradation may cause their habitat to crumble. It is, therefore, possible that the lack of pectin degradation in *Protea*-associated *Knoxdaviesia* species may be an adaptation to maintain the structural integrity of the *Protea* infructescence.

A comparison with *C. albifundus*, which uses stem wounds to establish on its hosts (Roux et al., 2007; Roux and Wingfield, 2009), illustrates the importance of cell wall-degrading enzymes in the saprotrophic *Knoxdaviesia* species. *Ceratocystis albifundus* used few monosaccharides and did not grow on either pectin or cellobiose. The limited repertoire of cell wall degrading enzymes in this pathogen may, instead, be focused on weakening the cell wall, as implied by the cankers and lesions that develop as the *C. albifundus* infection spreads through the vascular tissue (Kubicek et al., 2014; Morris et al., 1993; Roux et al., 1999). . As an example, *C. albifundus* may be capable of weakening the cell wall structure if its pectinolytic enzymes are functional. Pathogen polygalacturonase enzymes (PGs) that attack the pectin backbone are well-documented as virulence proteins (Huang and Allen, 2000; Roper et al., 2007; Rowe and Kliebenstein, 2007). In turn, plants often produce corresponding polygalacturonase inhibitor proteins (PGIPs) that elicit a defence response (Di et al., 2006; Howell and Davis, 2005). It is, therefore, possible that *C. albifundus* evades plant defences by not producing PGs, but rather attacking pectin sidechains to weaken the structure. In contrast, *Knoxdaviesia* species apparently use their range of cell wall-degrading enzymes to release monosaccharides from cellulose and hemicellulose. Gaining access to the cell is unnecessary for *Knoxdaviesia* and may negate the need for this species to degrade pectin.

4.2. Evolution of specialist and generalist *Protea*-associated *Knoxdaviesia*

Our phenome results did not provide strong support for the hypothesis that the generalist, *K. capensis*, has a greater capacity to utilize diverse substrates than its specialist counterpart. *Knoxdaviesia capensis* grew on only three more substrates than *K. proteae*. However, *K. capensis* typically showed greater biomass production on substrates that it shared with *K.*

proteae. Further, it also displayed substantial growth on substrates that were not shared with *K. proteae*, whereas *K. proteae* grew weakly on eight of the nine substrates that it did not share with *K. capensis*.

It is possible that the stable *P. repens* infructescence micro-niche has driven specialization in *K. proteae*, since stable habitats lacking temporal variation favour specialization (Wilson and Yoshimura, 1994). The host-exclusivity studies of Roets et al. (2011a) showed that *K. proteae* grows better than *K. capensis* on complete medium supplemented with *P. repens* flowers, implying increased fitness on this host. Therefore, the specialist nature of *K. proteae* may be a result of the generalist common ancestor of *Protea*-associated *Knoxdaviesia* increasing its efficiency on one host. Although sacrificing the ability to occupy other *Protea* hosts, the specialized *K. proteae* could have gained superior fitness on *P. repens* that makes it dominant and able to outcompete *K. capensis* on this host (Aylward et al., 2015).

In the context of the trade-off hypothesis, arguing that generalist species are capable of using many resources, while specialists use fewer resources with greater efficiency (Wilson and Yoshimura, 1994), it is necessary to understand why the *Knoxdaviesia* ancestor would specialize only on *P. repens* and not on each of its *Protea* hosts. One explanation may be that specialization on *P. repens* does not limit host availability, since it is the most widespread and common species in the African *Protea* clade (Rebelo, 2001). In contrast, the numerous hosts of *K. capensis* have either limited or patchy distributions. The phylogenetic distance between host species could also influence this phenomenon, since the fitness costs associated with being a generalists have been found to be more dependent on the taxonomic range than the actual number of hosts (Straub et al., 2011). Therefore, a generalist may increase its fitness by specialising on a host that is phylogenetically distant from the others. Following the *Protea* phylogeny (Mitchell et al., 2017), the hosts of *K. capensis* occur in three distinct groups, with *P. repens* occurring alone. Specialization may have been the mechanism

whereby the common ancestor of CFR *Knoxdaviesia* species increased its fitness on this lone host, whilst the generalist maintained its dominant presence in the rest.

In the *K. proteae* specialist niche, the lack of selective pressure to degrade diverse substrates could have led to loss of function, such as the inability of *K. proteae* to grow on glycogen. Although utilization of a ubiquitous plant storage polysaccharide would be advantageous for a generalist species, more abundant or preferable substrates are likely available to *K. proteae*. Xylose may be such an example as it was one of the few substrates on which *K. proteae* grew faster and produced more biomass than *K. capensis*. This may be linked to the host of *K. proteae* that has the highest concentration (up to 5%) of xylose in its nectar, compared to other *Protea* species (Nicolson and Van Wyk, 1998).

4.3. Unexpected carbon resources for *C. albifundus*

Glucose and fructose are regarded as the simplest available sources of energy, but these carbon sources were not used by the pathogen *C. albifundus*. This might be due to a lack of the necessary transporter proteins. In *S. cerevisiae*, strains deficient in the major transporters responsible for the uptake of glucose and fructose, cannot grow on these sugars (Batista et al., 2005). Additionally, the importance of the missing ketohexokinase enzyme in fungal metabolism of exogenous fructose remains undetermined. The inability of *C. albifundus* to use fructose and glucose was also demonstrated by its inability to grow on most polysaccharides comprised of glucose or fructose monomers. The single exceptions for each were dextrin and maltotriose (glucose polysaccharides) that induced much growth and inulin (a fructose polymer) that induced weak growth in *C. albifundus*, relative to other substrates. Growth on these polymers, coupled with an inability to utilize glucose and fructose, suggests that *C. albifundus* may transport short glucans and fructans and hydrolyse them

intracellularly. Such a scenario would be congruent with the access that this pathogen has to phloem through which soluble oligosaccharides are transported, but in which reducing sugars such as glucose and fructose are relatively scarce or even absent (Taiz and Zeiger, 2006). Additionally, *C. albifundus* may be able to access storage polysaccharides within plant cells. The ability to take up and utilize oligosaccharides rather than monosaccharides could reflect an adaptation to nutrient availability in the environment occupied by *C. albifundus.*

The most surprising result of the phenome assays was the inability to demonstrate *C. albifundus* growth on sucrose, the most common carbohydrate in plant phloem (Pritchard, 2007). This was further confounded by the recent description of two invertases predicted to facilitate sucrose uptake in *C. albifundus* (Van der Nest et al., 2015). It is possible that these invertases have affinities for storage carbohydrates other than sucrose, such as those mentioned above. The affinity of *C. albifundus* for these oligosaccharides implies that they may be abundant in the phloem of its plant hosts. The inability of this pathogen to metabolize sucrose, however, requires further confirmation.

5. Conclusions

CFR *Knoxdaviesia* species grow on simple sugars abundant in *Protea* nectar and can degrade complex cell wall polysaccharides once *Protea* inflorescences become senescent and nectar is depleted. The tree pathogen *C. albifundus* displayed a very different carbon usage profile and seems to rely predominantly on soluble plant storage polysaccharides for energy. Saprotrophic fungi that consistently occupy *Protea* infructescences are also known from the order Ophiostomatales (Roets et al., 2013). Future characterization of the carbohydrate metabolism genes in these species would verify whether the limited number of carbohydrate hydrolysing enzymes is due to the lack of diverse substrates in infructescences, as we

hypothesize here. Genomic characterization of transporters and experimental validation of substrate uptake is necessary to evaluate our hypothesis that these species are unable to grow on certain substrates due to ineffective substrate translocation. These experiments would specifically be important to determine the mechanism behind the perceived inability of *C. albifundus* to grow on glucose, fructose and sucrose.

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Supplementary Information

The following supplementary files are included with this submission:

Supplementary File 1:

Table S1, List of BioLog carbon sources tested.

Supplementary File 2:

Tables S2 and S3, KEGG categories and unique carbon-metabolising enzymes identified in the *Knoxdaviesia* and *Ceratocystis albifundus* proteomes.

Supplementary File 3:

Tables S4 and S5, Families of carbohydrate-active enzymes (CAZymes) identified in *Knoxdaviesia* and *Ceratocystis albifundus* proteomes.

Supplementary File 4:

Figures S1-S5,Growth curves and predicted carbohydrate utilization pathways in *Knoxdaviesia* and *Ceratocystis albifundus*.