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Carbon utilization and growth-inhibition of citrus-colonizing *Phyllosticta* species

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ABSTRACT

The genus *Phyllosticta* includes both endophytic and phytopathogenic species that occur on a broad range of plant hosts, including *Citrus*. Some pathogenic species cause severe disease, such as *Phyllosticta citricarpa*, the causal agent of Citrus Black Spot (CBS). In contrast, other species, such as *Phyllosticta capitalensis*, have an endophytic lifestyle in numerous plant hosts. Carbon utilization capabilities are hypothesized to influence both host range and lifestyle, and are in part determined by the set of Carbohydrate Active Enzyme (CAZyme) encoding genes of a species. In this study, carbon utilization capabilities of five *Phyllosticta* species were determined, as well as the CAZyme repertoire (CAZome) encoded in their genomes. Little variation was found among species in terms of carbon utilization capabilities and CAZome. However, one of the tested carbon sources, sugar beet pulp (SBP), inhibited growth of the plant pathogens, also when combined with another carbon source, while endophytic species remained unaffected.

1. Introduction

Fungal plant pathogens form an increasing threat to the agricultural sector and to our society, as they are responsible for enormous global crop losses and drive the dependency on environmentally harmful fungicides (Savary et al., 2019). In order to reduce the impact of these destructive diseases, an increased understanding of the underlying mechanisms of pathogenicity and the basic biology of the responsible species is of vital importance. One such disease is Citrus Black Spot caused by the fungus *Phyllosticta citricarpa*, which results in various disease symptoms including leaf and fruit lesions, and leads to economic losses and in severe cases crop loss due to early fruit drop (Brentu et al., 2012; Kotzé, 1981, 2000). Disease control includes leaf litter removal which can be highly effective but is a very labour-intensive and costly process (Truter, 2010). Successful management using fungicides is highly dependent on proper timing, the latter of which can reduce crop loss up to 77% (Eustáquio Lanza et al., 2018). However, neither of these approaches is fully effective, and the use of fungicides increases the risk of antifungal resistance developing.

The genus *Phyllosticta* includes many other species colonizing a broad range of host plants globally (Glienke et al., 2011; Guarnaccia et al., 2017; Wang et al., 2012; Wikee et al, 2013a, 2013b; Wulandari et al., 2009). Several *Phyllosticta* spp. have often been found to co-occur on a single *Citrus* host plant, with some being endophytic, while others are pathogenic. A species often found together with *P. citricarpa* is *Phyllosticta capitalensis*, an endophyte with a very broad host range (Wikee et al., 2013a). The co-occurrence and high morphological similarity of these species have led to some confusion in the past as to whether *P. capitalensis* is also a pathogen (Baayen et al., 2002; Glienke et al., 2011). Another endophytic species is *Phyllosticta citribraziliensis*, which was relatively recently described from *Citrus* hosts in Brazil (Glienke et al., 2011). Other pathogenic species include *Phyllosticta citriasiana*, causing tan spot on pomelos (*Citrus maxima*), and the recently described *Phyllosticta paracitricarpa*, which is both morphologically and genetically very

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similar to *P. citricarpa* (Guarnaccia et al., 2017). Their close genetic relationship combined with their varying lifestyles make these species ideal candidates to study whether the ability to utilize carbon sources differentiates pathogens from endophytes. The aforementioned species were chosen based on their phylogenetic relationship and distinct lifestyles; other species of *Phyllosticta* on citrus not used in this study include *P. citrichinaensis* and *P. citrimaxima*.

An important process during fungal colonization of a plant is the degradation of the plant cell wall, which consists largely of polymeric compounds (polysaccharides, lignin, protein) (Mäkelä et al., 2014). The enzymes involved in this process are referred to as plant cell wall degrading enzymes (PCWDEs), which are secreted by the fungus and often involved in pathogenicity (Douaiher et al., 2007; King et al., 2011; ten Have et al., 2002, for a review see Kubicek et al., 2014). Differences occur between the PCWDE arsenals of plant-pathogenic and endophytic species, supporting their role in pathogenicity. For instance, Lo Presti et al. (2015) reported a reduction of PCDWEs in symbionts and biotrophs (both facultative and obligate) compared to saprotrophs, necrotrophs and hemibiotrophs. Choi et al. (2013) also reported a higher number of PCWDE-encoding genes in pathogens as compared to saprotrophs, as well a difference in the type of genes that are present. According to their study, plant pathogens generally contain several genes encoding pectin lyases (PL) and polygalacturonases (PG), whereas most or even all of these genes are missing in wood-decaying fungi (Choi et al., 2013).

PCWDEs are a subset of the so-called Carbohydrate Active Enzymes (CAZymes), which have been extensively described for many fungal species, and perform the breakdown of the polymeric and oligomeric plant carbohydrates (Lombard et al., 2014). Plant cell wall differs in composition based on species and tissue, and its degradation therefore necessitates tailored sets of enzymes (van den Brink and de Vries, 2011; Houston et al., 2016). Fungi that colonize distinct hosts or employ dissimilar lifestyles possess varying CAZyme repertoires, which have even been proposed as a basis to classify phytopathogenic fungi into different lifestyles (Hane et al., 2020). Haridas et al. (2020) found that within the class *Dothideomycetes*, saprobes generally had a higher number of CAZyme encoding genes compared to pathogens.

Studies into the presence of CAZyme encoding genes in *Phyllosticta* have been restricted to a limited number of species and typically detected highly variable and relatively low numbers of CAZyme encoding genes (Rodrigues et al., 2019; Wang et al., 2020). For instance, Rodrigues et al. (2019) found 44 and 23 CAZyme encoding genes in *P. capitalensis* and *P. citricarpa*, respectively. Wang et al. (2020) studied the CAZome in *P. citriasiana* and found 267 genes, including many different CAZy families. Although this number is much higher than those in the study by Rodrigues et al. (2019), this number is still low compared to other *Dothideomycetes*, which on average contain between 300 and 400 CAZyme encoding genes (Ohm et al., 2012; Haridas et al., 2020). For *Phyllosticta,* Wang et al. (2020) hypothesized a correlation of the low number of CAZyme encoding genes with a relatively long infection time. However, an underestimation of gene numbers as a result of incompleteness of a genome assembly or the method used to detect CAZyme encoding genes cannot be excluded.

We hypothesize that there may be a difference in carbon utilization capabilities and CAZome content between pathogens and endophytes of the genus *Phyllosticta*. In this study, carbon utilization of five *Phyllosticta* species known to occur on *Citrus*, including pathogens and endophytes, are studied, and their CAZomes are compared to determine whether either or both of these factors play a role in determining lifestyle of *Phyllosticta* species on *Citrus*.

2. Materials and methods

2.1. Species and culture conditions

All *Phyllosticta* cultures (Table 1) were obtained from the Westerdijk Fungal Biodiversity Institute culture collection (CBS) or the working collection of Pedro Crous (CPC). Fungi were grown on malt extract agar (MEA) (Crous et al., 2019) at room temperature for 14 d before a plug was taken to inoculate test media. Initial test conditions used *Aspergillus niger* minimal medium (MM) containing 6 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl, 0.5 g/L $MgSO_4*7H_2O$ and 0.2 ml/L Vishniac solution with a standard pH = 6.0 at 25 °C (de Vries et al., 2004). For testing the effect of pH on growth, the pH of media was lowered to 4.9–5.1 (low) and increased to 7 (high); Bromocresol purple (Merck Netherlands) was added to monitor pH levels. For testing the requirement of supplements for growth, 1 mg/L thiamine (Merck Netherlands) or 12 μg/mL inositol (Merck Netherlands) was added to the media.

2.2. General carbon utilization studies

Assays for carbon utilization essentially followed those of the Fungal Growth Database (fung-growth.org), with the exception that a modified *A. niger* MM (mMM) was used (with lowered pH and added thiamine as described in the previous section). All mono-, di- and oligosaccharides were added to mMM at a final concentration of 25 mM, all controls and polysaccharides were added to a final concentration of 1%, and all crude plant biomass substrates were added to a final concentration of 2% (the complete set of carbon sources is shown in Suppl. Table S1). All experiments were performed in duplicate. A 1-mm-diameter plug was taken from the edge of a colony grown for 14 d on MEA. Cultures were incubated at 25 °C and grown until the largest colony of a species reached the edge of the plate (plate diameter = 35 mm). Colony diameters were measured at 2–3 d intervals by taking two perpendicular measurements of the colony. For non-circular and irregularly shaped colonies, both the longstest and the shortest width of the colony were measured, and the average of both was used. When the biggest colony of a species reached the edge of the plate, the experiment was concluded for that particular species and photos were taken of this species on all 35 sources using a standard camera setup. This means that the experiment duration was shorter for species with higher growth rates.

2.3. Sugar beet pulp growth study

Modified minimal medium, mMM + Glucose (25 mM) and mMM + 1.5% WB (wheat bran) were used as control media. A range of SBP concentrations (0.25%, 0.5%, 1%, 2%, 3%) was added to mMM + 1.5% WB to test whether SBP inhibits growth on other carbon sources (plate diameter = 35 mm). Acidity of all media was adjusted to pH 5. A 1-mm-diameter plug was taken from the edge of a colony grown for 14–21 d, inoculated on the media and incubated at 25 °C. All species from Table 1 were inoculated in triplicate. Culture diameters were measured after 3, 6, 8, and 10 d. All cultures were photographed after 10 d using a standard camera setup.

2.4. Statistical analysis sugar beet pulp tests

Measurements from d 8 were chosen for statistical analysis as none of the species had reached their maximum colony diameter on this d, but all had reached their maximum growth rate. A transformation was applied to all data to be able to compare species with different growth rates using the following method. For each

Table 1
Phyllosticta species and strains used in this study, and their most commonly reported lifestyle. Strains in bold were used for establishing minimal growth requirements and for these strains genome assemblies were available and used for studying CAZyme genes.

Species	CBS collection number	Pathogen (P) or endophyte (E)
Phyllosticta capitalensis	**CBS 128856 (CPC, 18848)**	E
	CBS 356.52	E
	CBS 111638	E
	CBS 117118	E
	CBS 123374	E
	CBS 173.77	E
	CBS 226.77	E
Phyllosticta citriasiana	**CBS 120486**	P
	CBS 120426	P
	CBS 123371	P
Phyllosticta citribraziliensis	**CBS 100098**	E
	CPC 17464	E
Phyllosticta citricarpa	**CBS 141350**	P
	CBS 127454	P
	CBS 102373	P
	CBS 122482 (CPC 14848)	P
	CBS 122670	P
	CBS 131864	P
	CBS 141352	P
	CPC 16586	P
	CBS 111.20	P
Phyllosticta paracitricarpa	**CBS 141357 (CPC 27169)**	P
	CBS 141358 (CPC 27170)	P

species, the average of all colony diameter measurements on WB was calculated. Species with a higher growth speed will have a higher average colony diameter on WB (Suppl. Fig. S1A). The average of one species was then subtracted from all measurements taken for that species. The effect of this data transformation can be seen in Suppl. Fig. S1B. This method allows for comparison of species with different growth rates while leaving any other variation, whether they be biological or technical, intact. The same transformation was applied to measurements taken on the WB + SBP combined source.

To determine any statistically significant differences between species of different lifestyles, species were assigned to one of two groups: endophytes or pathogens. *P. capitalensis* and *P. citribraziliensis* were assigned to endophytes, while *P. citriasiana, P. citricarpa,* and *P. paracitricarpa* were assigned to pathogens. All (transformed) measurements on WB were combined to use as reference. Colony sizes of the (transformed) lifestyle-specific measurements on WB + SBP and the (grouped) WB reference were then compared to determine any statistically significant difference using a t-test.

2.5. SBP fractionation studies

Differential centrifugations: 1.25 g SBP was added to 25 mL demineralized water, which was centrifuged at 100 g for 5 min. The supernatant was collected and centrifuged at 600 g for 10 min The supernatant was again collected and centrifuged at 15,000 g for 20 min. All pellets created by the centrifugation steps, as well as the supernatant of the last centrifugation, were added to mMM +1.5% WB to check for inhibitory properties. mMM, mMM + glucose (25 mM), mMM + 1.5% WB and mMM + 0.5% SBP were used as control media.

Autoclavation followed by centrifugation: 1.0 g of SBP was added to 80 mL of water, autoclaved, and centrifuged at 100 g for 5 min. The pellet and supernatant were separated and added to mMM +1.5% WB individually to check for inhibitory properties. mMM +1.5% WB and mMM +0.5% SBP were used as control media.

Ethyl acetate extraction: 1.0 g SBP was added to 20 mL demiwater and 20 mL ethyl acetate (VWR chemicals), mixed for 2 h at room temperature, followed by a centrifugation at 2500 rpm for 5 min. The upper ethyl acetate fraction, lower water fraction, and pellet were separated, and added to mMM +1.5% WB to test for inhibitory properties. The ethyl acetate in the upper fraction was vaporized under constant N_2-flow prior to addition to the medium. mMM +1.5% WB and mMM +0.5% SBP were used as control media.

Hexane extraction: 1.0 g SBP was added to 20 mL demiwater and 20 mL n-hexane (Merck), mixed for 2 h at room temperature, followed by centrifugation at 2500 rpm for 5 min. The upper hexane fraction, lower water fraction, and the pellet were separated, and added to mMM +1.5% WB to test for inhibitory properties. N-hexane in the upper fraction was vaporized under constant N_2-flow prior to addition to the medium. mMM +1.5% WB and mMM +0.5% SBP were used as control media.

Proteinase K digestion: 1.0 g SBP was added to 20 mL Tris EDTA (TE, pH 8.0) containing 4 mg proteinase K (Roche), incubated at 37 °C and 200 rpm for 3 h, and added to mMM + 1.5 WB to check for inhibitory properties. mMM +1.5% WB, mMM +0.5% SBP, and mMM +1.5% WB + 20 mL TE (in 200 mL) were used as control media.

2.6. CAZyme analysis

Carbohydrate Active Enzyme annotation was performed as part of the JGI annotation pipeline by the Henrissat lab (Lombard et al., 2014). To compare specific CAZyme genes across species, Orthofinder was used to make Ortholog Groups (OG), using all annotated genes (Emms and Kelly, 2019). All *Phyllosticta* gene sequences used were downloaded from the genomes available on the JGI website (Guarnaccia et al., 2019). The number of CAZyme genes (as annotated by JGI) present in each OG from each species was then determined. CAZyme genes involved in biomass degradation were selected manually. To determine which CAZymes are involved in degradation of specific types of biomass, the same classification as Vesth et al. (2018) and de Vries et al. (2017) was used. CAZyme-containing OGs comprising a different number of genes in pathogens compared to endophytes were assessed further. If genes distinctive to one lifestyle formed a separate clade in the phylogenetic trees automatically generated by Orthofinder, their

nucleotide sequences were collected and blasted against both the assembled genomes as well as the raw sequence data of the species of the opposite lifestyle, to exclude the possibility that these genes were merely missing because of an assembly error.

3. Results

3.1. Minimum growth requirements

To be able to study the effect of different carbon sources without the influence of other nutrients, a minimal medium first needed to be established for *Phyllosticta* spp. When provided with this minimal medium alone, the fungus should show only minimal or impaired growth, and upon addition of a common carbon source such as glucose this impairment should be lifted. *Aspergillus niger* MM (de Vries et al., 2004) was first tested, but appeared to be insufficient for growth of *Phyllosticta* spp (Suppl. Fig. S2). Several additional conditions and nutrients were tested using six *Phyllosticta* strains (Table 1, in bold). First, the pH of the media was stabilized at 7 (high) or 5 (low), and bromocresol purple was added to monitor pH levels. Interestingly, while the species showed improved growth at low pH, they had an alkalizing effect on the medium in almost all cases. One exception to this was *P. paracitricarpa* on MM + glucose at low pH, where it had an acidifying effect (Fig. 1A). The mechanisms or evolutionary advantages of this behaviour are unknown to us. Addition of inositol had no effect on growth of *Phyllosticta*. In combination with lowering the pH below 5, addition of thiamine to MM proved to be sufficient for growth of all tested *Phyllosticta* spp. (Fig. 1B, two representative species shown). Therefore, this modified MM (mMM) was used for the studies on carbon utilization.

3.2. Differences in carbon utilization by Phyllosticta species

On most of the 35 carbon sources tested, the different species reached comparable colony sizes (Suppl. Fig. S3, selected examples are shown in Fig. 2), with some variation in growth rate. Growth rates on the carbon sources generally followed one of four patterns, for each of which an example is shown in Fig. 2. Colonies are of equal (small) size for all species on mMM (Fig. 2), with the two endophytic species (in green shades) growing faster than the pathogenic species (in brownish shades). This difference in growth speed can be observed quite clearly on wheat bran (WB) (Fig. 2), on which the species reached maximum growth rates. The first two species to cover the plate containing WB were the endophytic *P. capitalensis* and *P. citribraziliensis*, followed 3 d later by the three pathogenic species *P. citriasiana*, *P. citricarpa* and *P. paracitricarpa*. On another rich medium such as citrus pulp, growth rates were more comparable, but the difference in growth speed could still be observed (Fig. 2). A striking difference between endophytes and pathogens was observed on sugar beet pulp (SBP, Fig. 2). All pathogenic species showed reduced growth on SBP compared to the endophytic species (Fig. 3). To confirm this, and to gain further understanding of this effect, tests using more strains of the aforementioned species and another carbon source (wheat bran) as reference were performed.

3.3. Growth inhibition by sugar beet pulp

The decreased growth rates of pathogenic species of *Phyllosticta* on SBP could have two underlying causes. Firstly, the pathogenic species could lack the genes necessary for utilization of SBP, in which case the effect should be lifted after addition of a source on which the species can grow (such as wheat bran). Alternatively, growth of pathogenic species is somehow inhibited by a component of SBP, in which case the effect would not be lifted after addition of another rich source such as wheat bran. To test which scenario is more likely to be true, SBP was added at different concentrations to wheat bran (WB) on which all species show a steady and reproducible growth. Growth on WB + SBP was then compared to growth on WB as a sole source to assess the presence of potential inhibitory effects of SBP.

The colony diameters of endophytic species on WB and SBP hardly differ from their colony diameters on WB alone (blue boxes in Fig. 4, Suppl. Fig. S4). At higher concentrations, a slightly but significantly larger colony diameter can be observed compared to WB alone, which is in line with expectations as addition of SBP leads to a higher nutrient availability. In contrast, a large negative effect on the colony diameters of pathogenic species can be observed on SBP and WB compared to on WB alone (red boxes in Fig. 4, Suppl. Fig. S4). Colony sizes are significantly smaller even at the lowest concentrations of SBP, with the effect seemingly becoming larger at higher concentrations. An exception to this is the highest concentration, where the effect seems to be decreasing. These results support an inhibitory effect of SBP on the growth of pathogenic species of *Phyllosticta*, while no effect is observed with endophytic species.

In an attempt to determine the nature of the inhibitory substance, several experiments were performed. Firstly (autoclaved) SBP was subjected to differential centrifugation, after which the separate fractions were tested for inhibitory activity. The substance always remained in the first (heaviest) fraction, and remained

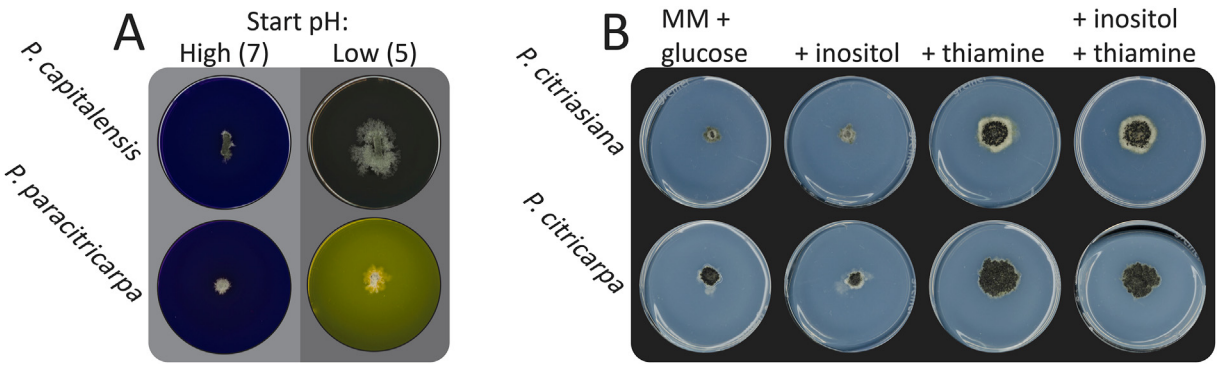

Fig. 1. Minimum growth requirements for *Phyllosticta* (A). One-mm-diam plugs were inoculated on *A. niger* MM + glucose with a pH = ~7 (high) or pH = ~5 (low). Bromocresol purple was added to monitor pH (yellow pH < 5.5, purple/blue = pH > 6.6). Photos were taken after 7 d (B). One-mm-diam plugs were put on *A. niger* MM + glucose with pH = ~5, and indicated ingredients were added (1 mg/L thiamine, 12 μg/mL inositol). Photos were taken after 11 d. The two species shown are representative of all *Phyllosticta* spp. tested in this study.

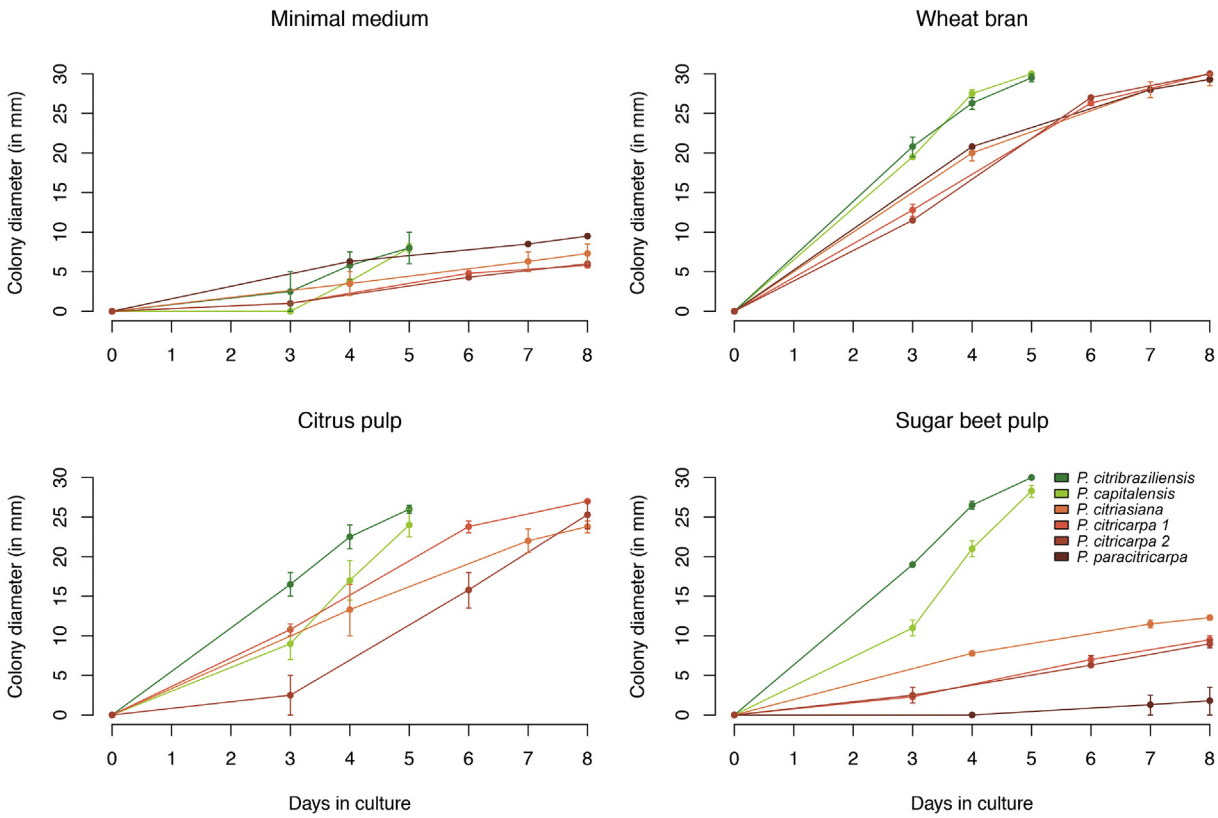

Fig. 2. Colony diameters of *Phyllosticta* spp. illustrated on four carbon sources. When the biggest colony of a species reached the edge of the plate, the experiment was concluded for that particular species (on all carbon sources). As a result, because endophytes grew faster, their experiments were concluded earlier. All measurements were done in duplicate. However, one of the *P. paracitricarpa* cultures on citrus pulp failed to grow. As we considered this to be due to technical reasons, *P. paracitricarpa* measurements on this source were omitted from the figure. Lines show averages with variance indicated with bars. All sources shown in the figure were added to an end concentration of 3%.

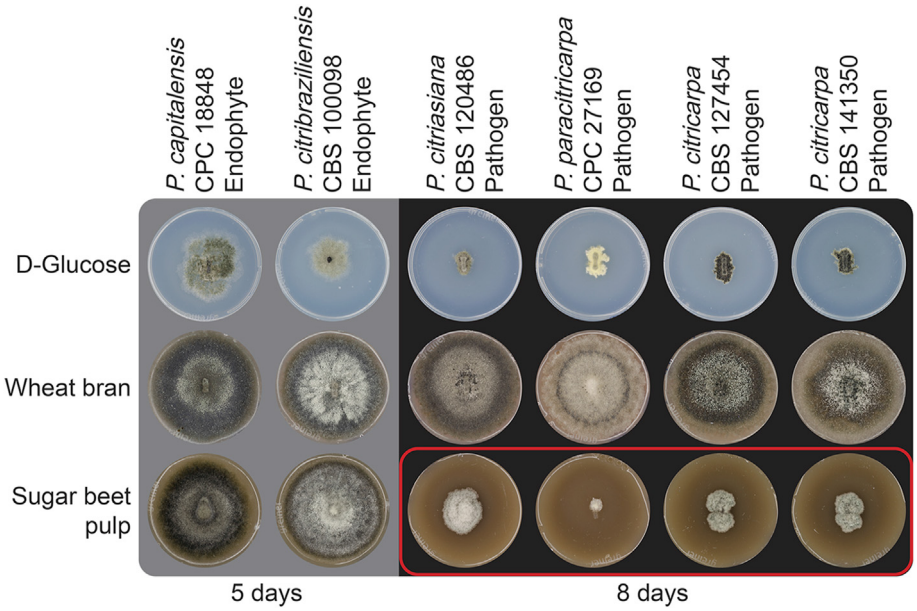

Fig. 3. Variable growth of *Phyllosticta* spp. on sugar beet pulp. Photos were taken after the number of d indicated below the figure. Species are ordered by lifestyle: endophytic on the left (grey background), pathogenic on the right (black background). Plate diameter = 35 mm.

active, indicating that it is either heat-tolerant or somehow protected. Secondly, two methods commonly used for extraction of bio-active compounds, namely ethyl-acetate extraction and n-hexane extraction, were used in an attempt to isolate the inhibitory substance. Again, the separate fractions were tested for inhibitory activity, which was detected for the pellet only, indicating that

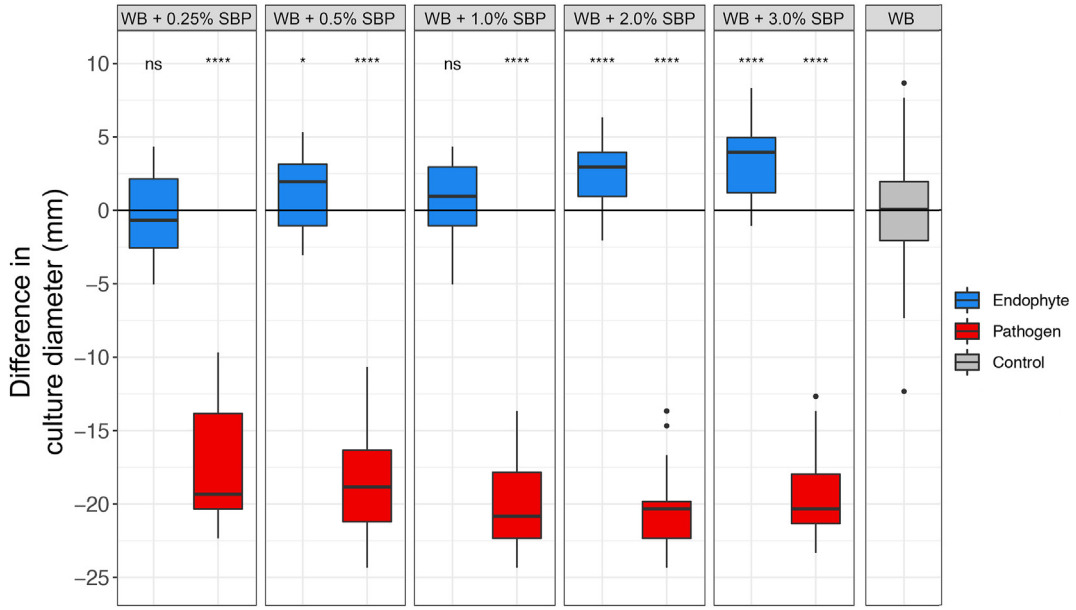

Fig. 4. Effect of sugar beet pulp (SBP) on growth of *Phyllosticta* spp. Culture diameters were measured on d 8. Per species, average culture diameter on 1.5% wheat bran (WB) was subtracted from each measurement on WB + SBP. Species were grouped together based on lifestyle (endophyte = blue, pathogen = red). The adjusted diameters were compared for a significant difference with growth on WB (both lifestyles together, grey). ns = not significant, * = $p < 0.05$, **** = $p < 0.00005$.

these extractions were not successful. Thirdly, a proteinase K digestion was performed, with no effect, indicating that the substance is not a protein. As these results provided only limited information and further attempts would require advanced chemical testing, a genetic approach was taken next.

3.4. CAZymes

In an attempt to find genes underlying the difference in growth rates on SBP, the presence of CAZyme encoding genes in the genomes of these species was examined. Two other descriptions of the CAZyme repertoire of *Phyllosticta* have previously been published (Rodrigues et al., 2019; Wang et al., 2020), but in both cases relatively few CAZyme genes were found (see Introduction), which could be due to the fact that the genome assemblies used were generally relatively fragmented (92–5748 contigs in one study (Wang et al., 2020), 11,080–19143 contigs in the other (Rodrigues et al., 2019) or due to the method used to identify the CAZyme encoding genes. In our study, CAZyme genes were annotated as part of the standard JGI annotation pipeline by the Henrissat lab (Lombard et al., 2014) for all tested species, except for one of the *P. citricarpa* genomes as no CAZyme annotation was performed for that genome. Between 381 and 396 CAZyme genes were found, with higher numbers of genes for the species with better genome assemblies. Most genes were in the Glycoside Hydrolase (GH) family (174–180 genes), followed by genes in the Glycosyl Transferase (GT) family (79–82 genes), Auxiliary Activity family (54–59 genes), Carbohydrate-Binding Module (CBM) family (34–45 genes), Carbohydrate Esterase (CE) family (15–17 genes), Polysaccharide Lyase (PL) family (8 genes), and genes distantly related to plant expansins (3–6 genes). The CAZyme genes found in this study were blasted to those found by Wang et al. (2020), and for 111 genes, no significant hits were found. This number corresponds well to the fact that we found on average 118 genes extra in this study. The 111 missing genes included genes from all six different types of CAZyme families (e.g. GH, GT, AA, CBM, CE and PL). Most striking was the difference in number found for the CBM family: while Wang et al. (2020) found a single gene in this family, we identified 34–45 genes. From the pool of genes without hits, 14 were randomly selected and blasted against the genome used by Wang et al. (2020). As no significant hits were found, the absence of these genes in earlier studies can likely be attributed to assembly quality.

Generally, all species seem to have similar CAZyme repertoires with some small differences in specific families (Table 2). CAzyme genes were filtered manually for those involved in plant biomass breakdown as described by (Vesth et al., 2018; de Vries et al., 2017). *Phyllosticta* genomes contain a lower number of genes in most categories (xylan, xyloglucan, mannan, pectin, starch) when compared to the numbers in several *Aspergillus* spp. as described by Vesth et al. (2018), except for cellulose, for which they contain a similar number of genes (Suppl. Table S2). However, *Phyllosticta* spp were able to grow on most carbon sources, albeit at a lower rate (Suppl. Figure 3). The only source on which growth was severely diminished for all species is D-xylose. This is surprising, as the enzymes required for D-xylose ultilization are also required for L-arabinose ultilization (Khosravi et al., 2015), on which growth was observed for most species.

Interestingly, significant differences were observed for growth in different substrates, but these could in most cases not be attributed to differences in genome content. Relative growth (compared to D-glucose) of *P. citribraziliensis* and *P. citriasiana* on xylan was better than that of the other species, but the genome content of known xylan-acting enzyme encoding genes was nearly identical between all species. Similarly, relative growth on calcium lignin was particularly good for *P. paracitricarpa* without any obvious difference in the number of laccases (CAZy family AA1) or other putative genes involved in lignin degradation. Also, the relative growth of *P. paracitricarpa* and both *P. citricarpa* strains on pectin is low compared to the other three species, without an obvious reduction in the number of putative pectinolytic genes. This suggests that the growth differences on these substrates are not due to differences in gene content, but rather to differences in gene expression. This has also been observed previously for various *Aspergillus* species (Mäkelä et al., 2018; de Vries et al., 2017; Benoit et al., 2015), indicating that genome content gives only a partial picture of the ability of a fungal species.

Table 2
Number of genes containing specific CAZy family modules in *Phyllosticta* (obtained from JGI Mycocosm).

	Phyllosticta capitalensis	*Phyllosticta citribraziliensis*	*Phyllosticta citriasiana*	*Phyllosticta paracitricarpa*	*Phyllosticta citricarpa*
Total	**396**	**382**	**382**	**382**	**381**
Auxiliary Activity	58	59	55	54	54
Carbohydrate-Binding Module	45	34	39	42	42
Carbohydrate Esterase	15	17	16	16	15
Distantly related to plant expansins	6	5	3	4	3
Glycosyl Hydrolase	180	178	177	174	175
Glycosyl Transferase	82	79	82	82	82
Polysaccharide Lyase	8	8	8	8	8

To gain a more precise understanding of the CAZyme repertoire of these species and to assess whether there are differences between endophytes and pathogens, all gene annotations from the available *Phyllosticta* genomes were grouped into Ortholog Groups (OGs) using orthofinder. Ortholog Groups containing annotated CAZyme genes were selected, and the number of genes annotated as CAZyme in each OG was counted for each species (for details see methods). Nearly all OGs contained similar or even the exact same number of CAZyme genes for each species (Suppl. Table S2). In six cases, an OG contained consistently more CAZyme genes in pathogens than in endophytes, or *vice versa*. However, as CAZyme genes were annotated for only one of the two *P. citricarpa* genomes, but both *P. citricarpa* genomes were included in the OG analysis, it was necessary to check whether the second *P. citricarpa* genome contained a similar number of genes in these OGs. In two OGs, this was not the case; the other four OGs and the putative function of the genes contained in them are listed in Table 3. These OGs included genes from the CBM18 family, which contain a chitin-binding domain, the GT31 family potentially involved in cell wall remodeling and the GH3 family which might play a role in cellulose degradation. The genes specific to one lifestyle formed a distinct clade from the other genes of the OG when assembled into a phylogenetic tree (Suppl. Fig. S5). The missing genes were not found when blasted against both the assembled genomes as well as the raw sequence data, indicating they are not missing due to an assembly error.

4. Discussion and conclusion

In this study, carbon utilization of *Phyllosticta* spp. occurring on *Citrus* was examined with particular attention to differences between endophytic and pathogenic species.

Firstly, the minimum growth requirements were established; *Phyllosticta* spp. have a thiamine dependency and only grow properly on a minimal medium supplemented with thiamine and at a low pH (~5). Wang et al. (2019) studied the effect of combinations of several nutrients on conidial germination and appressorium formation of *Phyllosticta citricarpa*. Their results suggest that absence of thiamine reduces appressorium formation with approximately 20%, an effect that becomes much stronger in the absence of salts (40%). They found no significant effect of solely thiamine on conidial germination, but in absence of citric acid the addition of thiamine had a positive effect on both conidial germination and appressorium formation. These results suggest that thiamine is involved in several complex processes in *Phyllosticta*, but what role it exactly plays in mycelial growth is unknown. *Ustilago esculenta*, a phytopathogenic species inducing edible galls in its host *Zizania latifolia*, has been reported to exhibit an obligate requirement for thiamine (Chung and Zheng, 2009), but further reports on the requirement of phytopathogenic species are very limited. A thiamine dependency of *Phyllosticta* spp. is not entirely surprising; many citrus fruits including oranges contain high levels of thiamine (Bailey and Thomas, 1942). We therefore hypothesize that the fungus has thiamine at its disposal in its natural surroundings and has lost the need and ability to produce it. The most important genes in thiamine biosynthesis seem to be present in the genomes of *Phyllosticta* spp. The inability to produce thiamine is therefore likely either the result of an unidentified mutation or due to altered transcriptional regulation.

All the species show improved growth at low pH (pH 5), while they are able to grow at a broader range. All species have been isolated from multiple host tissues, e.g. citrus leaves and citrus fruits. Citrus fruits are known to be acidic as they contain both citric acid and ascorbic acid. Therefore, it is quite possible that the preference for a low pH is an adaptation to growing in the acidic host fruit. These results are also in line with those of Wang et al. (2019), who observed that a pH between 3 and 5 is optimal for conidial germination and appressorium formation. Interestingly, despite a growth preference for low pH, *Phyllosticta* spp. generally alkalinized the surrounding media. Many phytopathogenic fungi are known to either acidify or alkalize their host environment (Alkan et al., 2013; Vylkova, 2017). Some species, such as *Botrytis cinerea*, release specialized sets of pathogenicity factors depending on the pH of the surroundings (Manteau et al., 2003). Furthermore, studies have shown that for certain species, such as *Plenodomus lingam* (*Leptosphaeria maculans*) and *Sclerotinia sclerotiorum*, the observed alkalinisation or acidification is directly related to canker length (Bousset et al., 2019). Several species of *Colletotrichum* are also known for preferring an environment with low pH, but have an alkalising effect on the surrounding host tissue (Alkan et al., 2013; Shnaiderman et al., 2013). For some of these, this alkalising effect has been shown to be directly correlated to pathogenicity (Alkan

Table 3
CAZyme genes in OGs with consistent differences between pathogens and endophytes.

OG number	Predicted function	Gene number in endophytes	Gene number in pathogens
OG2	GH3[a]	9	8
OG3	AA1/3, CBM18[b]	8	7
OG36	GT31[c]	3	4
OG468	CBM18[d]	1	0

[a] Glycoside Hydrolase family 3.
[b] Auxiliary activity family 1, subfamily 3 and Carbohydrate Binding Module family 18.
[c] Glycosyl Transferase family 31.
[d] Carbohydrate Binding Module family 18.

et al., 2008; Miyara et al., 2010). Whether a similar mechanism occurs *in planta* in *Phyllosticta* spp. needs to be addressed in future studies.

In general, all species show similar growth on 35 carbon sources, although a few differences were observed. Endophytic species appear to have higher growth rates than pathogenic species on all carbon sources, which is in line with the earlier finding that *P. capitalensis* grows faster than *P. citricarpa* (Baayen et al., 2002; Glienke et al., 2011). Small differences in growth, which were observed on several carbon sources, could be due to strain variation rather than species variation. A much broader sampling using a much larger number of globally distributed strains is needed to test whether these small differences are significant. The results of the growth profiles generally correspond well to what the species would be expected to rely on in their natural habitat. The species are often found on both the citrus fruit peel as well as citrus leaves, and sometimes twigs. On rich sources, such as citrus pulp, wheat bran, and cotton seed pulp, all species grew well, as expected. An exception is sugar beet pulp, which is discussed extensively in a later paragraph. Citrus peel contains large amounts of pectin, hemicellulose and cellulose, the latter two of which are present in smaller amounts than the first (Ting and Deszyck, 1961; Shan, 2016). All species grew well on pectin, and better on citrus pectin compared to apple pectin, which is to be expected for species with citrus as host. On cellulose as a sole source, most species grew poorly. Citrus leaves contain large amounts of starch, as well as sucrose, glucose, and fructose (the latter three to a lesser extent than the first) (Arbona et al., 2005; Iglesias et al., 2002, 2006). All species grew well on starch, with pathogens showing a slightly decreased growth rate compared to endophytes, and medium to well on glucose, fructose and sucrose.

The most closely related species for which a similar growth profiling has been performed is *Sphaeropsis sapinea* (www.fung-growth.org). Interestingly, similarly to *Phyllosticta*, this species shows reduced growth on D-xylose, while growth on L-arabinose was unaffected. This is surprising as utilization of both sources depends on nearly the same set of enzymes. The first step of the conversion of L-arabinose and D-xylose is mediated by a set of three pentose reductases in *A. niger* (Chroumpi et al., 2021), which however have different affinities for the two pentose sugars (Terebieniec et al., unpublished data). Possibly the reductase in *Phyllosticta* has a low affinity for D-xylose, causing the reduced growth on D-xylose. Alternatively, *Phyllosticta* may have a poor uptake ability for D-xylose. It could be that the molecular basis of the reduced D-xylose growth is shared within the *Bortyosphaeriales* and is not specific to the genus *Phyllosticta*. However, to be able to determine the extent of this effect, wider and more extensive sampling within the fungal order is required.

An inhibitory effect of SBP on pathogenic species was observed, also in presence of another carbon source (WB) and at low concentrations (0.25%). Which compound is responsible for this effect is unknown. Although a slight decrease in growth rate is observed for pathogens on starch, we consider this substance an unlikely candidate for causing the inhibitory effect, as SBP generally contains less starch compared to wheat bran (Onipe et al., 2015; Palmgren Karlsson et al., 2002) and a strong inhibitory effect is even observed on 0.25% SBP compared to 1% starch (data not shown). As the inhibitory effect was not observed on citrus pulp, it is not likely to be caused by pectin or cellulose, but rather a trace component of SBP. Trace elements such as iron (Fe), zinc (Zn), copper (Cu) and manganese (Mn) can have inhibitory effects when present in higher concentrations than optimum for the tested fungi (Thind and Mandahar, 1968). Sugar beet pulp has been shown to contain relatively high levels of Fe as well as several other trace elements (Škrbić et al., 2010; Organisation for Economic Co-operation and Development, 2002). However, the aforementioned trace elements have also been detected in wheat bran (Yu et al., 2018), which implies that the effect would be dose-dependent, and it is also very likely that the set and concentration of trace elements is strongly batch-dependent. To determine whether these or other elements are responsible for the inhibitory effect would therefore require an exact determination of the levels of the complete set of trace elements in both substances, and a detailed comparison to identify candidate compounds that may be responsible for the growth inhibition. This is a challenging task that requires specific expertise, but it could prove very worthwhile in future studies. Furthermore, optimum concentrations of the trace elements differ widely for different fungi and have not been determined for *Phyllosticta* spp.

Several experiments were performed in an attempt to identify the inhibitory substance, but with only limited results. Proteinase K digestion had no effect on the activity of the substance, indicating that it is not a protein. As the substance remains active after autoclaving, it is likely either thermotolerant, or protected by, for instance, embedment in rigorous cell wall components. Ethyl acetate and hexane extractions were not effective at extracting the inhibiting substance. After differential centrifugation, the substance consistently remains in the first fraction, even after spinning down at only 100 g. This indicates that the substance is likely either bound to something of substantial mass, or highly hydrophobic; perhaps it is associated with the cell wall or cell membrane. To determine the exact nature of the inhibitory substance, a precise analysis of the components present in SBP should first be performed in the future, after which the different components could be tested separately and in varying combinations.

At the highest concentration tested (3%), the inhibitory effect seemed to be decreasing. It is possible that the large increase in nutrient availability starts compensating for the inhibitory effect at this concentration. Interestingly, sugar cane residue was also reported to have an application in disease management of *P. citricarpa* (van Bruggen et al., 2017). Whether this is due to a similar mechanism is unclear.

Furthermore, we studied the presence of genes related to the carbon utilization capabilities of *Phyllosticta* spp. by analysing the CAZome of these species. Previous studies reported CAZyme encoding gene counts in *Phyllosticta* between 23 and 267 genes, which is low compared to other Dothideomycetes, which generally contain between 300 and 400 CAZyme genes (Haridas et al., 2020; Ohm et al., 2012; Rodrigues et al., 2019; Wang et al., 2020). In this study, we found much higher numbers of CAZyme encoding genes (381–396), which is in line with what is reported for other *Dothideomycetes*. The genome assemblies used in this study seem to be more complete than those used in previous studies. Several of the genes found in this study were blasted to the genome used by Wang et al. (2020), and no significant hits were found. We therefore think these data on CAZyme genes provide a more accurate account of what is present in these species.

Some notes on the method of generating gene counts in this study should be taken into account when comparing to gene counts generated by other pipelines. Genes are annotated as CAZyme when they possess a certain signature; as genes can possess more than one signature, some genes are included in more than one annotation. In fact, the numbers on the JGI Mycocosm table reflect the number of genes carrying a specific CAZy module, not the unique number of genes that have any CAZy module, which may cause differences with other approaches for CAZyme annotation. For instance, in the Auxiliary Activity family of *P. capitalensis* CBS 128856, two genes received a double annotation: 299,199 and 255,817, as they each contain the modules belonging to two different families. Functional research into these genes could provide further knowledge about their functions.

In contrast with earlier studies, we did not find a difference in the number of putative PCWDE genes between species with different lifestyles within this genus. The different *Phyllosticta* species assessed in this study possess similar CAZyme gene repertoires, with only small differences. *Phyllosticta* genomes contained fewer CAZyme genes for breakdown of plant biomass categories when compared to the numbers in several *Aspergillus* spp. as described by Vesth et al. (2018), with the exception of cellulose, for which they contain a similar number of genes. The species of *Aspergillus* studied by Vesth et al. (2018) are very diverse, including strains isolated from several different kinds of biomass, but *Aspergillus* species typically have a broad substrate range, which likely explains their larger set of CAZymes. This is not clearly reflected in the ability of *Phyllosticta* spp. to grow on a range of carbon sources, as the only source on which growth was severely diminished for all species was xylose. Other small but significant differences in growth on several sources were observed, but none could be attributed to a change in gene number. The ability to degrade biomass is therefore likely transcriptionally regulated.

In four cases, an OG was found which consistently contained a different number of genes in endophytes as compared to pathogens (Table 3). The lifestyle-specific genes formed a separate clade from other genes in the OG when assembled into a phylogenetic tree, indicating they are different to some extent from the other genes in the OGs (Suppl. Fig. S5). Although further research is necessary to confirm a role in pathogenicity for these genes, it is interesting to speculate about their putative functions.

Two of these OGs contain genes belonging to the CBM18 family (OG3 and OG468). CBM18 consists of enzymes containing a chitin-binding domain. Genes from this family have been hypothesized to prevent the activation of a host defence response by disguising fungal chitin in the two pathogenic fungal species *Batrachochytrium dendrobatidis* and *Verticillium nonalfalfae* (Liu and Stajich, 2015; Volk et al., 2019). A similar mechanism has been described for an endophytic *Pestalotiopsis* sp. by Landwehr et al. (2016). Determining whether such a mechanism is also present in *Phyllosticta* would be an interesting subject for future studies. Genes in OG36 belong to the GT31 family, and are likely important for cell wall remodelling. However, nothing is known about a potential role in pathogenicity for this gene family. Genes in OG2 belong to the GH3 family and are predicted to be β-glucosidases, important in cellulose degradation.

Overall, very few differences were found in the CAZome of *Phyllosticta* species with different lifestyles. Only four families showed consistent differences between pathogens and endophytes in this study, which are therefore interesting subjects for future studies. In general, *Phyllosticta* species with different lifestyles also showed only small variations in their ability to utilize different carbon sources, with two exceptions. Firstly, all endophytes grew faster than all pathogens. Secondly, growth of all pathogens was significantly inhibited by addition of sugar beet pulp to the medium. Despite attempts to identify the specific substance that is responsible for the inhibitory effect of sugar beet pulp, the substance and the mechanism associated with its inhibitory effect remain unknown. It is important to note that, while few differences in gene repertoire were found, variations could very well occur on the transcriptional level. Future studies on transcriptomic data of *Phyllosticta* spp. could be of great value in creating a better understanding the difference in lifestyles between these species, and could provide important clues into further understanding their pathogenicity.

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Appendix A. Supplementary data

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References

Alkan, N., Fluhr, R., Sherman, A., Prusky, D., 2008. Role of ammonia secretion and pH modulation on pathogenicity of *Colletotrichum coccodes* on tomato fruit. Mol. Plant Microbe Interact. 21, 1058–1066.

Alkan, N., Espeso, E.A., Prusky, D., 2013. Virulence regulation of phytopathogenic fungi by pH. Antioxidants Redox Signal. 19, 1012–1025.

Arbona, V., Marco, A.J., Iglesias, D.J., López-Climent, M.F., Talon, M., Gómez-Cadenas, A., 2005. Carbohydrate depletion in roots and leaves of salt-stressed potted *Citrus clementina* L. Plant Growth Regul. 46, 153–160.

Baayen, R.P., Bonants, P.J.M., Verkley, G., Carroll, G.C., van der Aa, H.A., de Weerdt, M., van Brouwershaven, I.R., Schutte, G.C., Maccheroni, W., de Blanco, C.G., Azevedo, J.L., 2002. Non-pathogenic isolates of the Citrus Black Spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology 92, 464–477.

Bailey, M.I., Thomas, A.W., 1942. The thiamine and riboflavin contents of citrus fruits. J. Nutr. 24, 85–92.

Benoit, I., Culleton, H., Zhou, M., DiFalco, M., Aguilar-Osorio, G., Battaglia, E., Bouzid, O., Brouwer, C.P.J.M., El-Bushari, H.B.O., Coutinho, P.M., Gruben, B.S., Hildén, K.S., Houbraken, J., Jiménez Barboza, L.A., Levasseur, A., Majoor, E., Mäkelä, M.R., Narang, H.M., Trejo-Aguilar, B., van den Brink, J., vanKuyk, P.A., Wiebenga, A., McKie, V., McCleary, B., Tsang, A., Henrissat, B., de Vries, R.P., 2015. Closely related fungi employ diverse enzymatic strategies to degrade plant biomass. Biotechnol. Biofuels 8, 107.

Bousset, L., Ermel, M., Soglonou, B., Husson, O., 2019. A method to measure redox potential (Eh) and pH in agar media and plants shows that fungal growth is affected by and affects pH and Eh. Fungal Biology 123, 117–124.

Brentu, F.C., Oduro, K.A., Offei, S.K., Odamtten, G.T., Vicent, A., Peres, N.A., Timmer, L.W., 2012. Crop loss, aetiology, and epidemiology of citrus black spot in Ghana. Eur. J. Plant Pathol. 133, 657–670.

Choi, J., Kim, K.-T., Jeon, J., Lee, Y.-H., 2013. Fungal plant cell wall-degrading enzyme database: a platform for comparative and evolutionary genomics in fungi and Oomycetes. BMC Genom. 14, S7.

Chroumpi, T., Peng, M., Aguilar Pontes, M.V., Müller, A., Wang, M., Yan, J., Lipzen, A., Ng, V., Grigoriev, I.V., Mäkelä, M.R., de Vries, R.P., 2021. Revisiting a 'simple' fungal metabolic pathway reveals redundancy, complexity and diversity. Microbial Biotechnology in press.

Chung, K., Tzeng, D., 2009. Thiamine (vitamin B1) plays a critical role on sugar utilization by the phytopathogenic fungus, *Ustilago esculenta*. Res. J. Microbiol. 4, 178–185.

Cord-Landwehr, S., Melcher, R.L.J., Kolkenbrock, S., Moerschbacher, B.M., 2016. A chitin deacetylase from the endophytic fungus *Pestalotiopsis* sp. efficiently inactivates the elicitor activity of chitin oligomers in rice cells. Sci. Rep. 6, 1–11.

Crous, P.W., Verkley, G.J.M., Groenewald, J.Z., Samson, R.A. (Eds.), 2019. Westerdijk Laboratory Manual Series 1: Fungal Biodiversity, second ed. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

de Vries, R.P., Burgers, K., van de Vondervoort PJI, Frisvad, J.C., Samson, R.A., Visser, J., 2004. A new black *Aspergillus* species, *A. vadensis*, is a promising host for homologous and heterologous protein production. Appl. Environ. Microbiol. 70, 3954–3959.

de Vries, R.P., Riley, R., Wiebenga, A., Aguilar-Osorio, G., Amillis, S., Akemi Uchima, C., Anderluh, G., Asadollahi, M., Askin, M., Barry, K., Battaglia, E., Ö, Bayram, Benocci, T., Braus-Stromeyer, S.A., Caldana, C., Cánovas, D., Cerqueira, G.C., Chen, F., Chen, W., Choi, C., Clum, A., Corrêa dos Santos, R.A., de Lima Damásio, A.R., Diallinas, G., Emri, T., Fekete, E., Flipphi, M., Freyberg, S., Gallo, A., Gournas, C., Habgood, R., Haimaut, M., Harispe, L., Henrissat, B., Hildén, K.S., Hope, R., Hossain, A., Karabika, E., Karaffa, L., Karányi, Z., Kraševec, N., Kuo, A., Kusch, H., LaButti, K., Lagendijk, E.L., Lapidus, A., Levasseur, A., Lindquist, E., Lipzen, A., Logrieco, A.F., MacCabe, A., Mäkelä, M.R., Malavazi, I., Melin, P., Meyer, V., Mielnichuk, N., Miskei, M., Molnár, A.P., Mulé, G., Ngan, C.Y., Orejas, M., Orosz, E., Ouedraogo, J.P., Overkamp, K.M., Park, H.-S., Perrone, G., Piumi, F., Punt, P., Ram, A.F.J., Ramón, A., Rauscher, S., Record, E., Riaño-Pachón, D.M., Robert, V., Röhrig, Ruller R., Salamov, A.,

Salih, N.S., Samson, R.A., Sándor, E., Sanguinetti, M., Schütze, T., Sepčić, K., Shelest, E., Sherlock, G., Sophianopoulou, V., Squina, F.M., Sun, H., Susca, A., Todd, R.B., Tsang, A., Unkles, S.E., van de Wiele, N., van Rossen-Uffink, D., Velasco de Castro Oliveira, J., Vesth, T.C., Visser, J., Yu, J.-H., Zhou, M., Andersen, M.R., Archer, D.B., Baker, S.E., Benoit, I., Brakhage, A.A., Braus, G.H., Fischer, R., Frisvad, J.C., Goldman, G.H., Houbraken, J., Oakley, B., Pócsi, I., Scazzocchio, C., Seiboth, vanKuyk PA., Wortman, J., Dyer, P.S., Grigoriev, I.V., 2017. Comparative genomics reveals high biological diversity and specific adaptations in the industrially and medically important fungal genus *Aspergillus*. Genome Biol. 18, 28.

Douaiher, M.-N., Nowak, E., Durand, R., Halama, P., Reignault, P., 2007. Correlative analysis of *Mycosphaerella graminicola* pathogenicity and cell wall-degrading enzymes produced in vitro: the importance of xylanase and polygalacturonase. *Plant Pathol.* 56, 79–86.

Emms, D., Kelly, S., 2019. OrthoFinder: phylogenetic orthology inference for comparative genomics. Genome Biol. 20, 238.

Eustáquio Lanza, F., Germano Metzker, T., Vinhas, T., Behlau, F., José Silva Junior, G., 2018. Critical fungicide spray period for citrus black spot control in são Paulo state, Brazil. Plant Dis. 102, 334–340.

Glienke, C., Pereira, O.L., Stringari, D., Fabris, J., Kava-Cordeiro, V., Galli-Terasawa, L., Cunnington, J., Shivas, R.G., Groenewald, J.Z., Crous, P.W., 2011. Endophytic and pathogenic *Phyllosticta* species, with reference to those associated with Citrus Black Spot. Persoonia 26, 47–56.

Guarnaccia, V., Groenewald, J.Z., Li, H., Glienke, C., Carstens, E., Hattingh, V., Fourie, P.H., Crous, P.W., 2017. First report of *Phyllosticta citricarpa* and description of two new species, *P. paracapitalensis* and *P. paracitricarpa*, from citrus in Europe. Stud. Mycol. 87, 161–185.

Guarnaccia, V., Gehrmann, T., Silva-Junior, G.J., Fourie, P.H., Haridas, S., Vu, D., Spatafora, J., Martin, F.M., Robert, V., Grigoriev, I.V., Groenewald, J.Z., Crous, P.W., 2019. *Phyllosticta citricarpa* and sister species of global importance to Citrus. Mol. Plant Pathol. 20, 1619–1635.

Haridas, S., Albert, R., Binder, M., Bloem, J., LaButti, K., Salamov, A., Andreopoulos, B., Baker, S.E., Barry, K., Bills, G., Bluhm, B.H., Cannon, C., Castanera, R., Culley, D.E., Daum, C., Ezra, D., González, J.B., Henrissat, B., Kuo, A., Liang, C., Lipzen, A., Lutzoni, F., Magnuson, J., Mondo, S.J., Nolan, M., Ohm, R.A., Pangilinan, J., Park, H.-J., Ramírez, L., Alfaro, M., Sun, H., Tritt, A., Yoshinaga, Y., Zwiers, L.-H., Turgeon, B.G., Goodwin, S.B., Spatafora, J.W., Crous, P.W., Grigoriev, I.V., 2020. 101 *Dothideomycetes* genomes: a test case for predicting lifestyles and emergence of pathogens. Stud. Mycol. 96, 141–153.

Houston, K., Tucker, M.R., Chowdhury, J., Shirley, N., Little, A., 2016. The plant cell wall: a complex and dynamic structure as revealed by the responses of genes under stress conditions. Front. Plant Sci. 7, 984.

Iglesias, D.J., Lliso, I., Tadeo, F.R., Talon, M., 2002. Regulation of photosynthesis through source: sink imbalance in citrus is mediated by carbohydrate content in leaves. Physiol. Plantarum 116, 563–572.

Iglesias, D.J., Á, Calatayud, Barreno, E., Primo-Millo, E., Talon, M., 2006. Responses of citrus plants to ozone: leaf biochemistry, antioxidant mechanisms and lipid peroxidation. Plant Physiol. Biochem. 44, 125–131.

Khosravi, C., Benocci, T., Battaglia, E., Benoit, I., de Vries, R.P., 2015. Sugar catabolism in *Aspergillus* and other fungi related to the utilization of plant biomass. Adv. Appl. Microbiol. 90, 1–28.

King, B.C., Waxman, K.D., Nenni, N.V., Walker, L.P., Bergstrom, G.C., Gibson, D.M., 2011. Arsenal of plant cell wall degrading enzymes reflects host preference among plant pathogenic fungi. Biotechnol. Biofuels 4, 1–14.

Kotzé, J.M., 1981. Epidemiology and control of citrus black spot in South Africa. Plant Dis. 65, 945.

Kotzé, J.M., 2000. Black spot. In: Timmer, L.W., Garnsey, S.M., Graham, J.H. (Eds.), Compendium of Citrus Diseases. American Phytopathological Society Press Inc, The American Phytopathological Society, pp. 23–25.

Kubicek, C.P., Starr, T.L., Glass, N.L., 2014. Plant cell wall-degrading enzymes and their secretion in plant-pathogenic fungi. Annu. Rev. Phytopathol. 52, 427–451.

Liu, P., Stajich, J., 2015. Characterization of the carbohydrate binding module 18 gene family in the amphibian pathogen *Batrachochytrium dendrobatidis*. Fungal Genet. Biol. 77, 31–39.

Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., Kahmann, R., 2015. Fungal effectors and plant susceptibility. Annu. Rev. Plant Biol. 66, 513–554.

Lombard, V., Golaconda Ramulu, H., Drula, E., Coutinho, P.M., Henrissat, B., 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 42, D490–D495.

Mäkelä, M.R., Donofrio, N., de Vries, R.P., 2014. Plant biomass degradation by fungi. Fungal Genet. Biol. 72, 2–9.

Mäkelä, M.R., DiFalco, M., McDonnell, E., Nguyen, T.T.M., Wiebenga, A., Hildén, K., Peng, M., Grigoriev, I.V., Tsang, A., de Vries, R.P., 2018. Genomic and exoproteomic diversity in plant biomass degradation approaches among Aspergilli. Stud. Mycol. 91, 79–99.

Manteau, S., Abouna, S., Lambert, B., Legendre, L., 2003. Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 43, 359–366.

Miyara, I., Shafran, H., Davidzon, M., Sherman, A., Prusky, D., 2010. pH regulation of ammonia secretion by *Colletotrichum gloeosporioides* and its effect on appressorium formation and pathogenicity. Mol. Plant Microbe Interact. 23, 304–316.

Ohm, R.A., Feau, N., Henrissat, B., Schoch, C.L., Horwitz, B.A., Barry, K.W., Condon, B.J., Copeland, A.C., Dhillon, B., Glaser, F., Hesse, C.N., Kosti, I., LaButti, K., Lindquist, E.A., Lucas, S., Salamov, A.A., Bradshaw, R.E., Ciuffetti, L., Hamelin, R.C., Kema, G.H.J., Lawrence, C., Scott, J.A., Spatafora, J.W., Turgeon, B.G., de Wit, P.J.G.M., Zhong, S., Goodwin, S.B., Grigoriev, I.V., 2012. Diverse lifestyles and strategies of plant pathogenesis encoded in the genomes of eighteen dothideomycetes fungi. PLoS Pathog. 8, e1003037.

Onipe, O.O., Jideani, A.I.O., Beswa, D., 2015. Composition and functionality of wheat bran and its application in some cereal food products. Int. J. Food Sci. Technol. 50, 2509–2518.

Organisation for Economic Co-operation and Development, 2002. Series on the Safety of Novel Foods and Feeds No. 3. Consensus Document on Compositional Considerations for New Varieties of Sugar Beet: Key Food and Feed Nutrients and Anti-nutrients. OECD Environmental Health and Safety Publications, Paris, France.

Palmgren Karlsson, C., Jansson, A., Essén-Gustavsson, B., Lindberg, J.E., 2002. Effect of molassed sugar beet pulp on nutrient utilisation and metabolic parameters during exercise. Equine Vet. J. 34, 44–49.

Rodrigues, C.M., Takita, M.A., Silva, N.V., Ribeiro-Alves, M., Machado, M.A., 2019. Comparative genome analysis of *Phyllosticta citricarpa* and *Phyllosticta capitalensis*, two fungi species that share the same host. BMC Genom. 20, 554.

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N., Nelson, A., 2019. The global burden of pathogens and pests on major food crops. Nature Ecology and Evolution 3, 430–439.

Shan, Y., 2016. Functional components of citrus peel contents. Comprehensive Utilization of Citrus By-Products, pp. 1–13.

Shnaiderman, C., Miyara, I., Kobiler, I., Sherman, A., Prusky, D., 2013. Differential activation of ammonium transporters during the accumulation of ammonia by *Colletotrichum gloeosporioides* and its effect on appressoria formation and pathogenicity. Mol. Plant Microbe Interact. 26, 345–355.

Škrbić, B., Durišić-Mladenović, N., Mačvanin, N., 2010. Determination of metal contents in sugar beet (*Beta vulgaris*) and its products: empirical and chemometrical approach. Food Sci. Technol. Res. 16, 123–134.

ten Have, A., Tenberge, K.B., Benen, J.A.E., Tudzynski, P., Visser, J., van Kan, J.A.L., 2002. The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens. In: Kempken, F. (Ed.), Agricultural Applications, first ed., vol. 11. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 341–358. The Mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research).

Thind, K.S., Mandahar, C.L., 1968. Trace element studies on some pathogenic fungi. Proc. Indian Acad. Sci. B 68, 37–51.

Ting, S.V., Deszyck, E.J., 1961. The carbohydrates in the peel of oranges and grapefruit. J. Food Sci. 26, 146–152.

Truter, M., 2010. Epidemiology of Citrus Black Spot Disease in South Africa and its Impact on Phytosanitary Trade Restrictions. Doctoral Dissertation, University of Pretoria.

van Bruggen, A.H.C., Sharma, K., Shin, K., 2017. Sugar cane processing residue, bagasse, enhances decomposition of citrus leaves and could contribute to citrus black spot management. Crop Protect. 93, 89–97.

van Den Brink, J., de Vries, R.P., 2011. Fungal enzyme sets for plant polysaccharide degradation. Appl. Microbiol. Biotechnol. 91, 1477–1492.

Vesth, T.C., Nybo, J.L., Theobald, S., Frisvad, J.C., Larsen, T.O., Nielsen, K.F., Hoof, J.B., Brandl, J., Salamov, A., Riley, R., Gladden, J.M., Phatale, P., Nielsen, M.T., Lyhne, E.K., Kogle, M.E., Strasser, K., McDonnell, E., Barry, K., Clum, A., Chen, C., LaButti, K., Haridas, S., Nolan, M., Sandor, L., Kuo, A., Lipzen, A., Hainaut, M., Drula, E., Tsang, A., Magnuson, J.K., Henrissat, B., Wiebenga, A., Simmons, B.A., Mäkelä, M.R., de Vries, R.P., Grigoriev, I.V., Mortensen, U.H., Baker, S.E., Andersen, M.R., 2018. Investigation of inter- and intraspecies variation through genome sequencing of *Aspergillus* section *Nigri*. Nat. Genet. 50, 1688–1695.

Volk, H., Marton, K., Flajšman, M., Radišek, S., Tian, H., Hein, I., Č, Podlipnik, Thomma, B.P.H.J., Košmelj, K., Javornik, B., Berne, S., 2019. Chitin-binding protein of *Verticillium nonalfalfae* disguises fungus from plant chitinases and suppresses chitin-triggered host immunity. Mol. Plant Microbe Interact. 32, 1378–1390.

Vylkova, S., 2017. Environmental pH modulation by pathogenic fungi as a strategy to conquer the host. PLoS Pathog. 13, e1006149.

Wang, N.-Y., Dewdney, M.M., 2019. The effects of nutrition and environmental factors on conidial germination and appressorium formation of *Phyllosticta citricarpa*, the causal agent of Citrus Black Spot. Phytopathology 109, 650–658.

Wang, X., Chen, G., Huang, F., Zhang, J., Hyde, K.D., Li, H., 2012. *Phyllosticta* species associated with citrus diseases in China. Fungal Divers. 52, 209–224.

Wang, M., Liu, B., Ruan, R., Zeng, Y., Luo, J., Li, H., 2020. Genomic sequencing of *Phyllosticta citriasiana* provides insight into its conservation and diversification with two closely related *Phyllosticta* species associated with citrus. Front. Microbiol. 10, 2979.

Wikee, S., Lombard, L., Crous, P.W., Nakashima, C., Motohashi, K., Chukeatirote, E., Alias, S.A., Mckenzie, E.H.C., Hyde, K.D., 2013a. *Phyllosticta capitalensis*, a widespread endophyte of plants. Fungal Divers. 60, 91–105.
Wikee, S., Lombard, L., Nakashima, C., Motohashi, K., Chukeatirote, E., Cheewangkoon, R., McKenzie, E.H.C., Hyde, K.D., Crous, P.W., 2013b. A phylogenetic re-evaluation of *Phyllosticta* (*Botryosphaeriales*). Stud. Mycol. 76, 1–29.
Wulandari, N.F., To-anun, C., Hyde, K.D., Duong, L.M., de Gruyter, J., Meffert, J.P., Groenewald, J.Z., Crous, P.W., 2009. *Phyllosticta citriasiana* sp. nov., the cause of Citrus tan spot of *Citrus maxima* in Asia. Fungal Divers. 34, 23–39.
Yu, X., Han, J., Li, H., Zhang, Y., Feng, J., 2018. The effect of enzymes on release of trace elements in feedstuffs based on in vitro digestion model for monogastric livestock. J. Anim. Sci. Biotechnol. 9, 1–8.