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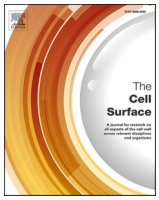

Study of fungal cell wall evolution through its monosaccharide composition: An insight into fungal species interacting with plants

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ABSTRACT

Every fungal cell is encapsulated in a cell wall, essential for cell viability, morphogenesis, and pathogenesis. Most knowledge of the cell wall composition in fungi has focused on ascomycetes, especially human pathogens, but considerably less is known about early divergent fungal groups, such as species in the Zoopagomycota and Mucoromycota phyla. To shed light on evolutionary changes in the fungal cell wall, we studied the monosaccharide composition of the cell wall of 18 species including early diverging fungi and species in the Basidiomycota and Ascomycota phyla with a focus on those with pathogenic lifestyles and interactions with plants. Our data revealed that chitin is the most characteristic component of the fungal cell wall, and was found to be in a higher proportion in the early divergent groups. The Mucoromycota species possess few glucans, but instead have other monosaccharides such as fucose and glucuronic acid that are almost exclusively found in their cell walls. Additionally, we observed that hexoses (glucose, mannose and galactose) accumulate in much higher proportions in species belonging to Dikarya. Our data demonstrate a clear relationship between phylogenetic position and fungal cell wall carbohydrate composition and lay the foundation for a better understanding of their evolution and their role in plant interactions.

1. Introduction

The kingdom Fungi is one of the most diverse clades of eukaryotes in terrestrial ecosystems, where they provide numerous ecological services ranging from decomposition of organic matter and nutrient cycling to beneficial and antagonistic associations with plants and animals (Hawksworth and Lücking, 2017; Spatafora et al., 2017). Most fungal cell is encapsulated in a cell wall, which forms the interface between the fungus and the environment (or host). Fungal cell walls are carbohydrate-based dynamic structures that are essential for cell viability, morphogenesis, and pathogenesis (Gow and Lenardon, 2023). The cell wall is much more than the outer layer of the fungus; it is a dynamic organelle whose composition is highly regulated in response to environmental conditions and imposed stresses and greatly influences the ecology of the fungus (Hopke et al., 2018; Pradhan et al., 2019). It has recently been suggested that a more appropriate terminology for this structure would be needed, as the cell wall metaphor does not accurately reflect the composition of the structure or its true role in partitioning and orchestrating the living cell (Casadevall and Gow, 2022). Despite its importance and decades of research, given its complexity and specific technical skills required, the detailed composition and structure of the fungal cell wall is only partially understood (Gow et al., 2023). Whilst we have a relatively detailed understanding of the cell wall in a handful of mammal-pathogens (Chakraborty et al., 2021; Erwig and Gow, 2016), we lack this knowledge in fungi-colonising plants (Geoghegan et al., 2017). Indeed, cell wall composition has only been determined quantitatively in a handful of species and using methodologies not always comparable (Blatzer et al., 2020).

The fungal cell wall can be divided into two main layers: the inner and outer cell walls. The inner "skeletal" layer is typically composed of

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chitin and linear β-glucans (Gow and Lenardon, 2023; Kang et al., 2018). It is relatively well conserved among all fungi genera and is protected from immune recognition by the outer layer, whose composition is more variable across different fungi (Hatinguais et al., 2020). While plants and bacteria are devoid of chitin, this polymer is present, with a few exceptions, along the whole fungal kingdom. Chitin is a linear polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues. It is typically crystalline and extraordinarily strong, and contributes to the stress-bearing property of the fungal cell wall (Brown et al., 2020). Glucans represent another family of wall polymers widely distributed across the fungal kingdom the most common type being those bonded between their carbons 1 and 3 (both α and β anomers), although β-1,6-, β-1,2-, β-1,4- and α-1,4-linked glucans also occur in some species (Kang et al., 2018; Ruiz-Herrera and Ortiz-Castellanos, 2019). In addition to glucans and chitin, fungal walls also contain variable proportions of homo- or hetero-polysaccharides, which can form part of glycoproteins or glycopeptides (Leal et al., 2010). If protein-bound, the carbohydrate moiety of such wall structural covalently-bound glycoproteins is actually much more abundant than the peptide one, and substantially varies depending on phylogenetic position. In addition, protein-free mannans and galactans are also frequently found. In ascomycete fungi, different types of complex heteromannans constitute up to 15 % of the cell wall dry weight, being usually α-1,2- and α-1,6-mannans (highly branched) the main backbones (Shibata et al., 2007; Leal et al., 2010). In other fungal species, the mannan backbone represents < 5 % (the core) of the whole polymer, to which long side chains of galactofuranose with different linkage types are attached. To these galactofuranose chains, single residues or short chains of other sugars may be attached (Leal et al., 2010). One type of galactans that has recently received increasing attention are the galactosaminogalactans. These are α-linked galactans containing galactosamine, whose proportion and degree of *N*-acetylation varies greatly between species (Briard et al., 2016). It should be noted that their interest arises in part from being the first polysaccharide described as a virulence factor in medical mycology (Fontaine et al., 2011).

The advent of molecular phylogenetics, and more recently phylogenomics, has greatly advanced our understanding of the patterns and processes associated with fungal evolution (Chang et al., 2022; James et al., 2020; Spatafora et al., 2017). Moreover, it is expected that during the course of fungal evolution, species have adapted their cell wall structures to the extremely diverse environmental conditions that they dwell. In line with this hypothesis, recently Schwerdt et al (2021) used phylogenomic analyses of the enzymatic pathways that synthesize and interconvert nucleotide-sugars to predict potential cell wall monosaccharide composition across almost 500 fungal taxa and found a significant reduction in monosaccharide diversity during fungal evolution. However, as cell walls are highly dynamic and complex, linking genomic repertories to their cell wall counterparts is not always possible and chemical analyses are mandatory. Our previous research conducted in a limited number of species showed striking differences in the cell wall monosaccharide composition of fungi in different phylogenetic lineages, especially in those placed at the early diverging groups (Mélida et al., 2015). This is consistent with a recent comparison of Microsporidia, Chytridiomycetes, and Zygomycetes with Ascomycetes and Basidomycetes that suggested that the most recent fungi in the phylogeny appear to have the most complex cell wall composition (Blatzer et al., 2020). Thus, these data challenge the dogma that fungal walls exhibit comparable structures regardless of their phylogenetic position and encourage to develop a more detailed and broad study of the fungal cell wall evolution. The aim of this work was therefore to shed light on the evolution of fungal cell wall carbohydrate composition, paying special attention to early divergent groups and species interacting with plants, groups that have been much less studied so far.

2. Material and methods

2.1. Biological materials and growth conditions

All analyses were performed on the mycelial cell walls from 18 different fungal strains representing saprobes and plant pathogens. The strains were from (I) the Centraal Bureau voor Schimmel Culture (CBS, The Netherlands) [*Mortierella elongata* (CBS 279.62); *Umbelopsis ramanniana* (CBS 219.47); *Rhizopus stolonifer* (CBS 263.28); *Gilbertella persicaria* (CBS 190.32); *Mucor mucedo* (CBS 109.16); *Fusarium graminearum* (CBS 123657) and *Fusarium verticillioides* (CBS 218.76)]; (II) the Agricultural Research Service Culture Collection (ARS-NRRL, USA) [*Basidiobolus meristosporus* (NRRL 2992); *Neoconidiobolus thromboides* (ARSEF 4968); *Capillidium pumilum* (ARSEF 6383); *Zoophthora radicans* (ARSEF 4784) and *Phycomyces blakesleeanus* (NRRL 1555 "-" mating-type)]; (III) the Spanish Type Culture Collection (CECT, Spain) [*Botrytis cinerea* (CECT 2100); and *Trichoderma harzianum* (CECT 2413)] or (IV) kindly provided by T. Engelsdorf (Philipps Marburg University, Germany) [*Ustilago maydis* (SG200); *Colletotrichum graminicola* (CgM2) and *Colletotrichum higginsianum* (MAFF 305635)] and (V) by A. Sánchez-Vallet (Universidad Politécnica de Madrid, Spain) [*Zymoseptoria tritici* (Swiss strain ST99CH_3D7)].

Fungi were grown in their appropriate media as specified in Table 1. Although it has been shown that wall composition can vary in response to different stimuli, such as different carbon sources (Ballou et al., 2016), we have chosen the described media as optimal for each species. These were, potato dextrose broth (PDB) or agar (PDA), malt extract broth (MEB) or agar (MEA), yeast extract peptone sucrose light (YEPSL) or yeast extract peptone dextrose (YPD) medium. Mycelia were

Table 1
Summary of strains used in this study and media on which they were grown.

Species	Phylum/Division (order)	Strain	Medium
Basidiobolus meristosporus	Zoopagomycota (Basidiobales)	NRRL 2992	PDB
Neoconidiobolus thromboides	Zoopagomycota (Entomophthorales)	ARSEF 4968	PDB
Capillidium pumilum	Zoopagomycota (Entomophthorales)	ARSEF 6383	PDB
Zoophthora radicans	Zoopagomycota (Entomophthorales)	ARSEF 4784	PDB
Mortierella elongata	Mucoromycota (Morteriellales)	CBS 279.62	MEB
Umbelopsis ramanniana	Mucoromycota (Umbelipsoidales)	CBS 219.47	MEA
Phycomyces blakesleeanus	Mucoromycota (Mucorales)	NRRL 1555	PDB
Rhizopus stolonifer	Mucoromycota (Mucorales)	CBS 263.28	MEA
Gilbertella persicaria	Mucoromycota (Mucorales)	CBS 190.32	MEA
Mucor mucedo	Mucoromycota (Mucorales)	CBS 109.16	PDA
Ustilago maydis	Basidiomycota (Ustilaginales)	SG200	YEPSL
Zymoseptoria tritici	Ascomycota (Capnodiales)	ST99CH_3D7	YPD
Botrytis cinerea	Ascomycota (Helotiales)	CECT 2100	PDB
Colletotrichum graminicola	Ascomycota (Glomerellales)	CgM2	PDB
Colletotrichum higginsianum	Ascomycota (Glomerellales)	MAFF 305,635	PDB
Trichoderma harzianum	Ascomycota (Hypocreales)	CECT 2413	PDB
Fusarium graminearum	Ascomycota (Hypocreales)	CBS 123,657	PDB
Fusarium verticillioides	Ascomycota (Hypocreales)	CBS 218.76	PDB

harvested by filtration on sterile cellulose-free filters (FP Vericel, Pall Corporation), extensively washed with distilled water to remove the excess of culture medium, frozen and freeze-dried.

2.2. Phylogenetic analyses

The phylogenetic tree was constructed using PHYling v2.0 (https://github.com/stajichlab/PHYling) to generate gene alignments for ~ 750 conserved genes in the fungi_odb10 BUSCO set. Individual gene trees were constructed with IQTREE2 v2.2.2.6 (Hoang et al., 2018; Kalyaanamoorthy et al., 2017) using ultrafast bootstrap, to score alignments and select best genes based on individual gene trees and alignment with treeness, toverr, and RCV statistics calculated by phykit (Steenwyk et al., 2021). Afterwards, a concatenated alignment of the top 50 previously selected genes was built and resolved the Maximum Likelihood tree with iqtree2 on partitioned dataset (Chernomor et al., 2016). The substitution models were determined using ModelTest-NG v0.1.7 (Darriba et al., 2020). The final tree was visualized with FigTree tool, rooting with the Zoopagomycota phylum. Tree building info can be retrieved from https://github.com/stajichlab/Yugueros_cellwall_fungiphylogeny. For the design of Fig. 7, the phylogenetic tree was edited using iTOL (https://itol.embl.de/) (Letunic and Bork, 2016).

2.3. Preparation of cell walls

Cell wall polysaccharides were extracted as previously described (Mélida et al., 2013; Mélida et al., 2015; Fig. 1). Fungal tissues were finely ground and homogenized using a coffee blender. The resulting powders were treated with 70 % (v/v) ethanol three times (1 h, overnight and 2 h) at 4 °C. The soluble materials after each step of centrifugation at 4000 rpm for 10 min at 4 °C were discarded. Mycelial pellets were then treated with 70 % ethanol twice (1 h each) at 85 °C. The pellets at this step were considered as the alcohol-insoluble residue (AIR). Proteins in this material were removed by heating the sample at 85 °C for 10 min (twice) in a 50 mM Tris-HCl buffer (pH 7.8) containing 2 % (w/v) sodium dodecyl sulfate (SDS), 40 mM 2-mercaptoethanol, and 10 mM EDTA. Glycogen/starch-like polymers were removed from the residue by treatments with a thermostable α-amylase from *Bacillus* sp. (Megazyme E-BSTAA) at 85 °C during 20 min and a subsequent treatment with amyloglucosidase (Megazyme E-AMGDF) at 50 °C during 40 min. The resulting pellets were washed three times with 70 % ethanol and with acetone. Once dried, these materials were considered the cell wall.

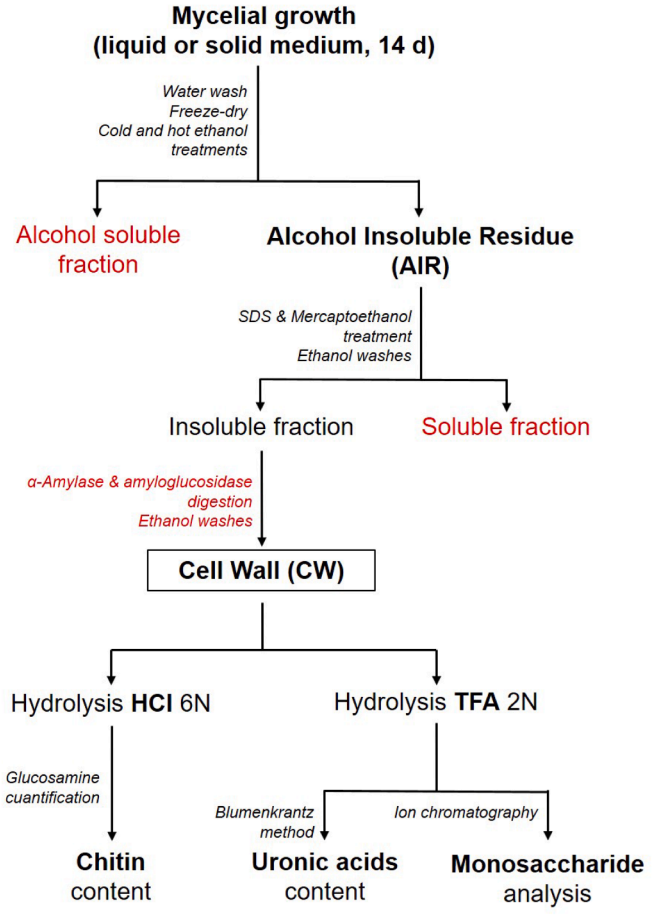

Fig. 1. Scheme of the preparation and analysis of fungal cell wall carbohydrates. Discarded fractions are shown in red.

2.4. Carbohydrate analysis

Purified cell walls were used to determine the amino sugars, the neutral sugars and the uronic acids content and, also, the monosaccharide composition of the different species. To determine the amino sugars content, samples (1 mg) were acid-hydrolysed with 6 N hydrochloric acid (HCl) at 100 °C for 17 h. After the hydrolysis, samples were dissolved in 1 ml of distilled water. Total amino sugars quantification was assayed following the method adapted from Clarke and Tracey (1956). The same hydrolysates were used to determine the ratio of glucosamine to galactosamine by liquid chromatography as described below.

For monosaccharides, total sugars and uronic acids analyses, cell walls (1 mg) were hydrolysed with 2 N trifluoroacetic acid at 120 °C for 3 h and then air dried. The hydrolysates were resuspended in 1 ml of distilled water and filtered through PTFE (polytetrafluoroethylene) syringe filters (0.45 μm). Monosaccharide composition was analysed by high-performance anion-exchange chromatography/pulsed amperometric detection (HPAEC-PAD) in a LC 930 Compact IC Flex (Metrohm) chromatography system with an IC pulsed amperometric detector (FlexiPAD). The eluents used were composed of 1 mM NaOH and 1 mM sodium acetate (NaAc) (eluent A) and 100 mM NaOH with 170 mM NaAc (eluent B). The chromatographic separation was carried out using a Metrosep Carb 2 250/4.0 (Metrohm) analytical column and a Metrosep Carb 2 Guard/4.0 (Metrohm) guard column at 25 °C. Standard curves of commercial standards were used to quantify the monosaccharide composition (in elution order: fucose, galactose, galactosamine, arabinose, glucose, glucosamine rhamnose, xylose, mannose, glucuronic acid and galacturonic acid). Sugars were separated with a flow rate of 0.5 ml/min with the following chromatographic method: 100 % eluent A (0–24 min), 100 % eluent B (24.1–55 min), and 100 % eluent A (55.1–75 min). The phenol–sulfuric method (DuBois et al., 1956) was used to determine the total sugars content and the analysis of uronic acids was performed by following the method adapted from Blumenkrantz and Asboe-Hansen (1973) adapted to 96-well plates. In this work we consider trace amounts of monosaccharides, those values lower than 0.5 % of the total cell wall dry weight (5 μg/mg).

2.5. Protein analysis

Total nitrogen was calculated using the Dumas method. Purified cell wall samples, weighing between 0.9–1.3 mg, were analysed in 5x9 mm pressed tin capsules. The determination was performed by instrumental analysis using the Euro Vector EA 3000 elemental analyser with 18–6 mm CHN reactor at 980 °C, GC SS 2 m 6x5 mm column at 100 °C and thermal conductivity detector. Total protein was then calculated by multiplying total nitrogen by the conversion factor 6.25 (Zeng et al., 2023).

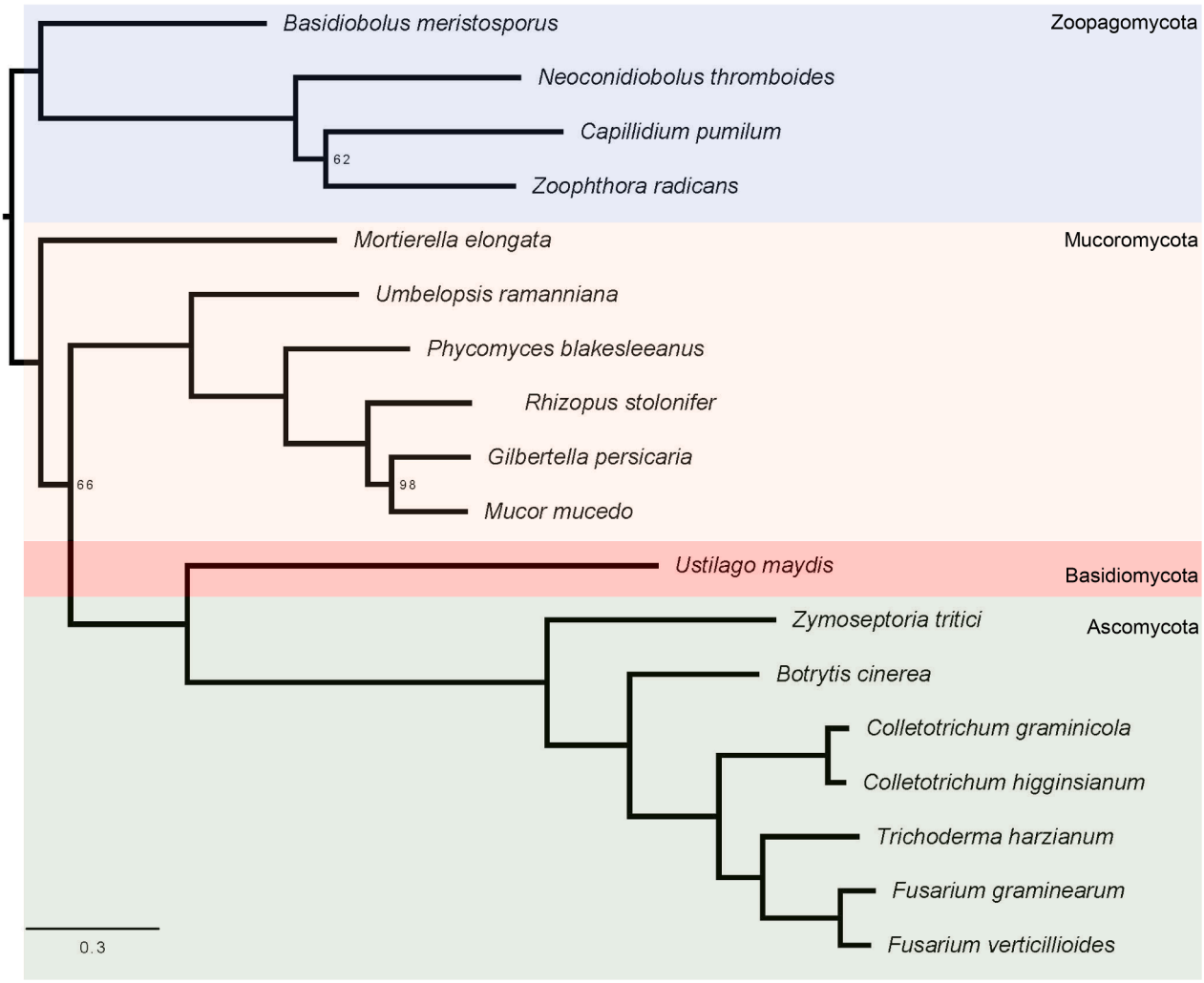

Fig. 2. Phylogenetic relations between fungal species from the Zoopagomycota, Mucoromycota, Basidiomycota and Ascomycota phyla analysed in this study.

3. Results

3.1. Phylogenetic relations among selected species

Cell walls from 18 fungal species were prepared by using a previously established protocol that avoids the use of alkaline solutions, which may lead to the structural alteration of the extracellular matrix and the loss of some of its components (Fig. 1). Among the 18 isolates, 10 of them belong to early divergent groups, namely the divisions Zoopagomycota and Mucoromycota (Fig. 2). Four species belonging to the Entomophthoromycotina (Zoopagomycota) subdivision were analysed: *Basidiobolus meristosporus*, *Neoconidiobolus thromboides*, *Capillidium pumilum* and *Zoophthora radicans*. Most of the species in this group occur commonly in soil and plant detritus but are best characterized as parasites of insects and mites (Spatafora et al., 2016). Among the Mucoromycota phylum, 5 species corresponding to the subdivision Mucoromycotina were sampled: *Umbelopsis ramanniana*, *Phycomyces blakesleeanus*, *Rhizopus stolonifer*, *Gilbertella persicaria* and *Mucor mucedo*. Some are isolated from decaying vegetables, fruits or grains while others have been identified as plant endophytes (Cruz-Lachica et al., 2016; Spatafora et al., 2016). Additionally, *Mortierella elongata* belongs to the same division, but to the Mortierellomycotina subdivision. The Mortierellomycotina are thought to include the first terrestrial fungi to develop distinct fruiting bodies and this species has been identified as a root endophyte (Gryganskyi et al., 2023; Uehling et al., 2017; Vandepol et al., 2020). The remaining 8 species belonged to Dikarya subkingdom and are all well-known plant interactors and most of them are considered pathogens (Fig. 2) (Dean et al., 2012). The maize pathogen *Ustilago maydis* was selected as a representative of the Basidiomycota, while 7 species form the Pezizomycotina subdivision were selected as Ascomycota representatives: *Zymoseptoria tritici*, *Botrytis cinerea*, *Colletotrichum graminicola*, *Colletotrichum higginsianum*, *Trichoderma harzianum*, *Fusarium graminearum* and *Fusarium verticillioides*.

3.2. Chitin and glucans are the main cell wall-glycosidic components along the four divisions

In addition to cabohydrates, fungal cell wall preparations may contain other biomolecules, such as proteins, lipids, pigments, nucleic acids and inorganic components (Gow and Lenardon, 2023). Although the aim of this study was to analyse the glycosidic part of the cell wall, we first decided to quantify the proteins that contribute to this structure. For this purpose, during the purification of the cell walls, chemical treatments were carried out to eliminate those non-covalently bound, so that only those covalently bound remained in the final cell wall residues. The results showed that this cell wall component was quite abundant, in all cases representing more than 10 % of the total weight of the residues and even more than 58 %, as was the case in *U. maydis* (Fig. 3). Thus, on average, proteins accounted for approximately 29 % of the total cell wall, taking into account the values obtained for the 18 species. Regarding the glycosidic counterpart, the sum of the three types of monosaccharides identified, amino sugars, neutral sugars and uronic

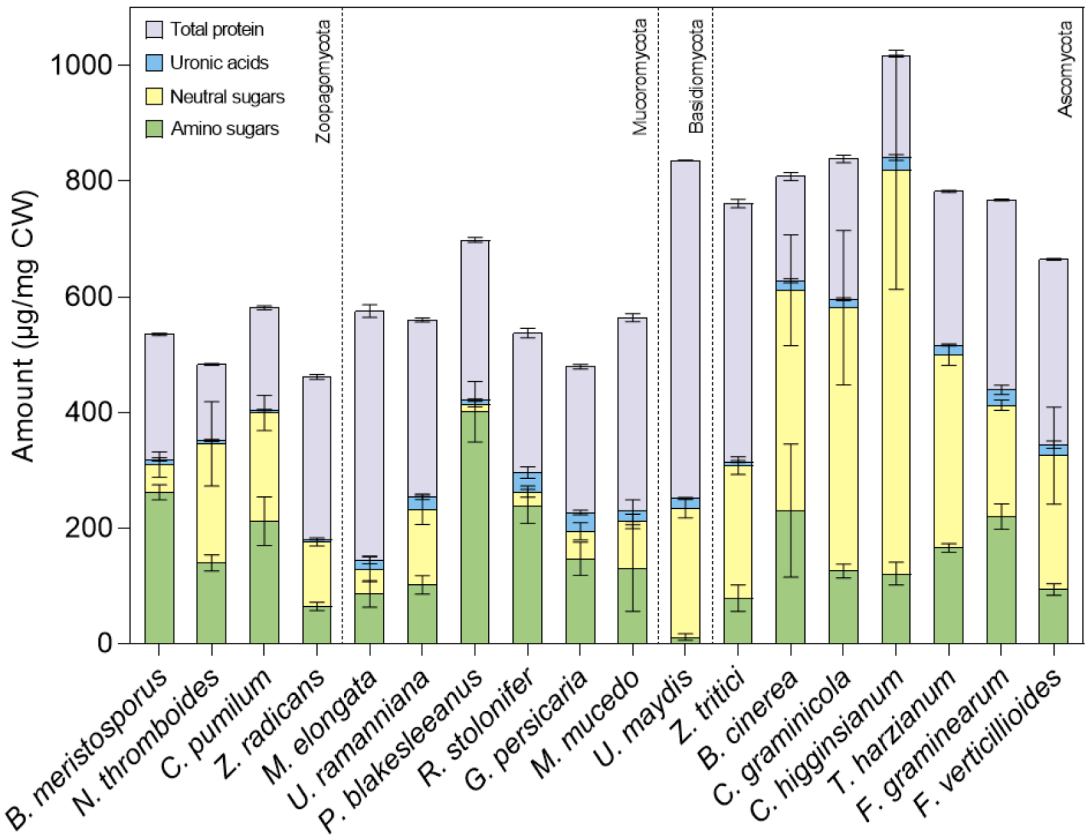

Fig. 3. Accumulative analysis of different types of monosaccharides and proteins in fungal cell walls. Absolute amounts (µg/mg cell wall dry weight) of amino sugars, neutral sugars, uronic acids and proteins in 18 fungal species are plotted. Data represent mean ± S.D. of 4 independent measurements.

acids, showed great variability in their abundance among the different species studied (Fig. 3). This ranged from *M. elongata* with less than 20 % of carbohydrates quantified in its cell wall to *C. higginsianum* with more than 80 %. The vast majority of early divergent ranged between 30 % and 40 % sugars. On the other hand, in general, a higher proportion of sugars in the cell wall could be observed for species belonging to Ascomycota, being in most cases higher than 40 % and in several cases higher than 50 % of total wall dry weight. This difference seems to be mainly explained by the higher abundance of hexoses found in the species belonging to Dikarya.

Among the 8 monosaccharides identified in the 18 species analysed, the only one that could be consistently detected in all species was glucosamine, the monosaccharide constituent of chitin (Figs. 3-6). Comparing between groups, the proportion of glucosamine was much higher for species belonging to Zoopagomycota and especially to Mucoromycota than to those encompassed in Dikarya. In fact, it was practically higher than 50 % and 75 % of the total sugars identified for Zoopagomycota and for most of Mucoromycota species respectively (Fig. 3). In particular *N. thromboides*, *Z. radicans*, *M. elongata*, *U. ramanniana*, *G. persicaria* and *M. mucedo* showed contents of around 100 µg of glucosamine per mg of cell wall (Fig. 4**A**). The rest of the species in these two groups showed values higher than 200 µg/mg. It is worth mentioning the case of *P. blakesleeanus* in which almost all the sugars identified corresponded to chitin (402 µg of glucosamine per mg of cell wall). At the other extreme we found *U. maydis*, containing only 12 µg of glucosamine per mg of cell wall. In the case of ascomycetes, their cell wall contained approximately between 80 µg/mg glucosamine in the case of *Z. tritici* and 220 µg/mg in *F. graminearum*. We then decided to investigate the possible presence of galactosamine in the cell walls of the species included in this study. Our HPAEC-PAC analysis detected its presence in amounts over trace only in *B. cinerea* (130 µg/mg; Fig. 4**A**). On the other hand, it could not detect its presence at all in *N. thromboides*, *C. pumilum*, *Z. radicans* and *U. maydis*. In the rest of the species it was detected, but in trace amounts (less than 1 µg/mg in all cases).

As expected, our analyses further demonstrated the presence of glucose in the extracellular matrices of certain groups of fungi (Fig. 4**B**). It was the main neutral sugar detected in Zoopagomycota, Basidiomycota and Ascomycota species but it was almost absent in most Mucoromycota species, which seemed a very defining feature of the cell wall of this group. In absolute terms, it was found to be more abundant in Dikarya walls, where both *Colletotrichum* species were more abundant, with more than 400 µg of glucose per mg of cell wall. Also noteworthy is the case of *U. maydis*, where we were able to quantify approximately 250 µg/mg in a total of less than 300 µg/mg, so its proportions were above 80 %. Finally, among the 4 species belonging to Zoopagomycota, in *B. meristosporus* we detected only 30 µg/mg of glucose, while for the rest of the species, glucose content was higher than 100 µg/mg in all cases. This divergence between *B. meristosporus* and the rest of Zoopagomycota was also observed for other monosaccharides, as will be shown below.

3.3. Other monosaccharides are less abundant and have a more marked phylogenetic distribution

The third most abundant monosaccharide was mannose, but its content displayed significant phylogenetic discrimination (Fig. 5**A**). Thus, while it was found to be relatively abundant in most of the ascomycete species, with several of them reaching around 80 µg/mg of dry wall weight, it was detected in much lower amounts in other divisions. Specifically, in most of the species of the other divisions, mannose was only present as traces or with a concentration of up to 15 µg/mg. Only in *N. thromboides* and *C. pumilum*, 40 µg/mg of mannose were found in the cell wall extracts.

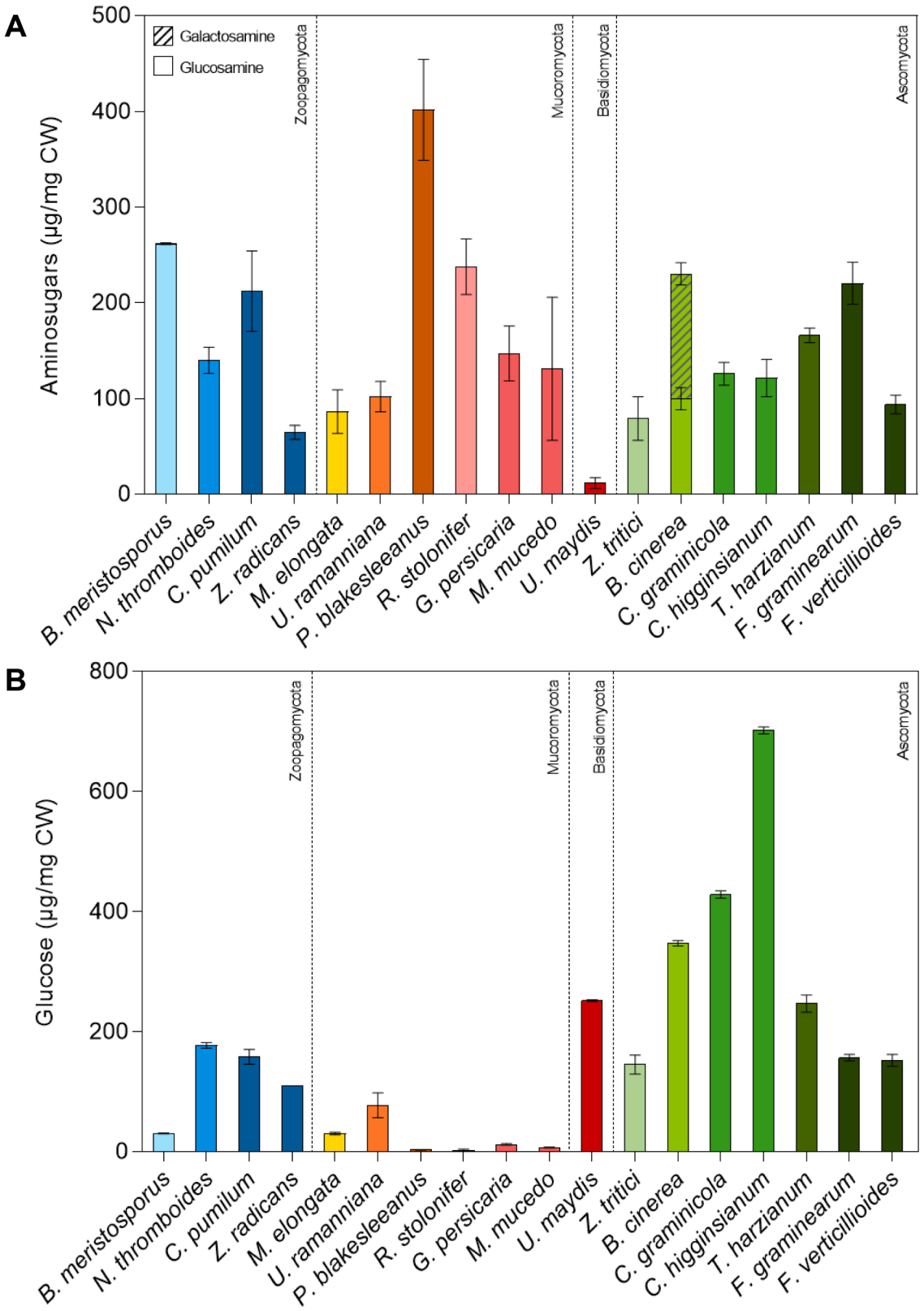

Fig. 4. Amount of glucosamine, galactosamine and glucose in the cell wall of selected fungal species. Data (µg/mg cell wall dry weight) represent mean ± S.D. of (A) glucosamine and galactosamine and (B) glucose from 4 independent measurements.

The galactose composition in the fungal cell walls was overall lower than that of mannose (Fig. 5**B**). Ascomycetes harbour the highest quantity of galactose with values ranging from 29 µg/mg in *Z. tritici* to 51 µg/mg in *T. harzianum*. The remaining species contained in general lower levels of galactose, with values ranging from 22 µg/mg in *U. maydis* and *M. mucedo* to trace amounts detected in *Z. radicans*. Therefore, in view of these results, it can be concluded that mannose and galactose can be found in the cell walls of most species throughout the

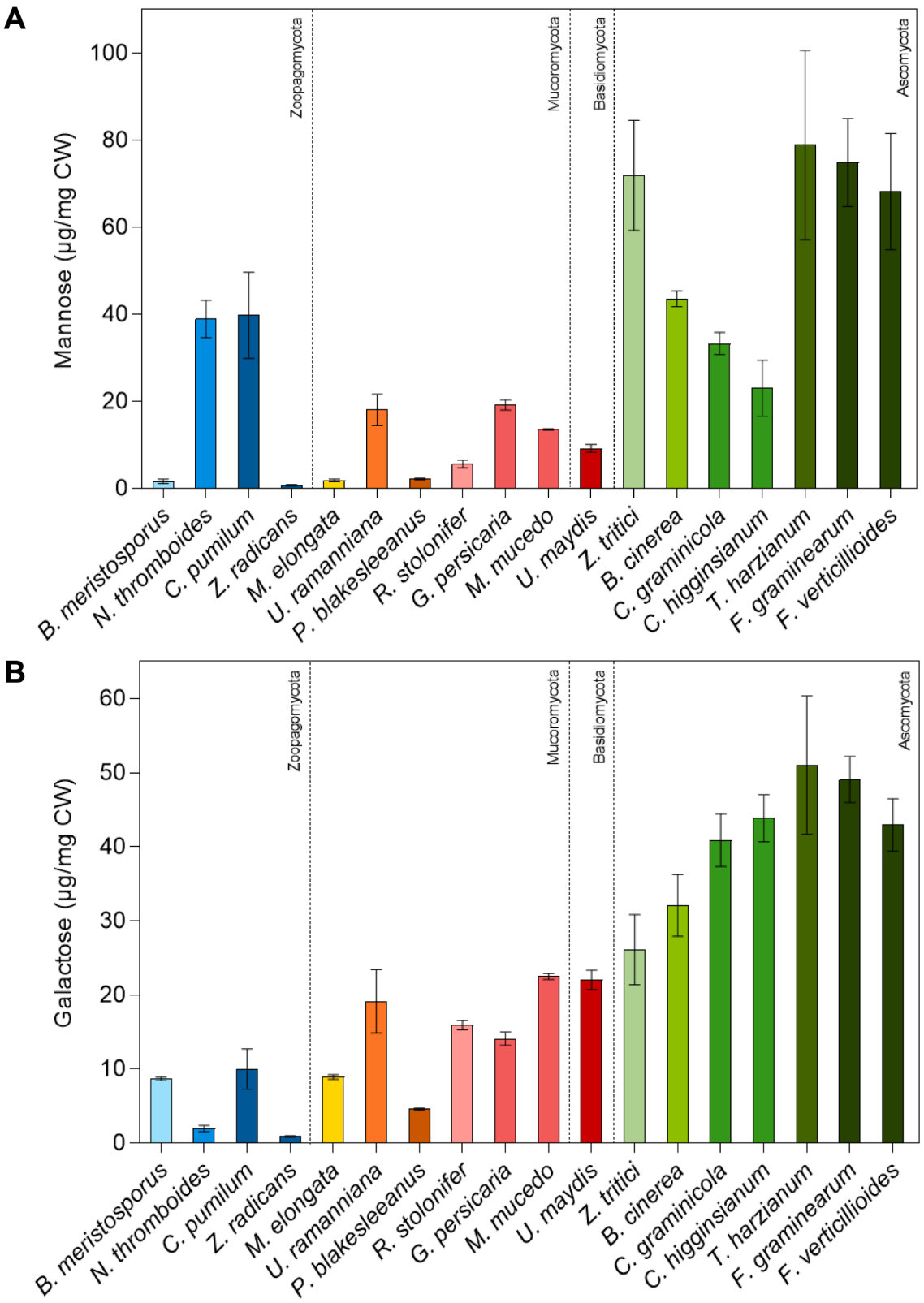

Fig. 5. Amount of mannose and galactose in the cell wall of selected fungal species. Data (µg/mg cell wall dry weight) represent mean ± S.D. of (A) mannose and (B) galactose from 4 independent measurements.

fungal kingdom, but with higher abundance in ascomycetes.

A last group of monosaccharides studied includes fucose and glucuronic acid (Fig. 6). In this case their distribution followed a marked phylogenetic trend. Both were found in all species of the division Mucoromycota, and in relatively similar amounts. Both showed the lowest abundance in *P. blakesleeanus* with values below 10 µg/mg and the highest in *U. ramanniana*, *R. stolonifer*, *G. persicaria* and *M. mucedo*, reaching a maximum of 48 µg/mg for fucose in *M. mucedo* and 38 µg/mg for glucuronic acid in *G. persicaria*. Regarding the other groups, they were only detected in *B. meristosporus* among the species analysed in

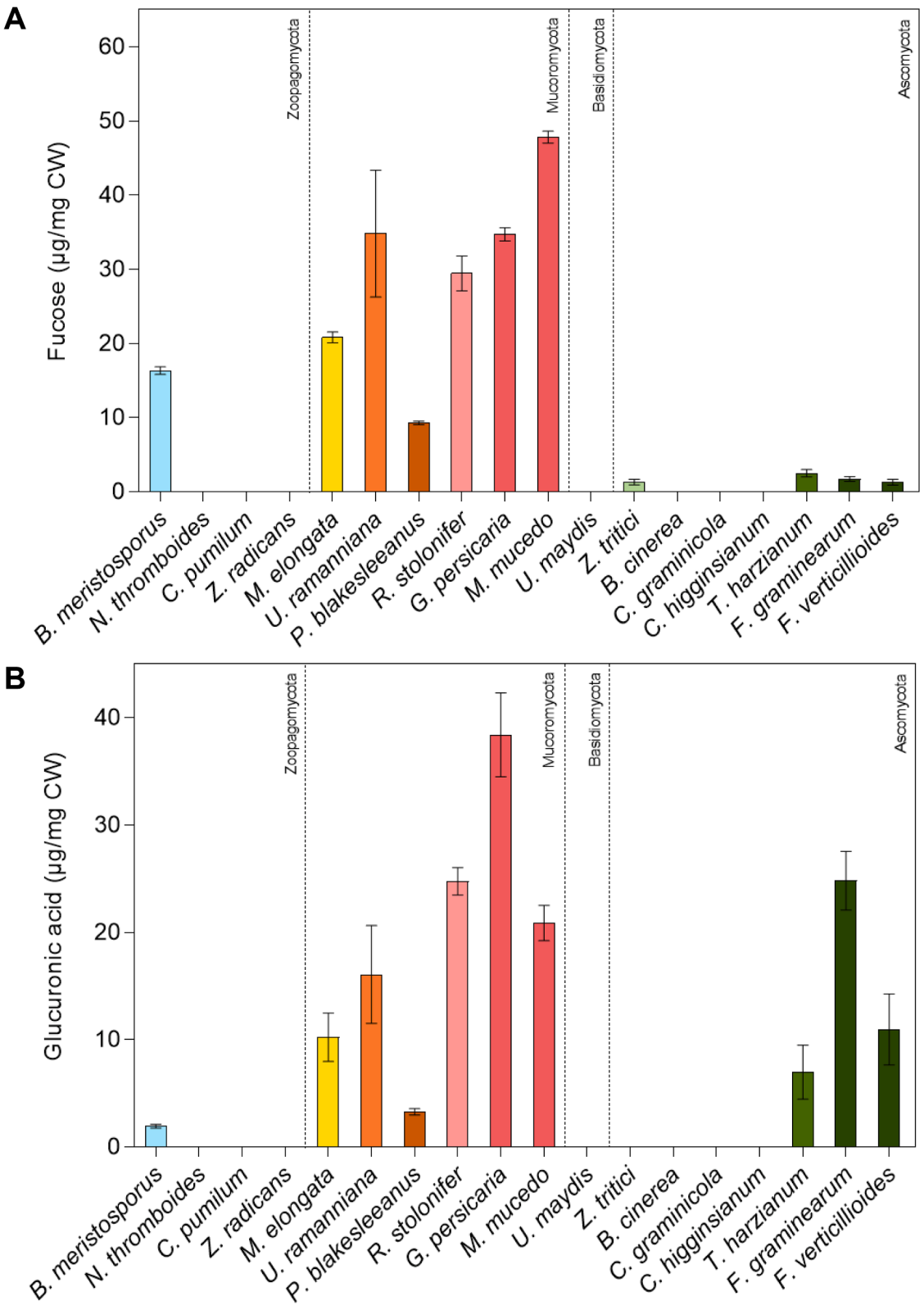

Fig. 6. Amount of fucose and glucuronic acid in the cell wall of selected fungal species. Data (µg/mg cell wall dry weight) represent mean ± S.D. of (A) fucose and (B) glucuronic acid from 4 independent measurements.

Zoopagomycota and in Ascomycota in the species grouped in the Hypocreales order, *T. harzianum*, *F. graminearum* and *F. verticillioides*. Finally, trace amounts of rhamnose were only detected only in *B. cinerea* cell wall (Fig. 7).

4. Discussion

While certain cell wall structures have been probably adapted by fungal groups during environmental adaptation, their hosts have co-evolutionarily selected such structures as a source of Microbe-

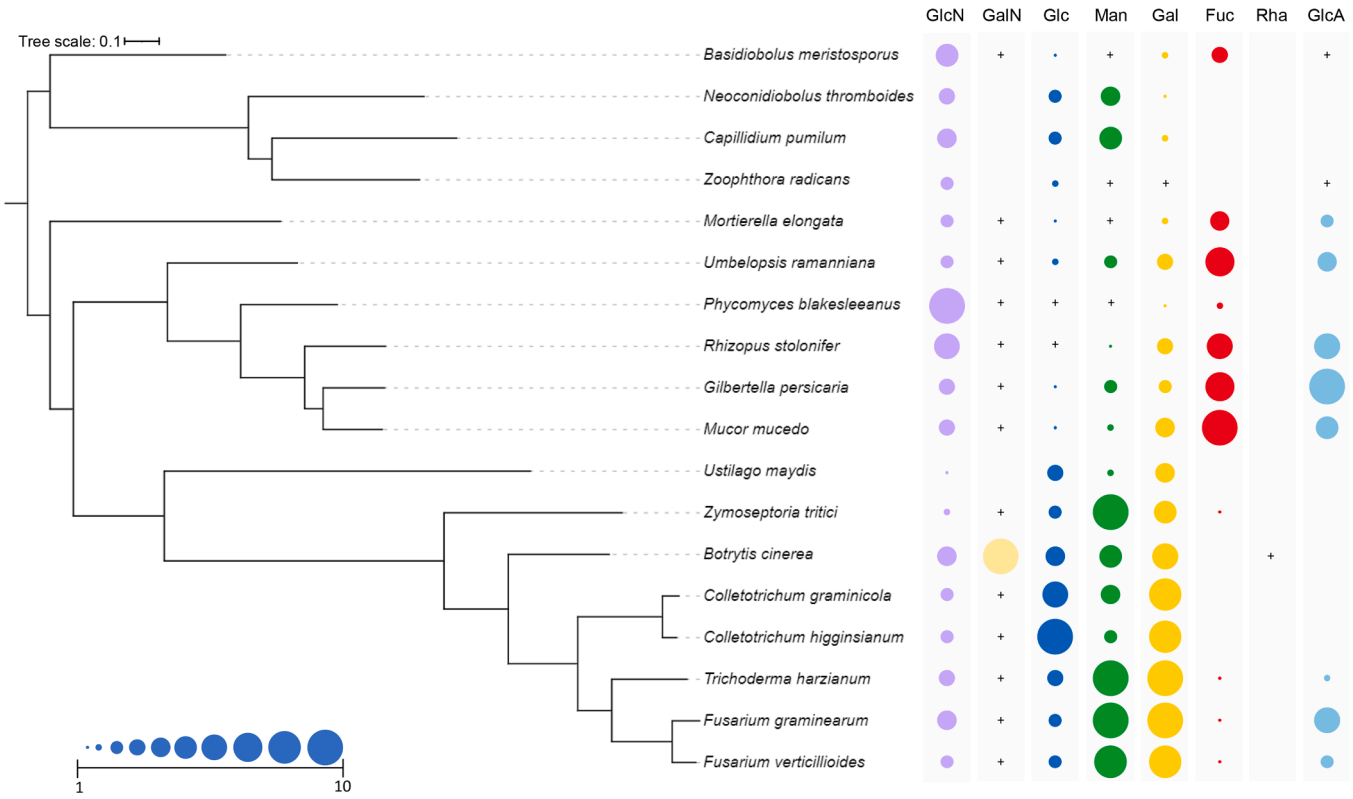

Fig. 7. Qualitative comparison of the presence of different monosaccharides in several fungal species. A phylogenetic tree including the species analysed is shown on the left-hand side. The size of the coloured circles is scaled from 1 to 10 and refer to the relative concentration for each monosaccharide, according to the concentrations obtained in each species. The symbol "+" means that the respective monosaccharide was detected only in trace amounts (less than 5 μg/mg cell wall dry weight), therefore, it was not scaled. Abbreviations: GlcN (Glucosamine), GalN (Galactosamine), Glc (Glucose), Man (Mannose), Gal (Galactose), Fuc (Fucose), Rha (Rhamnose), GlcA (Glucuronic acid).

Associated Molecular Patterns (MAMPs) (Hatinguais et al., 2020; DeFalco and Zipfel, 2021). During the initial steps of plant-fungi interactions, in the extracellular spaces of plant tissues, host-derived toxins and hydrolytic enzymes target pathogen cell wall integrity. As a consequence, wall fragments are released, recognised as MAMPs by host pattern recognition receptors (PRRs) and therefore activating immune responses (Rovenich et al., 2016). Chitin has been demonstrated to function as a fungal cell wall-derived MAMP in plants and the mechanisms behind its immune-activating capacities are partially characterized (Liu et al., 2012; Cao et al., 2014). However, chitin represents, on average, less than 20 % of the fungal cell wall carbohydrates, and therefore other fungal MAMPs could potentially be allocated in this structure. Despite pioneering work and recently regained momentum (Anderson, 1978; Ayers et al., 1976; Johnson et al., 2018; Mélida et al., 2018; Rebaque et al., 2021; Sharp et al., 1984; Wanke et al., 2020; Wawra et al., 2016), the specific mechanisms of plant defence activation by fungal carbohydrate-based MAMPs clearly lags behind the animal field (Erwig and Gow, 2016). Thus, as a first step towards a better understanding of plant-fungus interactions, we chose to carry out a comparative analysis of the cell wall monosaccharide composition of a wide variety of species belonging to different phylogenetic groups with special emphasis on plant interactors. However, this approach, despite its great informative value, has a weakness, which is that since a monosaccharide can be part of different polysaccharides with very different properties, when making more specific interpretations, the analyses should be completed with complementary technologies. Examples of such would be glycosidic linkage analysis and nuclear magnetic resonance (Kang et al., 2018; Mélida et al., 2015). Further work using these complementary technologies to characterise in detail the cell walls of early divergent species is highly needed to understand fungal cell wall evolution. In any case, the data presented in this work lay the groundwork for further work along these lines.

In this study, the mycelial walls of 18 fungal species, belonging to four divisions, were studied. In this regard, recent studies have demonstrated the enormous adaptability of the fungal cell wall to different stimuli, such as carbon sources (Ballou et al., 2016; Pradhan et al., 2019). In this study we have chosen a specific medium to grow each isolate under optimal conditions, but it would be interesting to extend the study using different media and conditions. For this purpose, cell walls were purified, following previously established protocols, and avoiding the use of alkaline solutions typically used in the final steps of these protocols (e.g. Fontaine et al., 2000). This way, we tried to avoid the possible loss of matrix polysaccharides different from chitin and alkali insoluble glucans (Casadevall and Gow, 2022). Surprisingly, we detected a relatively low proportion of carbohydrates in the investigated fungal cell wall fractions, which was of around 50 % carbohydrate to total weight on average. The most extreme case was observed for the cell wall of *M. elongata*, that harbour approximately 20 % of carbohydrate content. This value, which might seem low *a priori*, could be explained by the fact that alkaline treatments probably help to further purify the wall residue. However, the fact that the proportion of carbohydrates is lower than one would expect, especially in non-Dikarya species, suggests that this concept should be revised and we should stop talking about the fungal cell wall as a structure composed mainly of polysaccharides (Bowman and Free, 2006; Latgé, 2007). In fact, according to other authors, other components such as covalently bound proteins, lipids, pigments, nucleic acids and inorganic components seem to play a much more important role than the pioneering studies seemed to foresee (Bartnicki-Garcia, 1968; Gow and Lenardon, 2023). In this respect, in this study we have shown that the proportion of proteins covalently bound to the fungal cell wall can be as high as almost 60 % of the total weight of the residue in extreme cases, although the overall average is

about half of this value. On the other hand, it should be noted that most studies of cell wall composition represent the monosaccharide content as a normalised %, so it is difficult to get an idea of the proportion of carbohydrates in relation to the total weight of the cell wall (e.g. Henry et al., 2016; Lopes-Bezerra et al., 2018; Pettolino et al., 2009; Pham et al., 2019; Samalova et al., 2017).

Quantitatively, as expected, the most abundant monosaccharides identified are the constituents of the major fungal cell wall polysaccharides, glucans and chitin (Latgé, 2007). This is well known from numerous studies, and has been previously reported for different plant-pathogenic fungi (ascomycetes in all cases), such as, *Blumeria graminis*, *Magnaporthe oryzae*, *Plectosphaerella cucumerina* and *Rhynchosporium secalis* in which the glucan proportion ranged from 60 % to 75 % of total carbohydrates and chitin from 5 % to 20 % (Mélida et al., 2018; Pettolino et al., 2009; Pham et al., 2019; Samalova et al., 2017). Noteworthy are the extreme cases of *P. blakesleeanus* and *U. maydis* in which their cell walls were practically constituted by one or the other. Our results demonstrating the higher abundance of glucosamine in species belonging to Mucoromycota confirm previous work on *Rhizopus* and *Phycomyces* species (Mélida et al., 2015; Tominaga and Tsujisak, 1981; Van Laere et al., 1977). In addition, it would be interesting if future studies could determine the chitin/chitosan ratios, as there are some descriptions of the higher abundance of the deacetylated form in the early divergent groups (Bartnicki-Garcia, 1968; Mélida et al., 2015). The remaining monosaccharides quantified, in general descending abundance, were mannose, galactose, fucose and glucuronic acid. It should be noted that in our study we were able to detect the presence of galactosamine in 14 of the 18 species studied, although in quantities above those considered trace in only one. Bearing in mind that it is a very minor component in those species in which it has been studied previously (Gravelat et al., 2013), it is not surprising that we detected it in such low quantities in general. A remarkable fact is that it was completely absent all but one of the species belonging to Zoopagomycota, as it could only be detected in *B. meristosporus*, whose cell wall seems to differ greatly from that of the other three analysed members of this group in view of our results with other monosaccharides. We also were unable to detect it in *U. maydis*, the only basidiomycete, but it would be necessary to analyse more members of this group to be able to conclude whether it has any phylogenetic relationship. Finally, perhaps the most surprising fact was the large amount detected in *B. cinerea* where it accounted for more than 20 % of the total carbohydrates detected. Given the great importance of galactosaminogalactans in the pathogenesis of *Aspergillus* spp in humans demonstrated during the last decade (Briard et al., 2016; Speth et al., 2019), these data open the possibility of studying the immunomodulatory capacity of these polysaccharides on plant hosts.

In order to better understand the evolutionary relationships of each particular monosaccharide, we decided to plot a figure reflecting the relative abundance of each monosaccharide in each species compared to the rest (Fig. 7). This comparative figure shows firstly, that the most ubiquitous monosaccharide of all is glucosamine, with a relatively similar abundance distribution across the 4 divisions analysed. The highest abundance occurred in certain species belonging to early divergent groups, in agreement with the pioneering observations of Bartnicki-Garcia, who defined the cell walls of the previously named Zygomycota (now named as two phyla, Zoopagomycota and Mucoromycota) as rich in chitin and/or chitosan (Bartnicki-Garcia, 1968). Glucose, mannose and galactose showed a relatively similar distribution, highlighting their relative abundance in Dikarya species compared to early diverging ones. The highest abundance of glucose was found in the *Colletotrichum* branch and in *B. cinerea*. Similarly, a very high amount of glucose was found in cell walls of *B. cinerea* by other authors (Cantu et al., 2009). On the other hand, mannose showed highest levels in the Hypocreales order (*Fusarium* and *Trichoderma*), while galactose was higher in the Sordariomycetes and Leotiomycetes classes (*Botrytis*, *Colletotrichum*, *Trichoderma* and *Fusarium*). Remarkably, these three hexoses were practically absent in the cell walls of Mucoromycota. Instead, the members of this division replaced these components by fucose and glucuronic acid, previously described for some of these species, contributing to the formation of polysaccharides known as mucorans (Bartnicki-Garcia and Reyes, 1968; Mélida et al., 2015). Finally, it should be noted that there are no comprehensive studies on the cell wall composition of species belonging to Zoopagomycota. In this study, we found that the species phylogenetically most distant from the other three according to our phylogeny, *B. meristosporus*, was also found to have a more different cell wall. In this case it consisted mainly of chitin, and small amounts of other monosaccharides in which fucose stood out. In the other three species, *N. thromboides*, *C. pumilum* and *Z. radicans*, chitin was also the dominant component, but around 50 % of the total carbohydrates, but neither fucose nor galactosamine were present. On the other hand, the dominant hexose was glucose in all cases. Therefore, based on our results, it seems that the cell walls of Zoopagomycota species do not show great complexity in terms of monosaccharide diversity.

Regarding minority monosaccharides, as mentioned above, in our study we have been able to detect the presence of some of them. In particular, in *B. cinerea* we identified trace amounts of rhamnose. According to the predictions of the recent article by Schwerdt et al (2021), this monosaccharide could be present in species belonging to several groups covered in our study, including Pezizomycotina, to which *B. cinerea* belongs. In addition, these authors also predicted the presence of other minor monosaccharides, and their *in silico* predictions, based on 491 species, are mostly on accordance with our biochemical analyses on 18 species. In particular, we could not detect arabinose in any case, which agrees with their predictions for the groups included in our sampling, but neither could we detect xylose, which according to their predictions could be present, although in most cases with low probability. A similar situation as for arabinose occurred for galacturonic acid, which we did not detect in any case, and the predictions point to its presence only in Chytridiomycota, not included in our study (Schwerdt et al., 2021).

Our study shows that in addition to the polysaccharides already characterised as sources of fungal cell wall-derived MAMPs perceived by plants, chitin and β-glucans (Miya et al., 2007; Mélida et al., 2018; Johnson et al., 2018; Rebaque et al., 2021), there are other components whose immunostimulatory potential is worth studying. Mannans, galactans and mucorans are postulated as such, and therefore future research aimed at their purification and/or synthesis and subsequent evaluation of their immunostimulatory potential will be of great interest (Krylov and Nifantiev, 2020; Mélida et al., 2018). In any case, further structural characterisation using complementary techniques such as glycosidic linkage analysis or nuclear magnetic resonance will be necessary to better understand the precise structure of the MAMPs actually perceived by plants. Finally, it is worth noting that the scenario could be further complicated following research with animal fungal pathogens in which the existence of a cell wall reshuffling mechanism, allowing decreased host responses was demonstrated (Ballou et al., 2016; Pradhan et al., 2019). Thus, similar wall carbohydrate compositions may be subjected to different organizations dependent on different conditions, leading to construction of different ultrastructures, which largely affects to the interactions with the host. This is of particular interest for the fungal species described as plant endophytes, because the contribution of the cell wall components and ultrastructure in this process remains elusive. In addition, masking of cell wall polysaccharides may also be established by fungi that secret proteinaceous components known as effectors which may bind fungal cell wall carbohydrates to reduce its accessibility to host hydrolytic enzymes, or even enzymes that degrade oligosaccharides to monosaccharides, thus preventing the production of oligo-structures perceived by the host immune system (Liu et al., 2023; Sánchez-Vallet et al., 2015; Wawra et al., 2016). Thus, we propose that chemical data should be complemented by *in situ* visualization of specific substructures (epitopes)

localized among cell wall carbohydrates using suitably designed diverse molecular probes. In conclusion, the information provided in this study paves the way for future research into the evolution of the fungal cell wall and its role in plant-fungus interactions, but this approach needs to be completed with additional chemical and immunohistochemical analyses, as well as assays on the perception of these components by plant PRRs.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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