UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
Departamento de Botânica
Programa de Pós-Graduação em Biologia Vegetal

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CÍNTIA DE ALMEIDA GONÇALVES

USO DE INTERAÇÕES MOLECULARES, IMUNOCITOQUÍMICA E GENÔMICA PARA CARACTERIZAÇÃO DE REVESTIMENTOS DE CÉLULAS DE MICROALGAS

Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal do Departamento de Botânica do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Biologia Vegetal.

Área de Concentração: Fisiologia vegetal/ Ecologia

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Índice

Resumo

Microalgas são um grupo muito diversificado de organismos fotoautotróficos com importantes papéis ecológicos e sendo atualmente consideradas uma fonte de compostos de alto valor agregado e de matéria prima para produção de biocombustíveis. Tais compostos se encontram, na maioria das vezes, internamente à célula, fazendo com que o revestimento celular atue como uma primeira barreira para acessá-los. A ruptura das células algais nem sempre é um processo simples e de baixo custo, havendo um variado repertório de estruturas de revestimento encontradas nesses organismos. As estruturas rígidas de revestimento são pouco conhecidas ou o conhecimento é restrito a poucas espécies, sendo muito pouco representativo considerando o grande número de espécies. Porém, as superfícies celulares também possuem uma composição de biomoléculas que são muito variáveis dentre as microalgas e são ainda menos conhecidas. Sabe-se que dentre essas biomoléculas as mais importantes são os carboidratos e as lectinas, cuja interação entre si medeiam importantes processos fisiológicos. Assim, tanto as estruturas rígidas de revestimento quanto as superfícies celulares guardam informações importantes sobre a ecologia, morfologia e fisiologia das células. Nesse trabalho, os objetivos principais foram avaliar o conhecimento atual a respeito das estruturas de revestimento de microalgas, elucidar as composições da parede celular e dos receptores glicídicos das superfícies celulares de seis e cinco espécies de microalgas respectivamente. Para isso foram elaborados quatro capítulos, correspondentes a quatro manuscritos, os quais, em seu conjunto, foram baseados em uma aprofundada revisão da literatura sobre o tema, aplicação de técnicas de cito e imunocitoquímica, uso de glicodendrons como sondas, uso de microscopia confocal e também, para uma espécie (*Coelastrum microporum*), sequenciamento e análise genômica. A parede celular das espécies testadas apresentou uma

composição de celulose, pectinas e hemiceluloses que parece ser espécie-específica. Essa composição também variou de acordo com a idade das células, sugerindo que a composição da parede celular está associada ao momento fisiológico. *Coelastrum microporum* foi a espécie que mais se assemelhou à parede de plantas em termos de composição e dinâmica de esterificação de pectinas. Nas superfícies celulares foram detectados receptores glicídicos (lectinas) para os carboidratos de D-manose, L-fucose e *N*-acetilgalactosamina. A composição dos receptores também variou entre as espécies e de acordo com a idade das células. A análise do genoma de *C. microporum* mostrou a presença de uma lectina e muitas outras proteínas (137) com grandes chances de também serem lectinas devido às suas características. Estudos mais refinados, incluindo proteômica, são necessários para caracterização e confirmação da identidade e função dessas proteínas receptoras de carboidratos.

Palavras chave: Chlorophyta, *Chlorella vulgaris*, interação celular, parede celular, genoma.

Abstract

Microalgae are a very diverse group of photoautotrophic organisms with important ecological roles and are currently considered a source of compounds with high added value and raw material for biofuels production. Such compounds are found, most of time, inside the cell with the cell covering acting as a first barrier to access them. The rupture of algal cells is not always a simple and low-cost process due the varied repertoire of coating structures found in these organisms. Rigid coverings structures are poorly known or knowledge is restricted to a few species, being very unrepresentative considering the large diversity of microalgae. Cell surfaces also have a composition of biomolecules that are very variable among microalgae and are even less known. It is known that among these biomolecules carbohydrates and lectins are the most important, whose interaction between them mediate important physiological processes. Thus, both the rigid coverings structures and the cell surfaces hold important information about the ecology, morphology and physiology of cells. In this work, the main goals were to evaluate the current knowledge regarding the microalgae covering structures, to elucidate the cell wall and glycidic receptors compositions of the cell surfaces of six and five microalgae species respectively. For this, four chapters were prepared, corresponding to four manuscripts, which, taken as a whole, were based on a thorough review of the literature on the subject, application of cyto and immunocytochemistry techniques, use of glycodendrons as probes, use of confocal microscopy and also, for a species (*Coelastrum microporum*), sequencing and genomic analysis. The cell wall of the tested species showed a composition of cellulose, pectins and hemicelluloses that appears to be species-specific. This composition also varied according to the age of the cells, suggesting that the composition of the cell wall is associated with the physiological moment. *Coelastrum microporum* was the species that most resembled the plant wall in terms of composition and dynamics of pectin esterification. On cell

surfaces, glycidic receptors (lectins) for the D-mannose, L-fucose and *N*-acetylgalactosamine carbohydrates were detected. The composition of the receptors also varied between species and according to the age of the cells. The analysis of the *C. microporum* genome shows the presence of one lectin and many others encoding proteins (137) with a high chance of being lectins. More refined studies, including proteomics, are needed to characterize and confirm the identity and function of these carbohydrate receptor proteins.

Keywords: Chlorophyta, *Chlorella vulgaris*, cell interaction, cell wall, genome.

Introdução geral

O termo microalgas é frequentemente aplicado para descrever um vasto e diversificado grupo de organismos eucariotos e procariotos (cianobactérias) fotoautotróficos (Gigova and Marinova 2016), embora o ideal seja que as cianobactérias sejam tratadas como um grupo a parte. As microalgas e cianobactérias são onipresentes na natureza e desempenham importantes papéis ecológicos, como ciclagem de nutrientes, manutenção dos níveis de oxigênio (na água e na atmosfera) além de estarem na base da cadeia alimentar aquática (Hopes and Mock 2015). Além de sua importância ecológica, as microalgas e cianobactérias são vistas como uma fonte alternativa de matéria-prima para a produção de biodiesel (Wu et al. 2017) e extração de compostos de alto valor agregado (ácidos graxos, vitaminas e pigmentos) que podem ser utilizados em diversos segmentos industriais como de cosméticos, fármacos e nutracêuticos (Du et al. 2016).

Os compostos extraídos de microalgas encontram-se, na maioria das vezes, internamente à célula, sendo necessária à sua ruptura para acesso a tais compostos. (Dixon and Wilken 2018). No entanto, essa ruptura nem sempre é um processo fácil, pois depende da composição do envoltório celular de cada espécie. As algas possuem uma grande variedade de envoltórios celulares que por sua vez, são diversificados em termos de composição química e arquitetura. Essa diversificação das coberturas celulares algais é, inclusive, usada como carácter taxonômico em alguns grupos (Cavalier-Smith 2016, Eikrem et al. 2017). Apesar disso, poucas espécies têm a ultraestrutura e a composição de suas coberturas celulares bem elucidadas e o conhecimento sobre tais revestimentos ainda é muito limitado, principalmente considerando a grande diversidade do grupo. Em consequência, frequentemente são feitas muitas generalizações que sequer são aplicáveis,

pois as coberturas celulares algais podem ser muito variáveis até mesmo dentro de um mesmo grupo taxonômico (Baudelet et al. 2017).

Assim como as coberturas, as superfícies celulares das algas também possuem características que podem ser espécie-específicas, além de guardar informações importantes sobre a fisiologia das células. As superfícies celulares são compostas por biomoléculas que formam a primeira estrutura de contato entre o organismo e o ambiente. Dentre essas moléculas, destacam-se os carboidratos e as lectinas, que são os grandes protagonistas em processos em que o reconhecimento celular é necessário (Bulgakov et al. 2004). Lectinas estão presentes em células de todos os seres vivos e são proteínas de origem não imune, conhecidas por sua capacidade de reconhecimento de carboidratos de forma reversível e altamente específica (Santos et al. 2014). Lectinas de algas vem ganhando interesse entre os pesquisadores devido ao seu potencial para uma variedade de aplicações biomédicas (Singh et al. 2015, 2018) e, em adição, são moléculas importantíssimas para os organismos por mediarem uma série de processos fisiológicos. O conhecimento dessas moléculas, portanto, é fundamental e muito importante por ampliar os conhecimentos sobre a morfologia, fisiologia e ecologia dos organismos.

Nesse contexto, esse trabalho consiste em uma busca para ampliar o conhecimento sobre as coberturas e superfícies celulares de seis espécies de microalgas: *Chlorella vulgaris, Coelastrum microporum, Mougeotia* sp., *Pseudopediastrum boryanum* e *Spirogyra* sp. Para isso, foram utilizadas como ferramentas de trabalho: uma revisão da literatura bastante completa, técnicas de cito e imunocitoquímica, métodos de microscopia confocal e glicodendrons como sondas e, finalmente, sequenciamento e análise genômica para uma das espécies (*Coelastrum microporum*). O estudo é composto por quatro capítulos. No capítulo 1**,** foi feita uma compilação das informações disponíveis na literatura sobre coberturas celulares de microalgas com o objetivo de mostrar o que

realmente se sabe sobre essas estruturas. No capítulo 2 foi feito um estudo sobre a parede celular (um dos tipos de cobertura celular de algas) de seis espécies de microalgas. Foram utilizadas técnicas de cito e imunocitoquímica para detecção dos componentes da parede celular. Também foi avaliado se a composição da parede celular dessas espécies se altera com o tempo de crescimento da cultura após o repique. No capítulo 3, receptores glicídicos das superfícies de cinco espécies de microalgas foram detectados utilizando glicodendrons como sondas e microscopia confocal. As espécies foram testadas em quatro idades de cultivo diferentes para averiguação de possíveis mudanças na expressão desses receptores ao longo da vida dos organismos. Finalmente, no capítulo 4, foi feito um estudo dos receptores glicídicos encontrados na superfície de *Coelastrum microporum*. Foram utilizados três glicodendrons como sondas e sequenciamento e análise do genoma da espécie.

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Capítulo 1: What we really know about the composition and function of microalgae cell coverings? - an overview

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Abstract

Cell coverings can be observed in all major groups of organisms, which include animals, plants, fungi, protists and prokaryotes. They play a key role in guaranteeing the cell survival or adaptation to certain environmental conditions. Since the term algae refers to a polyphyletic and very artificial group, the cell coverings among these organisms show a very diverse molecular composition and different arrangements. Differences have taxonomic value since they allow to distinguish microalgae phyla or even minor taxonomic groups, such as classes, orders or families. Understanding the cell covering structure is also fundamental for the use of microalgae to obtain products of commercial value. Despite its importance, the composition and architecture of microalgae coverings is still poorly understood, mainly when considering the great diversity of organisms. Diatom frustules are the most studied covering due their uses in areas of bio and nanotechnology. There is a lack of information about cell wall, lorica, periplast, amphiesma and scales. This study is a review to synthesize literature information on the microalgae cell coverings, describing their compositions, arrangements, functions and industrial uses.

Key words: algal coverings variety, cell surface, molecular structures, taxonomical value, biological interactions.

1 - Introduction

Microalgae comprise eukaryotic and procariotic organisms (cyanobacteria) (Gigova & Marinova 2016), commonly studied together due to their similar photoautotrophic metabolism (Saad & Atia 2014). They show many differences in cell structure and physiology due to their polyphyletic origin and also because they evolved to adapt to many different environments, such as freshwater, seawater, salt lakes, soil, arctic environments, deserts (Raja *et al*. 2014; Zancan *et al.* 2006) and even in association with other organisms such as corals, plants and fungi (in lichens) (Sanders 2001, Wooldridge 2013). Among the particularities used to characterize the different groups of microalgae and cyanobacteria there are the cell coverings, which are the special structures that surround their cells.

The many types of cell coverings are related to the needs of different microalgae, which include interactions with chemical substances, connection between cells, fixation in substrates, protection, communication, reproduction and maintenance of the cell shape (Peterson & Quie 1981, Hoson 2002; Okuda 2002; Yoshimi *et al.* 2017). Microalgae cell coverings have been studied for decades, but details about their ultrastructure and composition are not well known, especially when compared to the higher availability of information about other structures, or even about pigments and reserve substances. Due to economic interests, the studies are focused on diatoms, chlorophytes and charophytes, with some generalizations being established for these phyla. There is a lack of recent studies about the cell coverings of other phyla. Further, generalizations have low value even for the well-known phyla since studies are concentrated in few species although species-specific particularities are commonly reported (Domozych *et al.* 2012).

Here, we present a review to synthesize the available information about composition, architecture and function of the different types of cell coverings present in cyanobacteria and microalgae.

2 – Types and composition of cyanobacteria and microalgae coverings

The cell coverings of autotrophic microorganisms have different names according to their particular structure, position in relation to cell surface and chemical composition (Okuda, 2002). Table 1 synthesizes information about these coverings' types, their major compounds and taxonomical groups in which they are present.

Table 1: Types and composition of cell covering found in algae and cyanobacteria.

Cell covering	Class	Major compounds	References
Cell wall	Cyanophyceae	Peptidoglycans	Rudolf *et al.* 2015
Amphiesma	Dinophyceae	Cellulose	Chan *et al*. 2019; Loeblich 1970
Periplast	Cryptophyceae	Proteins	Hoef-Emden & Melkonian 2003; Brett *et al.* 1994
Pellicle	Euglenophyceae	Proteinaceous strips	Cavalier-Smith 2017; Sommer 1965
Lorica		Mucopolysaccharides and minerals	Poniewozik 2017
Coccolith (calcified scale)	Haptophyceae	Calcium carbonate	Walker *et al.* 2018; Faber & Preisig, 1994
Frustule	Bacillariophyceae	Silica	De Tommasi *et al.* 2017; Nakajima & Volcani, 1969
Lorica	Chrysophyceae	Chitin and cellulose	Herth *et al.* 1977; Herth & Zugenmaier, 1979
Silica scales	Chrysophyceae	Silica	Leadbeter & Barker 1995
	Sinurophyceae	Silica	Sandgren & Hall 1996
Cell wall	Chlorophyceae	Cellulose	Okuda 2002
Organic scales		2-keto sugar acids	Becker *et al.* 1994
Cell Wall	Charophyceae	Cellulose	Okuda 2002

Some coverings are located internally in relation to the plasma membrane. They are found in Dinophyta, Cryptophyta and Euglenophyta and are called amphiesma, periplast and pellicle, respectively (Gantt 1971; Morril & Loeblich 1983; Leander *et al*. 2001). Cyanobacteria and some microalgae have coverings that are located externally the plasma membrane. Even the cell walls of Cyanobacteria and the eukaryotic green algae (Chlorophyta and Charophyta) being different in structure and composition, they are an example of external coverings with similar function. Other external structures are the lorica present in some Euglenophyta and Ochrophyta, the scales of some Ochrophyta and Haptophyta and the frustules of Bacillariophyta, which is one of the most particular algal coverings. Details on each covering type are presented in the following items.

2.1 - Inner cell coverings

2.1.1 – Amphiesma

Dinoflagellates can be divided into two major groups: naked organisms with no thick coverings and armored organisms, which have an amphiesma (Gómez 2007). This term (from Greek, amphi = around, esthma = clothing) was coined by Schutt (1895) and refers to the complete covering of armored dinoflagellates, which includes the plasma membrane as the outermost layer (Sekida *et al.* 2004; Morrill 1984), a layer of membranous vesicles, which may contain glucan tecal plates, and a pellicle (Pozdnyakov & Skarlato 2012) (Fig. 1). The term theca can be also used (Dodge & Crawford 1970), but not as a synonym for amphiesma since it refers only to the layer formed by the vesicles containing thecal plates in armored (thecate) dinophytes. The thecal plates are described originally as a cellulosic structure (Swift & Remsen 1970; Okuda & Sekida 2007), but a recent study (Wang *et al*. 2011) showed that proteins are also present. The number and disposition of thecal plates are important features for dinoflagellates classification (Dodge

1983). These membranous vesicles are empty or contain amorphous materials in athecate dinoflagellates (Dodge & Crawford 1970).

The innermost layer is a membrane that some authors consider as a part of the amphiesma, but others (see Morril & Loeblich 1983) consider it as a pellicle. This layer is present in some species and contains sporopollenin-like substances that confers resistance to it (Morril & Loeblich 1983; Okuda 2002). In some athecate dinoflagellates, the pellicle may be the most important layer to confer resistance to the cell surface, maintaining the cell's shape (Saldarriaga & Taylor 2017).

The amphiesma is a dynamic structure that undergoes many changes throughout the life cycle of the organisms (Sekida *et al.* 2004; Pozdnyakov & Skarlato 2012;). Despite all published studies, the structure and genesis of the amphiesma remain not fully understood (Pozdnyakov & Skarlato 2012). Sekida *et al.* (2001) showed that the vesicles are formed in the non-motile phase of the life cycle and after that the thecal plates are formed inside them in the motile phase.

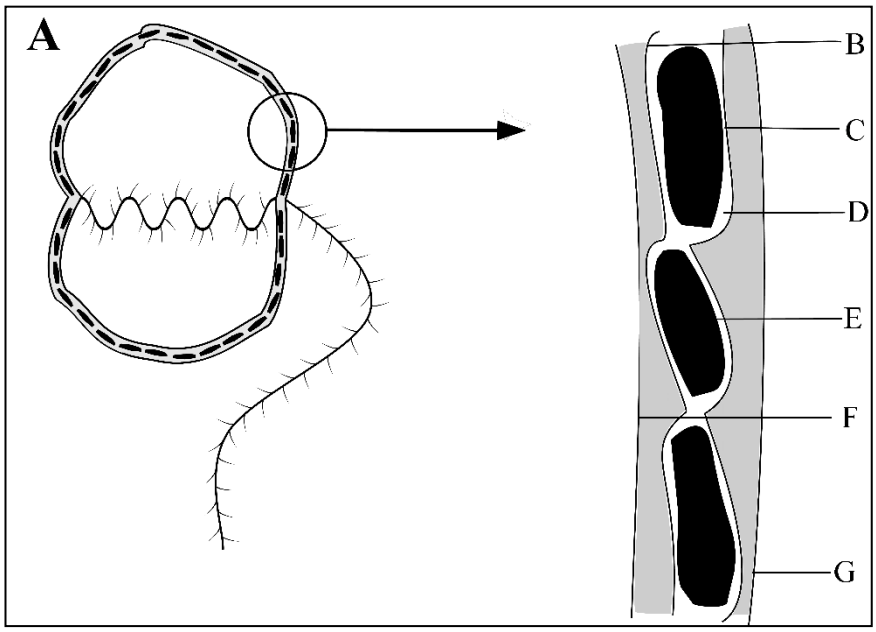

Fig. 1 - Schematic representation of a cell of Dinophyta (A), showing its two typical flagella. Representation of the structural components of the amphiesma (B): The pellicle layer; (C) Outer plate membrane; (D) Techal vesicle; (E) Techal plate; (F) Cytoplasmatic membrane and the outermost membrane (G). Adapted from Wang *et al.* (2011).

2.1.2-Periplast

The cryptophytes have an asymmetric cell shape with clearly defined dorsi-ventral/right-left sides (Hoef-Emden & Melkonian 2003) that has taxonomic significance. This shape partially results of the presence of a vestibule, which is a subapical invagination of plasma membrane, but it is mainly related to a rigid periplast, which is the typical cell covering in this phylum (Brett *et al.* 1994). It covers the entire cell, except the flagella and the vestibular/gullet region (Perasso *et al.* 1997). The vestibule (from where the flagella emerge) can extend internally to form a gullet or continue along the ventral surface to form a furrow (Kugrens & Lee 1991).

The periplast of cryptophytes is composed of two proteic layers, the inner periplast component (IPC) and the surface periplast component (SPC), with the plasma membrane sandwiched between them (Gantt 1971, Brett *et al.* 1994) (Fig. 2). Nevertheless, there are also some species that have a simpler periplast composed by only the plasma membrane and the inner layer (Kugrens & Lee 1987). The totality of characteristics and functions of the periplast are uncertain, but stiffness, flexibility and elasticity are commonly attributed to it (Faust 1974). A fourth possible function is to protect the integrity of the cell membrane during the explosive discharges of the ejectisomes, a type of extrusive organelles (Hausmann 1978, 1979).

The morphology and organization of the periplast are different among Cryptophyceae and more than one type of IPC were described (Brett & Wetherbee 1986). The IPC develops within specific regions called anamorphic zones that are located around the vestibule (Brett & Wheterbee 1996). The IPC is able to grow throughout the life cycle, allowing the elongation and expansion of the cell (Brett & Wheterbee 1996). Depending of the taxon, the IPC is formed by a unique continuous layer or it could be formed by several scales arranged internally in relation to the plasma membrane (Brett *et al.* 1994). The SPC

may appear as dense mats of an unidentified fibrillar material, complex rosulate scales or highly ordered surface plates (Brett & Wheterbee 1986; Brett *et al.* 1994;). The microarchitecture of these plates were described in detail by Brett & Wheterbee (1996b), who showed that these plates are formed by aligned tiny subunits. Studies suggest that these subunits of SPC are produced in the Golgi apparatus and secreted through the endomembrane system to be added at the edges of the periplast (Brett *et al.* 1994, Perasso *et al.* 1997).

The periplast is a complex and unique type of cell covering and some researchers dedicated their work to elucidate its formation, structure and composition by using refined techniques such as immunocytochemistry (Perasso *et al.* 1997), scanning electron microscopy, freeze-fracture and freeze-etch (See review: Brett *et al.* 1997). However, even with the necessity to better understand the periplast structure and functioning, there are few recent studies focused on this type of cell covering. As can be seen in this review, relevant researches are dated from the 1970s to the 1990s.

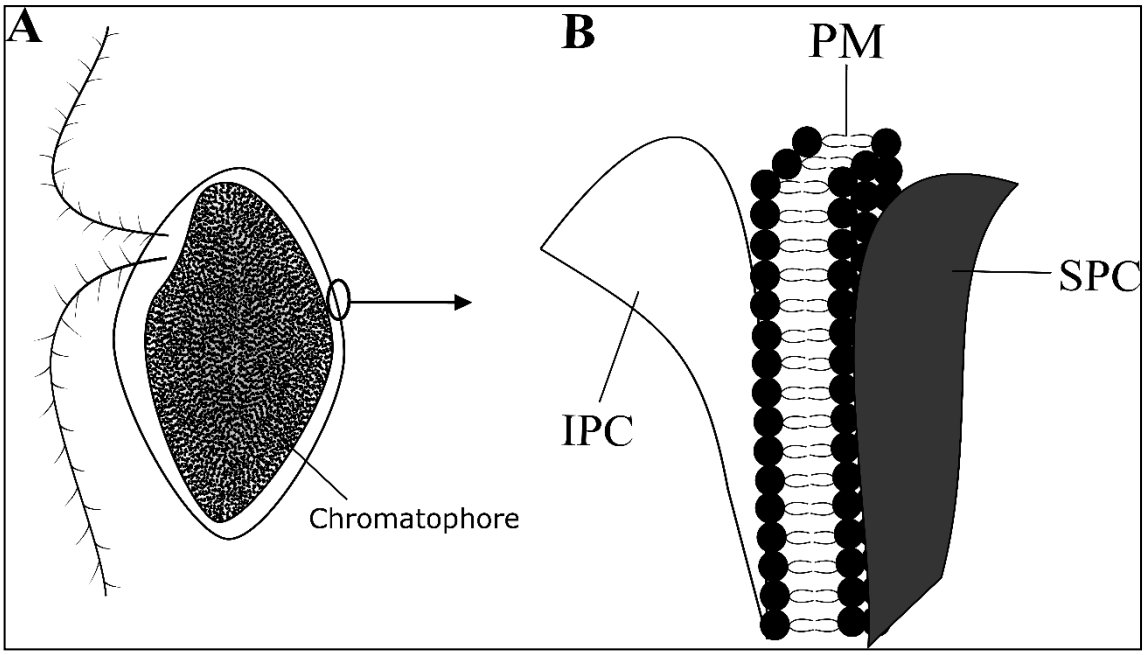

Fig. 2- A-Scheme of a cell of Cryptophyta. B-Representation of structural components of the periplast: Inner periplast component (IPC), surface periplast component (SPC) and the plasma membrane (PM). Adapted from Brec *et al*. (1999).

2.1.3-Pellicle

Although the pellicle can be found among the dinophytes, it is much more complex in Euglenophyta. It is the most important covering in this phylum and is the most rigid structure in the cell surface of most species. The pellicle of euglenophytes can be described as a complex region containing proteinaceous strips, microtubules and tubular cisternae of endoplasmic reticulum that runs along the length of the cell beneath the plasma membrane (Leedale 1964; Sommer 1965; Schwelitz *et al*. 1970; Vismara *et al.* 2000; Strother *et al*. 2019) (Fig. 3).

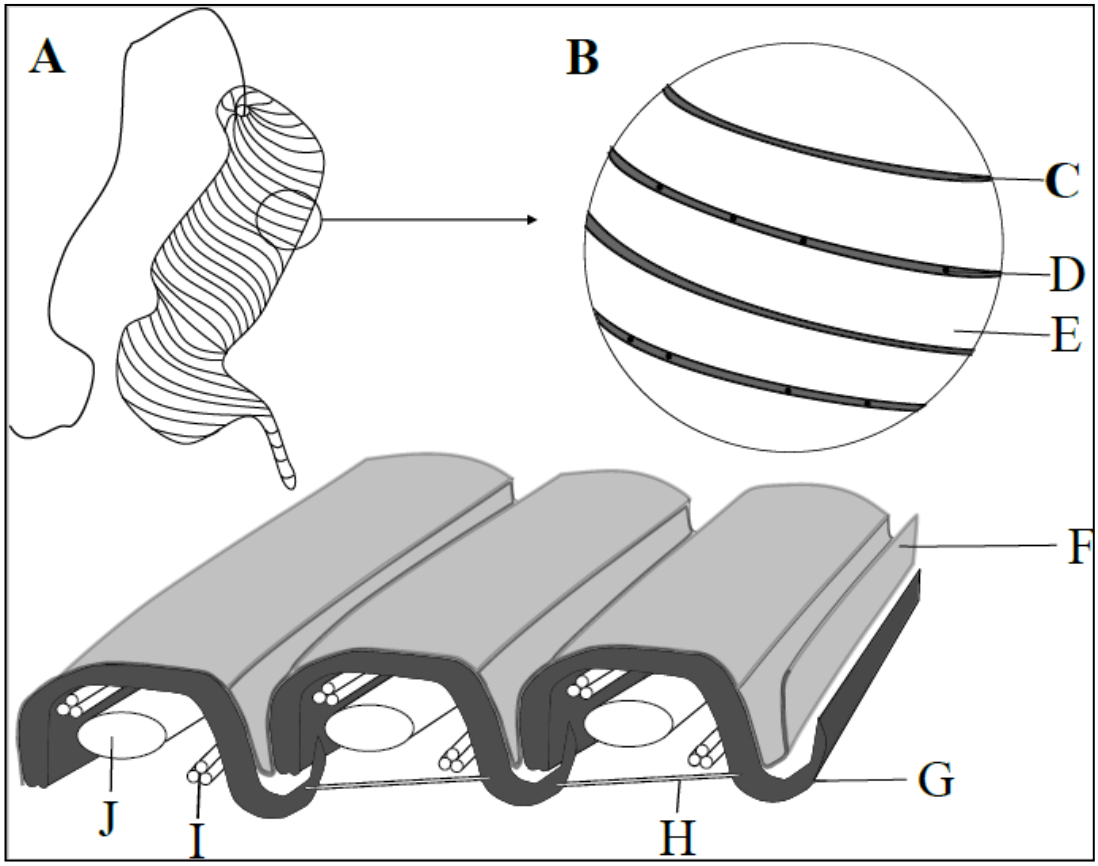

Fig. 3- Scheme of a cell of Euglenophyta (A). Magnification of the pellicle (B) showing the strips (C), the pores (D) and the spaces between the strips (E). Schematic representation of the pellicle's components and organization (F-J). The plasma membrane (F) covers the strips (G) that are connected by the centrines (H). In J and I the endoplasmic reticulum and the microtubules are represented respectively. Adaptaded from Cavalier Smith (2017) and Leander *et al.* (2001).

The strips are considered the major component of the euglenids pellicles, and their general ultrastructure is relatively well understood (Strother *et al.* 2019). They are composed by proteins named articulins which are arranged in parallel and result in a typical ultrastructure that can be used to differentiate species or genera (Cavalier-Smith 2017). The quantity of strips has taxonomic value since it varies widely among species, but is conserved within them (Cavalier-Smith 2016). Another important aspect of strips is how they are organized in cell surface, since they can be arranged in longitudinal rows or helically twisted (Leander *et al.* 2007). When arranged longitudinally, the strips make the pellicle rigid and prevent changes in the cell's shape, as observed in the most primitive euglenoids that form the classes Entosiphonea, which has fewer strips (12 or less), Stavomonadea and Ploeotarea (superclass Rigimonada) (Cavalier-Smith 2016; Cavalier-Smith 2017). The pellicles with helical strips are found in euglenoids of the superclass Spirocuta, comprising heterotrophic *Peranema* Stein, 1859, and ancestrally photosynthetic Euglenophyceae, which in turn have several strips (14–80) (Cavalier-Smith 2016; Cavalier-Smith 2017). These pellicles show higher malleability and are often associated with a peculiar mode of cellular locomotion called metabolic or "euglenoid movement" (Leander *et al.* 2001).

At the junctions between the strips there are the pellicle pores. They are small openings whose function is to provide access for two different ejectile organelles (muciferous bodies and mucocysts) to the cell surface (Leander *et al.* 2001). The strips are connected each other by oblique traversing fibres of centrin, which is a calcium-sensitive contractile protein closely related to the body's reorientations during photophobic responses and flagellar contractions (Höhfeld *et al.* 1988). Intimately associated with each strip there is still the cisterna of endoplasmic reticulum, that pump and store calcium for release when centrin contractions are required (Cavalier-Smith 2017).

2.2 - Extracellular coverings

2.2.1-Cyanobacterial cell wall

Cyanobacteria are a special type of bacteria since they are the only group of prokaryotes that can perform oxygenic photosynthesis (Zhang *et al.* 2018). They are ubiquitous organisms mainly due to their adaptation to various types of environments and their tolerance to extreme conditions (Gaysina *et al.* 2019). Their cell wall is part of their adaptive success.

Bacteria are generally classified as gram positive or negative according to the chemical and physical properties of their cell walls (Hiremath & Bannigidad 2011). Cyanobacteria are gram-negative bacteria, with the cell wall located externally to the plasma membrane. This wall consists of a peptidoglycan layer that is involved by a superficial layer, also called outer membrane (Fig. 4).

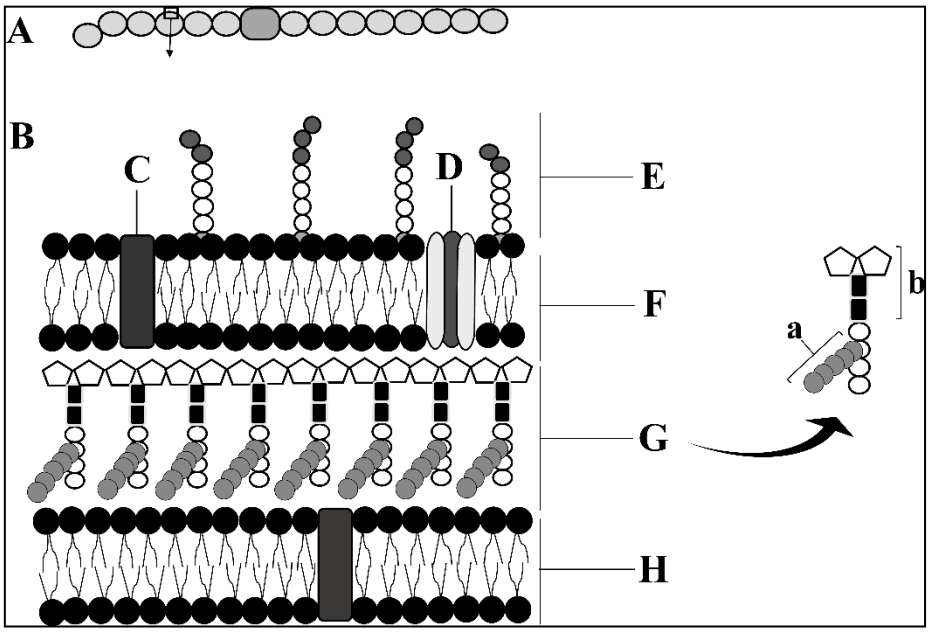

Fig. 4- A- Representation of a filamentous Cyanobacteria. B-Scheme showing the components of the gram-negative cell wall of Cyanobacteria. The outermost layer is composed of lipopolysaccharides (LPS) (E) that are found on the surface of the outermost plasma membrane (F). In this membrane are located some integral proteins (C) and porins (D). Just below there is a peptidoglycan layer (G), composed of pentaglycine cross-links (a) and alternating polymers of *N*-acetylmuramic acid and *N*-acetylglucosamine (b). Below this layer is another layer of phospholipid membrane (H). Adapted from Aiad *et al.* (2016).

The plasma membrane is also commonly called inner membrane by some authors, who consider it as a third layer composing the cell wall (Silhavy *et al.* 2010). The outer membrane is a particularity of Gram-negative bacteria and it is formed by an asymmetric bilayer, in which the inner face is composed of phospholipids, while the outer face is composed of lipopolysaccharides (LPS) (Zhang *et al.* 2013) that play a key role in bacterial pathogenicity (Maldonado *et al.* 2016). The outer membrane is a selective permeation barrier (Nikaido 2003) that is involved in cell nutrition and also confers resistance to a variety of detergents and antibiotics (Doerrler 2006).

The peptidoglycan layer is composed by repeated units of the disaccharide *N*-acetylglucosamine and by *N*-acetylmuramic acid, which are cross-linked by pentapeptide side chains (Vollmer *et al*. 2008). This layer gives rigidity to the cyanobacterial cells, maintaining their shape. It also confers a protection against differences in osmotic pressure between the external and internal media, and also serves as a scaffold for anchoring proteins and teichoic acids (See review: Irazoki *et al.* 2019). Despite its rigidity, the peptide glycan layer is sufficiently dynamic to allow cell growth, division and morphogenesis (Zhang *et al.* 2018).

Although the general structure of cyanobacterial cell walls is the same observed in gram-negative bacteria, some characteristics of gram-positive walls and other particularities are also present (Hoiczyk & Hansel 2000). Their peptidoglycan layer, for example, is considerably thicker (reaching 700 nm in larger species, like *Oscillatoria princeps* Gomont, 1892) than those observed in most gram-negative bacteria (5-10 nm) (Hoiczyk & Hansel 2000). In *Synechocystis* Sauvageau, 1892, the degree of cross-linking between peptidoglycan chains is greater than that usually found in heterotrophic gram-negative bacteria and is more similar to the reported values for gram-positive bacteria (Hoiczyk & Hansel 2000). Further, cyanobacteria cell walls have components that are

absent in the cell walls of other gram-negative bacteria. For example, they have carotenoids (Resch & Gibson 1983) and the fatty acid b-hydroxypalmitic as a substitute for the hydroxymyristic acid commonly found in other gram-negative bacteria (Jurgens & Weckesser 1985).

2.2.2- Eukariotic microalgae cell wall

Among the microalgae coverings, the term "cell wall" is reserved for a thick, rigid and continuous structure that is mainly composed of cellulose microfibrils (Okuda 2002). This kind of cell covering is found in the green algae (Chlorophyta and Charophyta). The molecular structure of the cellulose of these algae is the same of the cellulose found in plants, which is a linear polymer of β-(1-4)-linked d-glucose (Baldan et al. 2001, Fry 2003). However, the structure of the cellulosic walls is not the same for the both groups (Okuda 2002), except for few algal taxa more related to plants (Sørensen *et al*. 2011; Domozych *et al*. 2012). Beyond cellulose, other polymers like xylans and mannans may also occur as the major structural polymer in some algae (Okuda 2002) and some species of Chlorophyta have no cellulose (Iman *et al.* 1985).

The structural polymers are embedded in an amorphous matrix composed by polysaccharides, which varies among different green algae taxa (Domozych *et al.* 2012). Then, together with cellulose, hemicellulose, pectins, and other polysaccharides composing this matrix were described in cell walls of various microalgae (Sorensen *et al.* 2011, see table 2) (Fig. 5). However, there is a lack of information about the complete structure and composition of algae cell walls. Recently, more attention has been paid to algal cell wall due to the new tools and techniques allowing to do very detailed studies and mainly due to the need to better understand these structures. This knowledge is fundamental to solve questions about the morphology and physiology of these organisms and their interaction with the environment. It is also necessary for the development of methods to disrupt cells

for the extraction of various compounds of economic interest (Baudelet *et al*. 2017), such as pigments and fatty acids.

Table 2: Chemical composition of green-algae cell walls.

Phylum	**Cell wall componentes**	**References**
Charophyta	Uronic acids	Pooper and Fry 2003
	Cellulose, hemicelluloses and pectins	Domozych *et al*. 2010 Sorensen *et al.* 2011
	Pectins	Domozych *et al.* 2007 Eder and Lutz-Meindl 2008 Eder and Lutz-Meindl 2010 Domozych *et al.* 2011
	Cellulose	Wurdack 1923 Herburguer and Holzinger 2015
	AGPs and hemicelluloses	Eder *et al.* 2008
	Chitin	Wurdack 1923
Chlorophyta	Cellulose	Parker 1964 Yamada and Sakaguchi 1982 Baldan *et al.* 2001 Nemcová 2003
	Glycoproteins	Goodenough and Heuser 1985
	Sporopollenin	He et al. 2016
	Aminoacids and saccharides	Abo-Shady et al. 1993
	HRGP and hemicelluloses	Estevez et al. 2009
	Cellulose and other polysacharides	Piro et al. 2000
	Chitin	Wurdack 1923
	D-glucose, D-mannose	Parker 1964
	Pectins	Wurdack 1923 Bisalputra and Weier 1963 Yamada and Sakaguchi 1982

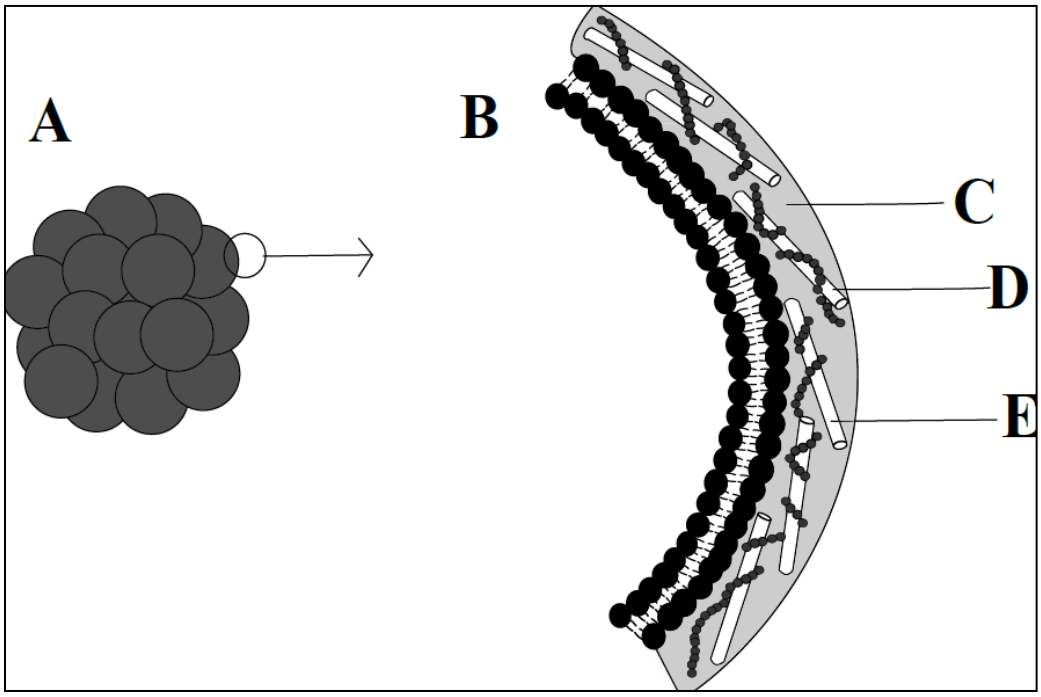

Fig. 5 - A- Representation of a colony of Chlorophyta. B- Magnification showing the main components of the green algae cell wall. In C is represented the layer of pectic compounds in which the microfibrils of cellulose (D) and hemicellulose (E) are immersed.

2.2.3- Organic scales

Members of Prasinophyceae (Chlorophyta) have their cells covered by organic scales. These scales are mainly composed of acid polysaccharides (2-keto sugars), with some proteins being present in lesser amount (Becker *et al.* 1994). Prasinophyceae scales are synthesized in the Golgi cisterns and transported to the cell surface by exocytosis (Moestrup & Walne 1979). Interestingly, a cell can show many types of scales arranged in several layers (1-5) on the surface of the cell body and even of the flagella (Becker *et al.* 1994) (Fig. 6). Less common, it is also observed the scales fused in one piece, as occurs in the genera *Tetraselmis* Stein, 1878, and *Scherffelia* Pascher, 1912 (Arora *et al.* 2013). In the order Pyramimonadales the scales are arranged in 3 layers. These are considered the most complex among the Prasinophyceae. In the innermost layer, the scales are small, square or pentagonal; in the middle layer the scales are naviculoid, have the form of a spider web or the form of a box, while the scales of the outer layer have the shape of a crown (Daugbjerg *et al.* 2000). The scales morphometry is widely varied and very

important as a taxonomic character to differentiate between orders, families and genera (Becker *et al.* 1994).

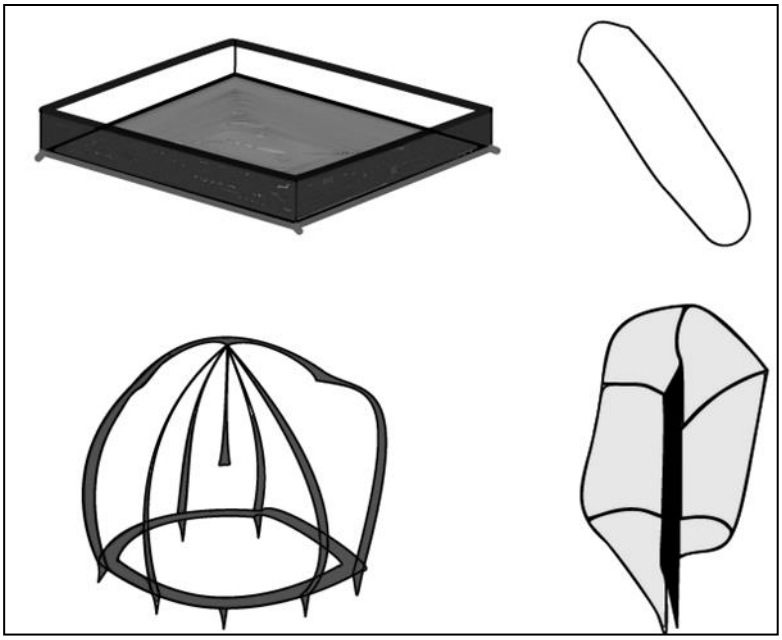

Fig. 6- Scheme of a cell of *Pyramimonas diskoicola* (A) and its types of organic scales (B). Adapted from Harðardóttir *et al.* (2014.)

Some haptophytes (such as *Phaeocystis* Scherffel, 1899, *Prymnesium* Carter, 1937, *Pavlova* Butcher, 1952, and *Chrysochromulina* Lackey, 1939) have their cells covered with organic scales (Young & Henriksen 2003). Composed by cellulose, these scales are produced in the Golgi apparatus and transported through vesicles to the cell surface (Jordan & Chamberlain 1997). In some cases, they can also cover the haptonema or one of the flagella (De Vargas *et al.* 2007). Scales morphology varies among taxa within this phyllum and then these structures are commonly used as a taxonomic character (Eikrem *et al.* 2016). In Pavlovophyceae, for example, the scales are structurally simpler and have a knoblike form, while in the Prymnesiophyceae, the scales are more ornamented and shaped like plates (De Vargas *et al.* 2007). For many species, the organic scales serve as a

calcification matrix for the formation of rigid scales that are named coccoliths (Houdan *et al*. 2004, Liu *et al.* 2010). These structures are special scales that are only observed in Haptophyta and a detailed description will be presented in the next item.

2.2.4-Coccoliths

Coccoliths are the most common cell covering found in haptophytes. They are calcified plates ($CaCO_3$ as calcite) that cover the cells forming a coccosphere (Taylor *et al*. 2016; Müller 2019) (Fig. 7). The arrangement of these plates is a taxonomic character that is used for even distinguish organisms in the species level (Chrétiennot-Dinet *et al.* 2014). The coccoliths are formed in the cisterns of the dicytiosomes (Mantoon 1966) and are released to the cell surface by fusion of the plasmalema with the cisternal membrane (Eikrem *et al*. 2017).

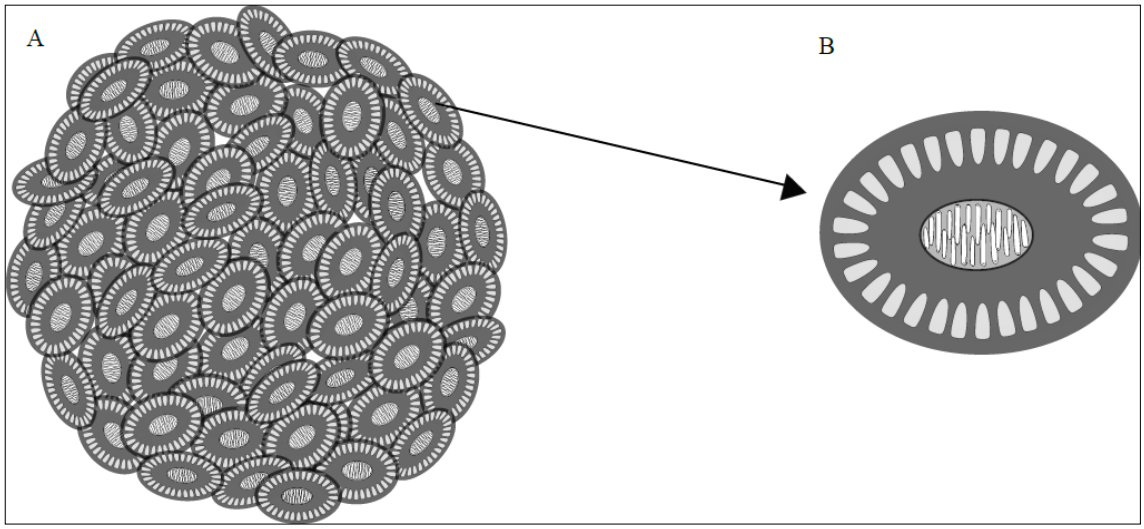

Fig. 7- Schematic representation of a coccolithophore (A) and an amplification of the structural unit that form its covering, the coccolith (B).

There are two main types of coccoliths, the heterococcoliths and the holococcoliths, based on their ultrastructure and morphology (Braarud *et al.* 1995). Some possible functions attributed to coccoliths are protection against predation and virus attack (Monteiro *et al.* 2016), optimization of light absorption by the cell (Young 1994), dissipation of excessive absorbed light energy to avoid photo damage under nutrient

limitation (Paasche 2002), regulation of buoyancy (Young 1994) and carbon concentration mechanism (Sikes *et al.* 1980). However, these hypotheses have not yet been proven (Eikrem *et al*. 2017) and none of them has sufficient and consistent evidence to be scientifically accepted (Müller 2019).

The species of coccholite-coated haptophyta are commonly called coccolithophore. The oldest recorded coccolithophores are from the upper Triassic sediments, approximately 225 Ma. (Bown *et al.* 2004). They were and are abundant in the marine phytoplankton and show a historical and current very important role in carbon cycling. Biomineralization of coccolithophores controls the alkalinity, chemistry of photic zone carbonates of the oceans, and the carbonate precipitation (through the calcification reaction) is a short-term source of CO_2 to the high ocean and atmosphere (De Vargas *et al*. 2007).

2.2.5- Lorica

The particularity of lorica in relation to other coverings is that it is not adhered to the plasma membrane, being similar to an envelope or armor. Lorica can be found in some Euglenophyta and Ochrophyta. For Euglenophyta, pellicle is their typical covering but some genera such as *Strombomonas* Deflandre, 1930, *Trachelomonas* Ehrenberg, 1834, and *Ascoglena* Stein, 1978, additionally have lorica (Duangjan & Wolowski 2013). This covering is a rigid and mucilaginous protective envelope composed by mucopolysaccharides and minerals (mainly iron and sometimes manganese) (Poniewozik 2017) that surround the cell and have a gap from which the flagellum emerges (Fig. 8-A). The lorica surface can be smooth, but it usually presents granular or rough appearance due to the agglutination of particles from the environment. The lorica shape and its ornamentation are very important taxonomic characters to differentiate genera and species

among the euglenophytes (Brosnan *et al.* 2005). Lorica can be colorless, but they generally have a yellow-brown or orange color due to the impregnation of minerals (Leedale 1975).

Although chrysophyceans (Ochrophyta) lorica are similar to euglenophytes lorica in relation to minerals impregnation, colors and microarchitecture (Dunlap *et al.* 1987), their coverings are mainly composed of chitin and cellulose (Herth & Zugenmaier 1979). In some Chrysophyceae, the organization of lorica can be simplified in foot, stalk and cup (Fig. 8-B). These structures have species-specific features (see Peck 2010) with evident taxonomic significance, such as shape, size and ornamentation (Belcher 1969; Kapustin 2019). Composition and architecture are also important (Dunlap *et al.* 1987). *Dinobryon* Ehrenberg, 1834, for example, has a lorica with a vase or beaker-shaped form, while *Chrysococcus* Klebs, 1892, has globular and *Lagynium* present a flask-shaped lorica (Kristiansen & Škaloud, 2017). As observed in euglenophytes, manganese and iron compounds can be present and are responsible for the dark and opaque color of some chrysophycean lorica (Dunlap *et al.* 1987).

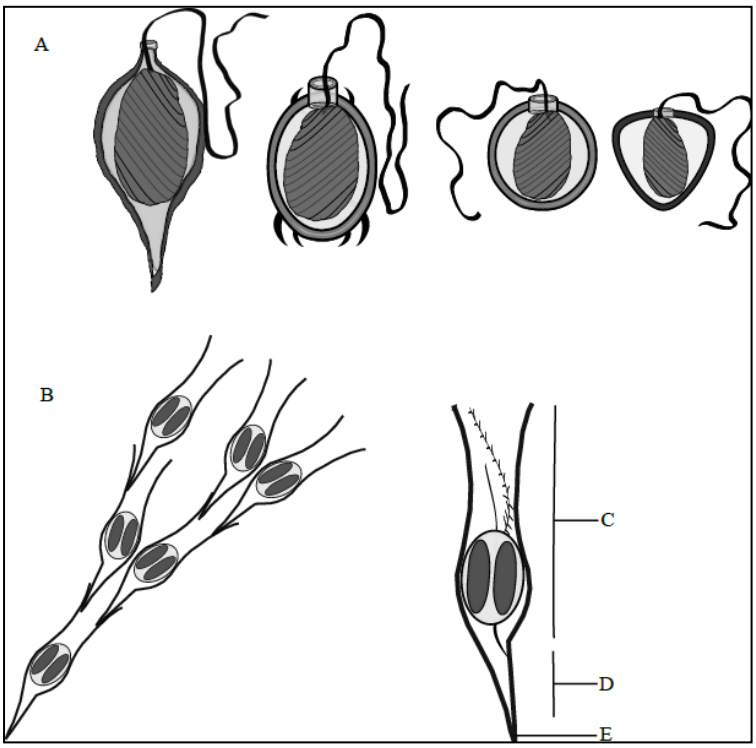

Fig. 8- Schematic representation of some types of Euglenophyta lorics (A). A *Dinobryon* colony and a detailed vision of the main regions (B): The cup (C), the stalk (D) and the foot (E). Adapted from Conforti (2010).

The formation of a new lorica is not well understood for many species, but it is better understood for *Dinobryon.* It was observed that the formation of a new lorica in this genus begins after cell division. The daughter cell moves to the edge near the opening of the parent lorica, where it connects and fixes. After that, it will first secret the small basal cone and then the complete cup-shape of the lorica (Karim & Round, 1967).

2.2.6- Silica scales

Chrysophyceans (Ochrophyta) of the family Paraphysomonadaceae, mainly the genera *Chrysosphaerella* Lauterborn, 1896, and *Paraphysomonas* De Saedeleer, 1930, *do* not have lorica, but silicified scales covering their cells (Kristiansen 2008). They are attached outside to the plasma membrane (Nemcová & Pichrtová 2012) with no defined pattern. Silica scales are radially or biradially symmetrical and their sizes vary from about 1 to 10 μm (Škaloud *et al.* 2013).

The scales of chrysophyceans have an endogenous origin and are formed inside a vesicle of deposition of silica, which is in turn derived from the endoplasmic reticulum (Lee 2008). The scales are extruded from the cell and placed in the correct position on its surface (Kristiansen 1986). The covering formed by scales is a dynamic structure that allows the addition of new scales during both growth and division (Škaloud *et al.* 2013). Techniques of electron microscopy allowed to know much about the structure of the scales, which is highly variable among species (Kristiansen & Skaloud 2017) and therefore have taxonomic significance. However, a basic structure is common for all species, and it can be described as a perforated plate that can have ribs, spines and other ornaments (Kristiansen 1986).

Silica scales are also found in Synurophyceae (Ochrophyta) cells. They are formed internally in silica deposition vesicles and then they are transported to the cell surface

(Wee 1997). Interestingly, several scale types can occur on the same cell and each type show a particular distribution on the cell surface (Neustupa *et al* 2010; Skaloud *et al.* 2012) (Fig. 9). The genus *Synura* Ehrenberg, 1834, for example, has three distinct scales by cell: body, apical and rear scales that are characterized by their different length to width ratios (Skaloud *et al.* 2012). Abiotic factors in the environment like pH (Siver 1989) and temperature (Řezáčová-Škaloudová *et al.* 2010) seems to have some influence on the morphological differentiation of the scales. The morphology is specie-specific and have highlighted taxonomy significance (Kristiansen 2002), especially the body scales, that exhibit the most highly developed and complex characters (Skaloud *et al.* 2012).

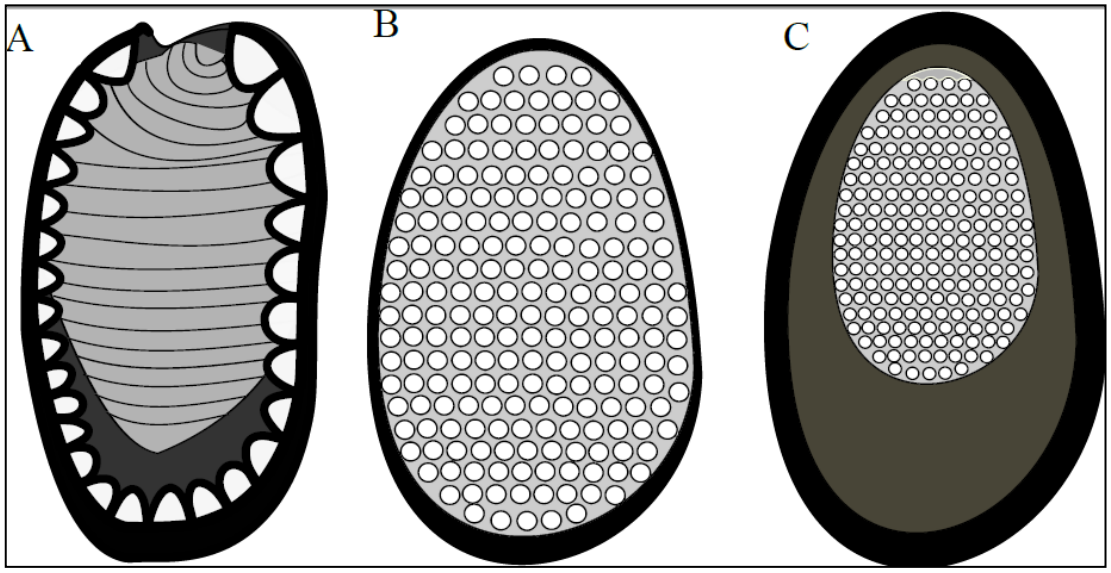

Fig. 9- Scheme of silica scales found in the genus *Mallomonas*. Scale found in the species *M. flora* (A), *M. matvienkoae* (B), *M. ouradion* (C). Adapted from Peck (2010).

2.2.7-Frustule

Diatoms are one of the most easily recognizable groups among the algae due to the presence of its characteristic silicified cell wall covering (Reimann *et al*. 1965). This special cell wall is named frustule and is formed by two parts of similar size that are called valves. One valve is slightly larger than the other, with the smaller valve fitting inside the

larger one. This fitting is connected by structures called girdle bands that allow preciselly link these valves around the protoplasm (Kröger & Poulsen 2008; Tesson & Hildebrand 2010). The large valve is named epitheca and the smaller is the hypotheca (Fig. 10).

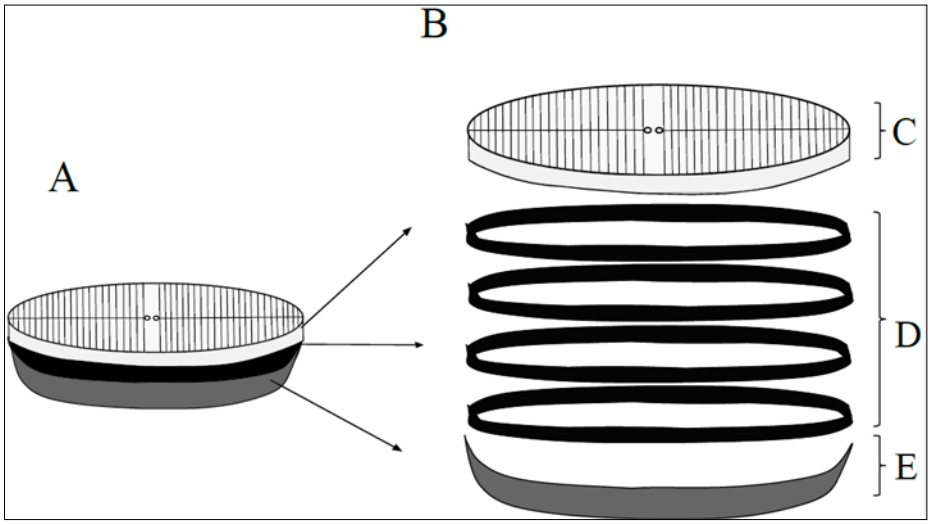

Fig. 10- A-Scheme of a closed frustule of a penate diatom. B- The main components of the frustule: The epitheca (C), the girdle bandes (D) and the hypotheca (E). Adapted from Cox (2014).

A symmetrical structure of the leaflets divides the diatoms into two generic groups: centric, with radial symmetry and as pennates with bilateral symmetry, sometimes with a transverse groove, the raphe.

The frustule is mainly composed by silica, that gives rigidity to its structure, but it is in association with an organic wall composed by proteins, polyamines and polysaccharides (Nakajima &Volcani 1969; Swift & Wheeler 1992; Krögger 1999; Gügi *et al*. 2015). The organic matter seems to play a role in cell adhesion to surfaces and protection to cell desiccation (Kröger & Poulsen, 2008). Four families of proteins have been described in diatom's cell walls: frustulins, pleuralins, p150 family and silaffins (Kröger *et al*. 1994; 1996; 1997; 1999; 2000; 2001; Kröger & Poulsen 2008). Silaffins are suggested to be the molecule involved in silica formation (Kröger & Poulsen 2008). In relation to the carbohydrates (mono or polysaccharides), there are many types that were

observed in frustule (Table 3). Chitin, for example, is a polysaccharide that was detected in association with silica (Gügi *et al*. 2015). A detailed study of *Phaeodactylum tricornutum* Bohlin, 1898, showed the presence of glucuronic acid and mannose (Ford & Percival 1965). These carbohydrates are common in high amounts in frustules of several species of diatoms while the quantity of fucose and xylose is more variable (Gügi *et al*. 2015).

The knowledge on frustule composition, structure and synthesis has not only taxonomical importance, but this cell covering has also commercial value and many industrial uses. The ease of cultivation in artificial environments (culture media) and the availability of fossilized frustules (diatomite) make diatom silica a promising natural alternative to synthetic materials for biomedical, environmental, agricultural, and energy applications (Maher *et al.* 2018; Terraciano *et al.* 2018). Diatoms have been currently studied for biotechnological and nanotechnological purposes, being involved in techniques of nanofabrication, chemo and biosensor, classification and control of particles in micro and Nano fluid (Jamali *et al.* 2012). Dolatabadi and La Guardia (2011) for example, present in their review the applications of silicious diatoms and nanomaterials in biosensing (drug and gene delivery) and their utility to form complex metallic nanostructures.

Table 3- Main components of the frustule.

Chemical component			References
Inorganic	silica		Nakajima &Volcani 1969
Organic	Proteins	Frustulins p150 family silaffins pleuralins,	Kröger *et al*. 1994; 1996; 1997; 1999; 2000; 2001; Kröger & Poulsen 2008
	Polyamines		Krögger 1999
	Monosaccharides	Rhamnose	Hecky *et al*. 1973
		Fucose	Hecky *et al*. 1973 Gügi *et al*. 2015
		Ribose	Hecky *et al*. 1973
		Arabinose	Hecky *et al*. 1973
		Xylose	Hecky *et al*. 1973 Gügi *et al*. 2015
		Mannose	Hecky *et al*. 1973 Ford & Percival 1965
		Galactose	Hecky *et al*. 1973
		Glucose	Hecky *et al*. 1973
		Glucuronic acid	Ford & Percival 1965
	Polysaccharide	Chitin	Gügi *et al*. 2015

3- Coverings affect commercial exploration of microalgae: limitations and tools to disrupt cells and assess products

Algal cell walls are one of the main products of exploitation among marine macroscopic algae, from which sulfated polysaccharides and other compounds are extracted and used in a wide variety of industrial segments (Jönsson *et al*. 2020). In relation to the microscopic ones, diatom frustules, as already mentioned, have been studied for use in biotechnological and nanotechnological purposes (Jamali *et al*. 2012). Other products not related to covering composition are also of commercial interest. Algae, and especially microalgae, has been increasingly targeted as a sustainable source of high added

value compounds used by the industry of pharmaceuticals, cosmetics and nutrition, and some are also alternative feedstocks for biofuel production (Wu *et al.* 2017; Dixon & Wilken 2018). Various microalgae and cyanobacteria are known to produce these targeted compounds, but commercial exploration and research are concentrated in few genera, as summarized in the table 4.

Table 4: Most commonly cultivated microalgae and cyanobacteria for commercial purposes or for researches to support a possible future exploration. The major compounds of interest by genus or species are also presented.

Phyllum	**Genus or species**	**Compounds of interest**	**References**
Chlorophyta	*Botryococcus*	Hydrocarbons	Metzger & largeau 2005
	Chlorella	Pigments and lipids	Safi *et al*. 2014
	Scenedesmus		Wiltshire *et al.* 2000
	Dunaliella	β-Carotene	Raja *et al.* 2007
	Haematococcus	Astaxanthin	Shah *et al*. 2016
	Tetraselmis	Eicosapentaenoic acid (EPA), Vitamin E	Pereira *et al*. 2019
Haptophyta	*Isochrysis galbana*	Fatty acids	Bandarra *et al*. 2003
Cyanobacteria	*Limnospira maxima*	nutritional supplements	Priyadarshani & Rath 2012
	Arthrospira platensis	Human nutritional supplements	Colla *et al*. 2007
Bacillariophyta	*Odontella aurita*	Fatty acids	Pasquet *et al.* 2014
	Phaedactylum tricornutum		Yongmanitchai & Ward 1991
Euglenophyta	*Euglena gracilis*	Vitamin E	Takeyama *et al.* 1997
Rhodophyta	*Porphyridium cruentum*	Polysaccharides	Balti *et al.* 2018

Although many microalgae and cyanobacteria are cultured, only four species have been the focus for biotechnological application through the last decades: *Arthrospira*

platensis Gomont, 1892 (commercially known and marketed as *Spirulina*), *Chlorella vulgaris* Beyerinck, 1890), *Dunaliella salina* Teodoresco, 1905, and *Haematococcus pluvialis* Flotow, 1844 (Mobin & Alam 2017). More recent studies expanded the attention to other microalgae with potential for biofuel production, but large-scale cultivation is still rare. Among the four commercially important species mentioned above, *Arthrospira platensis* is a Cyanobacteria, but all the other are chlorophytes. Chlorophytes have a thick cell wall that exhibits a wide variety of chemical composition and morphology within the group (Rashidi & Trindade 2018). In fact, a great variability has been reported for the cell wall composition and structure among chlorophyte genera, species and even among lineages or the life stage of the cell (Domozych *et al*. 2012).

As mentioned before in this review, little is known about the cell wall structure for many species of microalgae (Scholz *et al*. 2014) and generalizations are frequently made based on few studies considering a very small number of species. This is frequently a problem, since the compounds of interest are mostly found within the cells (Baudelet *et al*. 2017) and the cell wall can be acting as a barrier to access these products (Kim *et al*. 2016). Taxonomy, although being a strong tool, is not enough to appropriately deduce the composition of the algae wall in order to reduce costs and time for development of rupture processes for them (Baudelet *et al*. 2017). In this context, knowledge of the composition and architecture of algal cell coverings is essential to optimize the extraction and recovery of the compounds of commercial interest (Dixon & Wilken 2018).

The lack of knowledge on algal cell coverings contributes with the difficulty to disrupt algal cells to extract compounds, which is one of the biggest obstacles to the industrial use of microalgae on a large scale (Wu *et al*. 2017). Several methods of disrupting microalgae cells were developed and their applications depend on the characteristics of the cell and on which compounds are of interest (Dixon & Wilken 2018).

These methods can be mechanical or non-mechanical. Mechanical methods include treatments with high pressure homogenization techniques, high speed homogenization, ultrasound and pulsed electric field (Aarthy *et al*. 2018). Non-mechanical methods can be thermal (microwave, autoclave or freezing), chemical (organic solvent, osmotic shock and acid-alkaline reactions) and biological (microbial or enzymatic degradation) (Dixon & Wilken 2018). Despite all these methods, the disruption of algae cell coverings remains a problem since they are often expensive and inefficient. Cell disruption is crucial for the valorization of algal biomass, however, obtaining efficient and economically attractive cell disruption methods and for all species of interest is still a challenge (D'Hondt *et al.* 2017).

4 - Conclusion

As mentioned in this review, cell coverings generally play important roles in cellular physiology and each type of covering must meet the specific needs of each group. This study tried to organize the knowledge about the several types of coverings of microalgae and cyanobacteria and highlighted how diverse they are. Cell coverings can be intra or extracellular and have a variety of mainly components depending of group and most of them are considered a taxonomic feature due to their group or species-specific morphology. This review allows us to notice that despite all the research cited, very little is known about microalgae coverings, considering the diversity of species. Some few groups, such as diatoms, are in general the most studied group due to their possibilities of use in the areas of bio and nanotechnology. However, algae in general are a very diverse group and have been increasingly studied for different purposes, which requires a better understanding of various aspects of these organisms, especially the morphological ones.

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Capítulo 2: How similar are microalgal and plant cell walls? Applying methods developed for plants to elucidate microalgal cell wall composition

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Abstract

Polysaccharides are molecules commonly found in cell walls, but little is known about the exact composition of these compounds and the level of taxonomic specificity in different algal groups. Here, we used calcofluor white to detect cellulose and the monoclonal antibodies JIM 5, JIM 7, LM5, LM 6, LM 19 and LM 20 to identify epitopes of pectins and LM 10, LM 15 and LM 21 to identify epitopes of hemicelluloses in living cells of six microalgae: *Mougeotia* sp., *Spirogyra* sp., *Chlorella vulgaris*, *Coelastrum microporum*, *Pseudopediastrum boryanum* and *Pediastrum duplex*. All experiments were performed in two different growth phases: phase I, at the beginning of the exponential growth phase, and phase II, at the end. Cellulose was detected in all species. The epitopes of pectins were labeled by JIM 5 and JIM7 in *C. vulgaris*, by JIM 5, JIM 7, LM 5, LM6 and LM 20 in *C. microporum*, and by JIM 7, LM 19 and LM 20 in *P. boryanum*. The epitopes of hemicelluloses were labeled by LM 10 and LM 15 in the cell walls of *C. microporum*, and by LM 21 in the cell walls of *P. duplex*. The results also showed that the six investigated species have different cell wall compositions, and some components appear to vary according to the stage of growth. *C. microporum* cell walls had the highest similarity to plants regarding the polysaccharide composition.

Key words: cellulose, hemicelluloses, pectins, monoclonal antibodies, growth phase, green algae.

1-Introduction

Algal cells have different types of coverings with varied biochemical composition, which can be found internally or externally to cell membrane depending on the taxonomic group (Okuda 2002). Nevertheless, definitions of algal cell walls are usually simplified, and their simplest characterization describes them as thick, rigid and continuous extracellular coverings, mainly made up of cellulose microfibrils (Okuda, 2002). Cellulose is a linear polymer of β-(1-4)-linked d-glucose that is indeed the major component in the cell wall of some algae (Baldan *et al.,* 2001) and plants (Festucci-Buselli *et al*. 2007). However, there are many components in algal and plant cell walls and their exact composition varies among taxon, tissue and cell types. Besides cellulose, other structural polysaccharides, such as hemicelluloses and pectins can be found (Costa & Plazanet 2016), as well as lignin in the secondary walls of plants (Anderson & Kieber 2020).

In plants, the cellulose presents individual glucose molecules closely aligned and connected to each other, resulting in highly ordered microfibrils, which contribute to the rigidity and strength of the walls (Thomas *et al*. 2013). These microfibrils are connected by hemicelluloses, which are flexible polysaccharides whose major function is to tether the cellulose microfibrils together while maintaining the wall strength (Scheller & Ulvskov, 2010). Xylans, xyloglucans and mannans are the most commonly found hemicelluloses in plants (Obel *et al.,* 2006). The network composed by cellulose and hemicelluloses is embedded in a hydrated gel phase composed by pectins (Alberts *et al.* 2002). Pectins are polysaccharides rich in galacturonic acid (GalA), which are involved in several cytological processes, such as cell expansion, growth regulation, defense, adhesion, signaling and porosity (McCann *et al.*, 2001). There are four major types of pectins in plant cell walls: homogalacturonan, rhamnogalacturonan-I, rhamnogalacturonan-II and xylogalacturonan (Ridley *et al.*, 2001). Homogalacturonan (HGA) is a linear chain of 1,4-linked a-d-

galactopyranosyluronic acid (GalpA) (Ridley *et al.,* 2001). HGA GalpA residues may be methyl-esterified at the C-6 carboxyl or acetylated at the O-2 or O-3, and the pattern and degree of methylesterification varies depending on the stage of development of the plant cells and tissues (Caffall & Mohnen, 2009). Rhamnogalacturonan-I (RG-I) is a family of pectic polysaccharides that contain a backbone of the repeating disaccharide [→4)-a-d-GalpA-(1→2)-a-l-Rhap-(1→] and that can also have side chains that differ in length and structure (Mikshina *et al*. 2015) , while RG-II is much more complex with 12 different types of glycosyl residues, including the rare sugar species 2-O-methyl xylose, 2-O-methyl fucose, 32 aceric acid,33 2-keto-3-deoxy-D-lyxo heptulosaric acid (Dha),34 and 2-keto-3-deoxy-D-manno octulosonic acid (Caffall & Mohnen 2009; O'Neill *et al.,* 2004).

Contrasting with the reasonable knowledge available on plants, there is a lack of information on algal cell walls. Some data are available for chlorophytes, charophytes, ochrophytes and rhodophytes (see table 1), but even these groups have not been thoroughly studied (Popper and Fry 2003), and the chemical profile of their cell walls is still poorly understood (Baudelet *et al.,* 2017). Some green algae show similar cell wall composition to that of plants, with presence of cellulose, hemicelluloses and pectins (Domozych *et al.,* 2012), but with quite different organization of these molecules (Okuda, 2002). Some cell wall peculiarities within algal groups could even prove as valuable tools for their classification (Popper and Fry 2003). Certain species of the Chlorophyceae class, for example, produce coverings that are structurally and biochemically unique (Domozych *et al.,* 2012). Additionally, many polysaccharides are uniquely found as components of algal cell walls and differ from those observed in plants (see reviews: Baudelet *et al.,* 2017; Kinnaert *et al.,* 2017), such as the agar and carrageenan present in seaweeds (Rhein-Knudsen *et al.,* 2015).

"Algae" is defined as a polyphyletic group that includes about 72,500 species (Guiry, 2012). The limited knowledge about algal cell wall structure and composition is a consequence of the taxonomic complexity of these organisms, added to the little focus given on this topic by phycologists. Studies may be even more limited if we consider that algal cell wall composition can vary depending on the cell's life stage (Hagen *et al.,* 2002; Takeda 1991). Algal cell wall research has especially focused on species of ecological and commercial importance (Popper & Tuohy 2010). Therefore, a deeper understanding on this topic would be useful for taxonomical support (Popper & Fry 2003), for the proper treatment and handling of algae of industrial importance (Li *et al.,* 2013), and to understand how the diversity of these compounds may affect the algal physiology and its interactions with other organisms and the environment (Popper *et al.,* 2014).

Many tools that have been employed for the study of plant cell walls, such as electronic and light microscopy, enzymatic techniques, and different probes and histochemical methods (Costa & Plazanet 2016; Paniagua *et al.,* 2014; Hervé *et al.,* 2011) and these techniques have also been used for studies on algal cell covering (see the review by Domozych *et al.,* 2012). Plant monoclonal antibodies, for example, originally used on plants, have also be employed for macroalgae (Domozych *et al.,* 2010) and microalgae (Eder *et al.,* 2008; Estevez *et al.,* 2008), generating some new information. In the present study, we aimed to characterize the cell wall composition of living cells of six green microalgae species by using probes and cytochemical techniques generally used for plants. Calcofluor white and eight monoclonal antibodies for the detection of the epitopes of the cell wall polysaccharides (cellulose, pectins and hemicelluloses) were used in two growth phases of the algal cultures. With the use of these techniques, we wanted to address the following questions: (1) how different or similar are these components in algal and plant cell walls? (2) Can the algal cell wall constituents change according to the cells age? (3)

Are the techniques developed for the study of plant cell walls also suitable for algal studies? To our knowledge, this is the first study using this combination of methods to evaluate the cell wall composition in various microalgal species at different growth phases.

Table 1- Main cell wall components already identified in algae.

Phylum	Species	Methods	Cell wall components	References
Charophyta	*Chara corallina*	Enzymatic methods, HPLC and chromatographic analysis	Uronic acids	Popper and Fry 2003
		Cytochemistry and immunocytochemistry	Cellulose, hemicelluloses and pectins	Domozych et al. 2010
		Immunocytochemical and biochemical approaches	Cellulose, hemicelluloses and pectins	Sorensen et al. 2011
	Chlorokybus atmophyticus *Coleochaete nitellarum* *Cosmarium turpini* *Klebsormidium flaccidum* *Micrasterias furcata* *Pleurotaenium trabecula* *Spirogyra* sp.	Immunocytochemical and biochemical approaches	Cellulose, hemicelluloses and pectins	Sorensen et al. 2011
	Closterium acerosum	Cytochemistry	Pectins	Bisalputra and Weier 1963
	Coleochaete scutata	Enzymatic methods, HPLC and chromatographic analysis	Uronic acids	Popper and Fry 2003
	Klebsormidium crenulatum, *K. nitens*, *Zygnema* sp.	Cytochemistry (Calcofluor White)	Cellulose	Herburguer and Holzinger 2015
	Micrasterias denticulata	Immunocytochemistry and TEM	Pectins	Eder and Lutz-Meindl 2008
		Immunocytochemistry and TEM	AGPs and hemicelluloses	Eder et al. 2008
	Netrium digitus	Immunocytochemical and biochemical approaches	Cellulose, hemicelluloses and pectins	Sorensen et al. 2011
		Immunocytochemistry	Pectins	Eder and Lutz-Meindl 2010
	Penium margaritaceum	Immunocytochemistry	Pectins	Domozych et al. 2007
		Immunocytochemistry	pectins	Domozych et al. 2011
		Immunocytochemical and biochemical approaches	Cellulose, hemicelluloses and pectins	Sorensen et al. 2011
	Spirogyra fluviatilis, *Zygnema cruciatum*	Biochemical approaches	Cellulose, pectins and chitin	Wurdack 1923

Table 1: continued

Phylum	Species	Methods	Cell wall components	References
Chlorophyta	*Chaetomorpha aerea*	Enzyme-gold affinity	Cellulose	Baldan et al. 2001
	Chlamydomonas reinhardtii	Quick-freeze, deep-etch technique	Glycoproteins	Goodenough and Heuser 1985
	Chlorella protothecoides	TEM and FTIS	Sporopollenin	He et al. 2016
	Chlorella. vulgaris (3 strains)	Cytochemistry (Calcofluor White and Ruthenium Red)	Cellulose and pectins	Yamada and Sakaguchi 1982
	C. vulgaris (2 strains)	Biochemical approaches	Aminoacids and saccharides	Abo-Shady et al. 1993
	C. vulgaris	Celulase-gold complex	Cellulose	Nemcová 2003
	Chlorella ellipsoidea (3 strains) and *Chlorella saccharophila*	Cytochemistry (Calcofluor White and Ruthenium red)	Cellulose and pectins	Yamada and Sakaguchi 1982
	Codium fragile	Immunocytochemistry and SR-FTIR	HRGP and hemicelluloses	Estevez et al. 2009
	Enteromorpha intestinalis	enzyme-gold affinity	Cellulose	Baldan et al. 2001
	Kirchneriella lunaris	Biochemical approaches	Aminoacids and saccharides	Abo-Shady et al. 1993
	Koliella Antarctica	Biochemical approaches	Cellulose and other polysacharides	Piro et al. 2000
	Gonium sociale, Oedogonium cardiacum	Electron microscopy, X·ray diffraction, and chromatographic Analysis	Cellulose	Parker 1964
	Oedogonium crissum, O. irregular, Cladophora glomerata, Draparnaldia plumosa	Biochemical approaches	Cellulose, pectins and chitin	Wurdack 1923
	Pediastrum tetras	Electron microscopy, X·ray diffraction, and chromatographic Analysis	D-glucose, D-mannose	Parker 1964
	Scenedesmus quadricauda	Celulase-goldcomplex Cytochemistry	Cellulose pectins	Nemcová 2003 Bisalputra and Weier 1963
Ochrophyta	*Cystoseira barbata, Dictyota dichotoma, Fucus virsoides, Ulva laetevirens*	Enzyme-gold affinity	Cellulose	Baldan et al. 2001
	Vaucheria geminata	Biochemical approaches X-ray analysis and electron microscopy	Cellulose, pectin and chitin Glucose, ribose, xylose and arabinose	Wurdack 1923 Parker et al. 1963
	Vaucheria sessilis (2 strains)	X-ray analysis and electron microscopy	Glucose, ribose, xylose and arabinose	Parker et al. 1963
Rhodophyta	*Calliarthron cheilosporioides*	Immunolabeling and DFRC	Lignin	Martone et al. 2009
	Gracilaria verrucosa	Enzyme-gold affinity	Cellulose	Baldan et al. 2001

Abbreviations: HPLC: High performance liquid chromatography; TEM: Transmission electron microscopy; FTIS: Fourier transform infrared spectroscopy; SR-FTIR: synchrotron radiation Fourier transform infrared; DFRC: Derivatization followed by reductive cleavage.

2- Materials and methods

2.1- Species and culture conditions

All analyses were performed on monoclonal cultures of six green algal species belonging to three different classes. The strains were obtained from the culture collection of the Phycology Laboratory of the Federal University of Minas Gerais, here indicated by their register numbers in the collection as: 35-*Mougeotia* sp. and 7-*Spirogyra* sp. (Zygnematophyceae), 56-*Chlorella vulgaris* (Trebouxiophyceae), 18-*Coelastrum microporum*, 29-*Pediastrum duplex*, and 105-*Pseudopediastrum boryanum* (Chlorophyceae). The species were chosen due to their close phylogenetic relationship with plants, since our major goal was to evaluate the efficiency of methods firstly developed for studies on plants. All species are very common in natural freshwater systems, and *C. vulgaris* is one of the most studied microalgae due to its economic value (Safi *et al.,* 2014). Cells were grown in 50 mL of sterile CHU10 medium in erlenmeyer flask (volume, 125 mL), and incubated at 20°C (±1) °C under fluorescent light (22 ±0,1 μmol photon m^{-2}. s^{-1}), photoperiod of light 12 h: dark 12 h. To verify if there were effects of culture aging, cell wall composition was evaluated in two different growth stages: phase I, at the beginning of the exponential growth phase (3 days of experiment), and phase II, at the end (of the exponential growth phase) (19 days of experiment). All experiments and analyses were performed in triplicate, using living cells, and with no fixation procedure. All the results presented here are representative of more than 90% of the individuals observed on the slides.

2.2- Cytochemistry: Detection of cellulose by Calcofluor white

Calcofluor white (CFW), a cellulose detection dyer (Herburger & Holzinger 2016), was used to identify cellulose. For this purpose, 1 mL of each algal culture was centrifuged during ten minutes at 12000 rpm in a microcentrifuge (Eppendorf 5424). The supernatant

was discarded. The pellet was resuspended in 40 μL of 50% CFW for five minutes, at room temperature. Finally, the treated cells were placed on a glass slide containing 50% glycerin, which were analyzed and photographed in a fluorescence microscope with excitation wavelength of 405 and 415-480 nm emission filter (Adapted from Hughes & McCully 1975). Positive results were evidenced by the blue color of the dye.

2.3- Immunocytochemistry

All the immunofluorescence labeling procedures (for pectins and hemicelluloses) used here were modified from Formiga *et al.* (2013). Antibodies commonly employed to identify pectin and hemicellulose epitopes in higher plants were tested in the six algal species. Briefly, we tested the following antibodies for pectins: JIM5, which is specific to low-methyl-esterified (up to 40%) homogalacturonans (Knox *et al.*, 1990); JIM7, specific to high methyl-esterified (15–80%) homogalacturonans (Willats *et al.*, 2000); LM19, which binds to non-esterified HGAs; LM20, which labels high methyl-esterified HGA (Verhertbruggen *et al.*, 2009); LM5, which is specific to (1→4) β-D-galactan (Jones *et al.*, 1997), and LM6, which labels the (1→5) α-L-arabinans (Verhertbruggen *et al.*, 2009). For hemicelluloses, three monoclonal antibodies were applied: LM15, specific to xyloglucan epitopes (Marcus *et al.*, 2008); LM21, which labels the β-(1→4)-manno-oligosaccharides (Marcus *et al.*, 2010), and LM10, specific to (1-4)-β-D-xylosyl residues (McCartney *et al.*, 2005).

2.2.1- Detection of pectins

For the analyses of pectins by immunocytochemical techniques, 1 mL of the culture of each algal species was centrifuged (microcentrifuge Eppendorf 5424) during ten minutes at 12000 rpm. The supernatant was discarded and the pellet was blocked with a 3% solution of powder milk for 30 minutes. Then, the pellet was resuspended with a sterile PBS solution (Phosphate buffered saline, 10x) and centrifuged again. Afterwards, the

pellets were incubated for 2 hours, at room temperature, in a solution of PBS containing the primary antibodies JIM5, JIM7, LM5, LM6, LM19, and LM20 produced at the Centre of Plant Sciences, University of Leeds, UK. Then, the pellet was washed with PBS again and incubated, for 2 h in the dark, in a PBS solution containing secondary antibody anti-rat IgG FITC (Sigma). Finally, the pellet was washed with PBS, and placed on a glass slide containing 50% glycerin and analyzed in a Confocal Zeiss 5 LIVE microscope (belonging to the image processing acquisition center; CAPI-UFMG) under excitation wavelength of 488 and detection of emission by using a 505-610 nm filter. Secondary antibodies bind to the primary antibodies; thus, primary antibodies were omitted for labeling procedure to serve as control.

2.2.2- For detection of hemicelluloses:

For detection of hemicelluloses, 1 mL of culture was centrifuged (microcentrifuge Eppendorf 5424) during ten minutes at 12000 rpm. The supernatant was discarded and the pellet was pre-treated with pectolyase at 10 µg/mL for 2 h at room temperature in 50 mM *N*-cyclohexyl-3-aminopropane sulfonic acid (CAPS), 2 mM CaCl2 buffer at pH 10 (Marcus *et al.,* 2008). The high pH of the enzyme buffer removes the homogalacturonan (HG) methyl esters in cell walls, which could mask the epitopes of hemicellulose compounds. After two hours, the pellet was washed with PBS solution, and blocked with a 3% solution of powder milk for 30 minutes. Then, the pellet was washed with sterile PBS solution (Phosphate buffered saline, 10x) and incubated, for 2 h at room temperature, in PBS solutions containing the primary antibodies LM10, LM15, and LM21 produced at the Centre of Plant Sciences, University of Leeds, UK. After the incubation, we followed the same steps described in the protocol for detection of pectins (see above).

3- Results

3.1- Cytochemistry

The cell walls of the six algal species were stained by calcofluor white. No differences were detected between the growth stages (Phase I and II) (Figure 1).

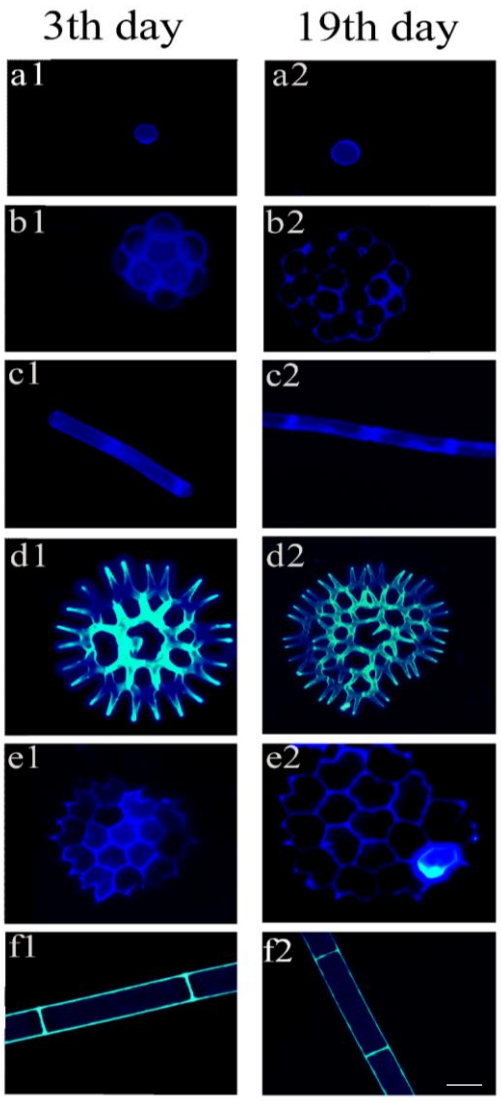

Fig. 1- Cellulose stained by calcofluor white (in blue) in cell walls of living microalgae in two stages of growth evidenced by fluorescence microscopy. a) *C. vulgaris*; b) *C. microporum*; c) *Mougeotia* sp. d) *P. duplex*; e) *P. boryanum*; f) *Spirogyra* sp. Phase I: a1 to f1. Phase II: a2 to f2. Scale bar: 10µm.

3.2- Imunocytochemistry

3.2.1- Detection of pectins

The results for the interactions between the six monoclonal antibodies used to detect pectins in the six algal species are compiled in the figures 2 (Phase I) and 3 (Phase II). The epitopes of HGAs were labeled by JIM5 and JIM7 in the cell walls of *Chlorella vulgaris* with 3 days of growth, but no labeling occurred in phase II (19 days old). The epitopes of HGAs were labeled by JIM5 and JIM7 in the cell walls of *Coelastrum microporum* in phase I, and by LM20 in phase II. Also, the epitopes of galactans and arabinans were labeled by LM5 and LM 6 in the cell walls of *Coelastrum microporum* in phase II. The strains *Mougeotia* sp., *Pediastrum duplex* and *Spirogyra* sp. were not labeled by any antibody in both phases of growth. The epitopes of HGAs were labeled only by JIM5 and LM19 in *P. boryanum* in phase I, and by LM19 and LM20 in phase II.

3.2.2- Detection of hemicelluloses

All results related to the monoclonal antibodies used to detect hemicelluloses are compiled in the figures 4 (3 days of growth) and 5 (19 days of growth). The epitopes of xylans, xyloglucans and mannans were not labeled by the antibodies LM10, LM15, and LM21 in both phases of growth in *Mougeotia* sp., *P. boryanum*, *Spirogyra* sp. and *Chlorella vulgaris*. The epitopes of (1-4)-β-D-xylosyl residues were labeled by LM10 in the cell walls of *Coelastrum microporum* in both phases of growth, and the epitopes of xyloglucans were labeled by LM15 only in phase I (3 days). The epitopes of β-(1→4)-manno-oligosaccharides were labeled by LM21 in cell walls of *P. duplex* cultures also in phase I. No labeling occurred for *P. duplex* cultures in phase II (19 days).

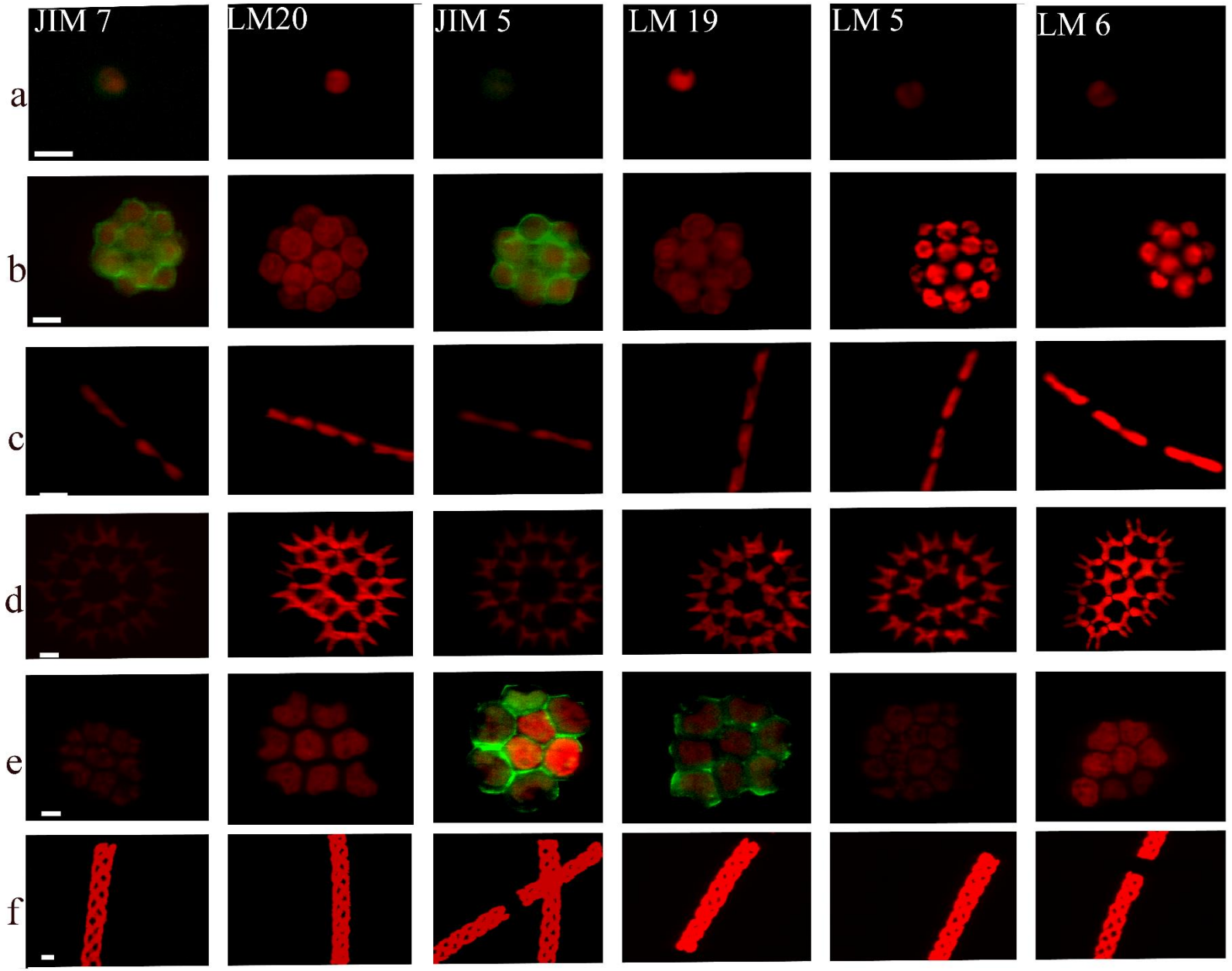

Fig. 2- Confocal images of immunocytochemistry for pectin epitopes labeled by JIM 5. JIM 7, LM 19, LM 20, LM 5 and LM 6 in living microalgae cell walls in phase I of growth. a) *C. vulgaris*; b) *C. microporum*; c) *Mougeotia* sp. d) *P. duplex*; e) *P. boryanum*; f) *Spirogyra* sp. Positive immunolabeling evidenced by green fluorescence. In red, chlorophyll autofluorescence. Scale bar: 10µm

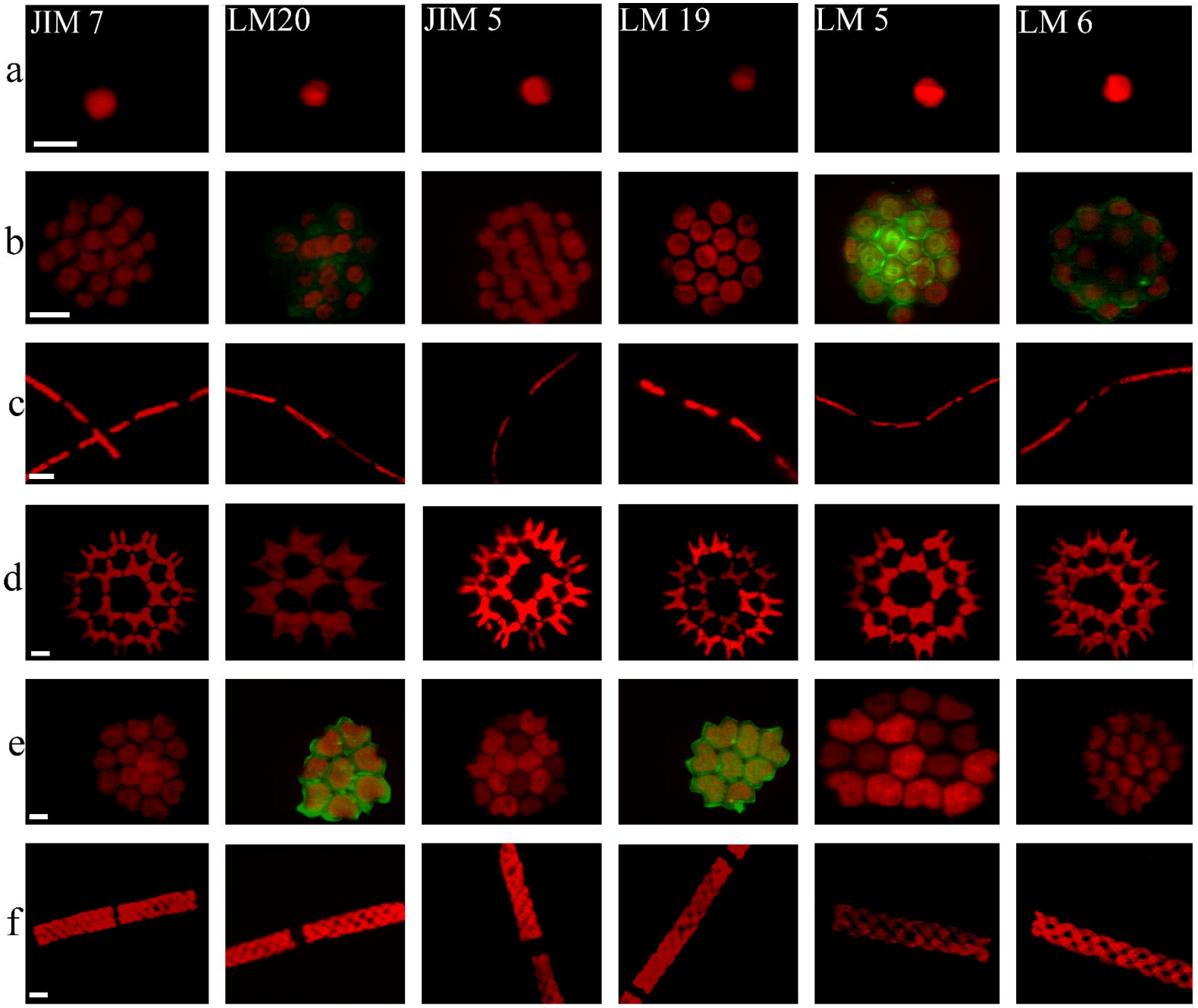

Fig. 3- Confocal images of immunocytochemistry for pectin epitopes labeled by JIM 5. JIM 7, LM 19, LM 20, LM 5 and LM 6 in living microalgae cell walls in phase II of growth. a) *C. vulgaris*; b) *C. microporum*; c) *Mougeotia* sp. d) *P. duplex*; e) *P. boryanum*; f) *Spirogyra* sp. Positive immunolabeling evidenced by green fluorescence. In red, chlorophyll autofluorescence. Scale bar: 10µm.

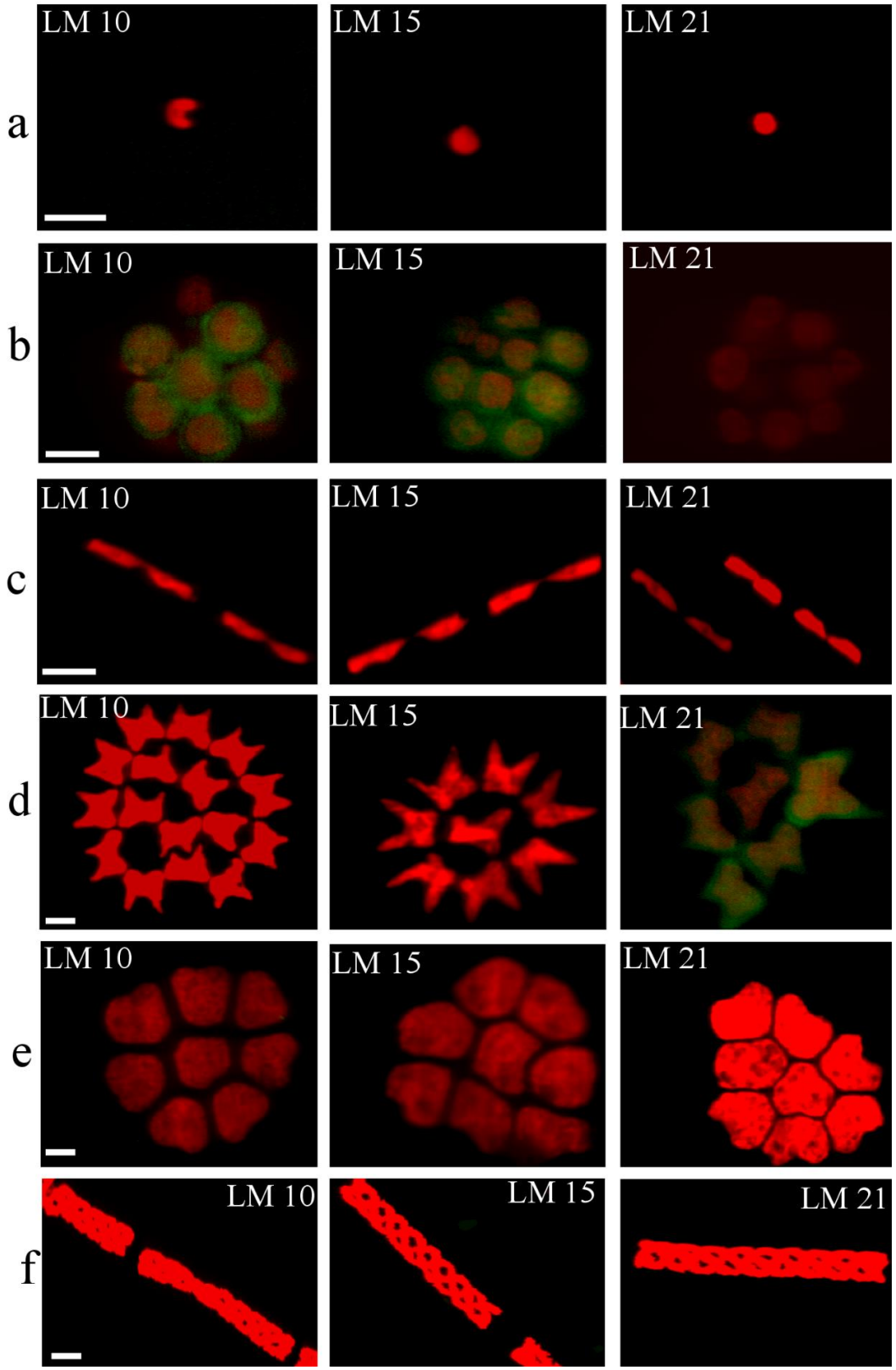

Fig. 4- Confocal images of immunocytochemistry for hemicellulose epitopes labeled by LM 10, LM 15 and LM 21 in living microalgae cell walls in phase I of growth. a) *C. vulgaris*; b) *C. microporum*; c) *Mougeotia* sp. d) *P. duplex*; e) *P. boryanum*; f) *Spirogyra* sp. Positive immunolabeling evidenced by green fluorescence. In red, chlorophyll autofluorescence. Scale bar: 10µm.

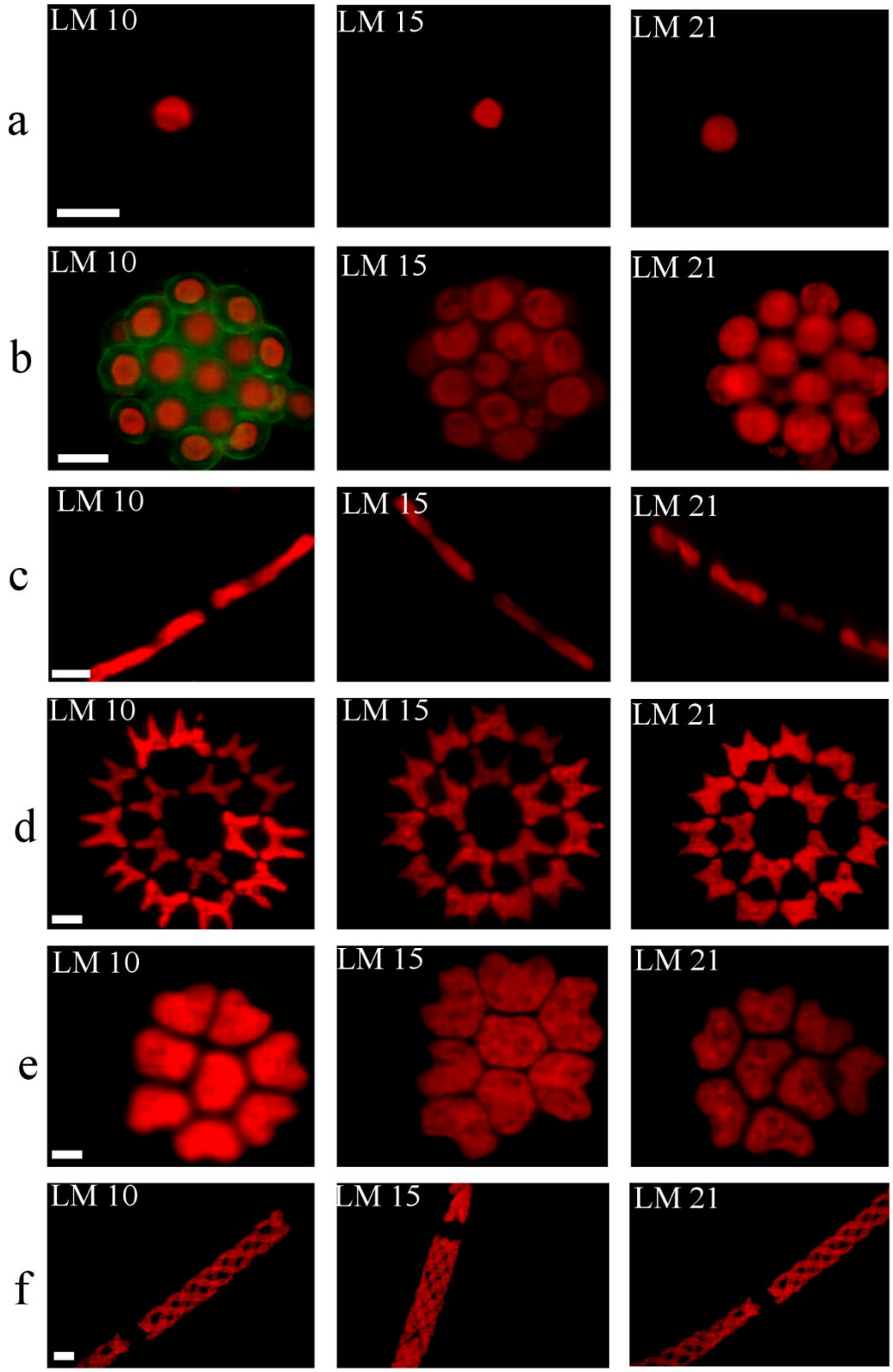

Fig. 5- Confocal images of immunocytochemistry for hemicellulose epitopes labeled by LM 10, LM 15 and LM 21 in living microalgae cell walls in phase II of growth. a) *C. vulgaris*; b) *C. microporum*; c) *Mougeotia* sp. d) *P. duplex*; e) *P. boryanum*; f) *Spirogyra* sp. Positive immunolabeling evidenced by green fluorescence. In red, chlorophyll autofluorescence. Scale bar: 10µm.

The synthesis of all results is presented in table 2, which allows the comparison of the species according to their interactions with calcofluor white and the monoclonal antibodies.

Table 2- Summary of the interactions between the probes and the algal species. The symbols (+) and (-) represent positive (presence) and negative (absence) of labeling, respectively. CFW: calcofluor white, JIM 5, JIM 7, LM 5, LM 6, LM 19 and LM 20: MAbs for pectins; LM 10, LM 15 and LM 21: MAbs for hemicelluloses.

		Chlorella vulgaris		***Coelastrum microporum***		***Mougeotia* sp.**		***Pediastrum duplex***		***Pseudop. boryanum***		***Spirogyra* sp.**	
		Ph. I	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II	Ph. I	Ph. II
Cellulose	**CFW**	+	+	+	+	+	+	+	+	+	+	+	+
Pectins	**JIM 5**	+	-	+	-	-	-	-	-	+	-	-	-
	JIM 7	+	-	+	-	-	-	-	-	-	-	-	-
	LM 19	-	-	-	-	-	-	-	-	+	+	-	-
	LM 20	-	-	-	+	-	-	-	-	-	+	-	-
	LM 5	-	-	-	+	-	-	-	-	-	-	-	-
	LM 6	-	-	-	+	-	-	-	-	-	-	-	-
Hemicelluloses	**LM 10**	-	-	+	+	-	-	-	-	-	-	-	-
	LM 15	-	-	-	+	-	-	-	-	-	-	-	-
	LM 21	-	-	-	-	-	-	+	-	-	-	-	-

4- Discussion

Our results partially highlighted the differences on cell wall composition among the six studied species of algae. The knowledge on these differences about the structure and composition of their cell wall can help clarifying some aspects of the organism structure, physiology and adaptations. In some algae, for example, cell wall composition confers vulnerability or resistance to interactions with the environment and other organisms (Dunker & Wihelm 2018). Additionally, as many algal compounds of industrial interest are located in protoplast, a better knowledge of cell wall composition may lead to the development of methods for a more accurate cell wall rupture and extraction of compounds (Li *et al.,* 2013). Cellulose is one of the main molecules of algal cell walls and other polysaccharides, such as chitin and carrageenan, may also be present (Domozych, 2011). The proportion of cell wall components and the presence or absence of certain molecules are species-specific characteristics (Baldan *et al.,* 2001). In the present work, we used innovative tools commonly applied in plant research for the detection of cell wall polysaccharides in algae. Calcofluor white is a blue fluorescent dye commonly used to detect cellulose in plant cell walls (Flores-Félix *et al.,* 2015) or chitin in fungi walls, but it is rarely used for studies on algae. As expected, the cell walls of all species were stained with CFW in both growth stages, corroborating the phylogenetic relationship of green algae and plants, as far as cellulose as cell wall component is concerned (Baudelet *et al.,* 2017; Domozych *et al.,* 2010; Park *et al.,* 2015; Estevez *et al.,* 2008). The positive staining of the cell walls of *C. vulgaris* by CFW corroborates that cellulose is abundant in cell walls of green algae (see Yamada & Sakagushi 1982; He *et al.,* 2016), but contradicts the absence of cellulose in *Chlorella*, previously proposed by Kapaum *et al.,* (1992).

Pectins correspond to approximately 35% of the dry weight of plant cell walls (Oliveira *et al.,* 2014; Voragen *et al.,* 2009; Jones *et al.,* 1997), and are the major components of the mucilage of some algae (Eder & Lutz-Meindl 2008). In our study, the detection of pectins in algal cell walls by monoclonal antibodies (MAbs) revealed some peculiarities. MAbs labeled pectins in *Chlorella vulgaris, Coelastrum microporum* and *Pseudopediastrum boryanum.* The epitopes of the most common pectins, homogalacturonans (HGAs), in the cell walls of the last two species were labeled by JIM5 and JIM7 in both growth phases (I and II), but the labeling of pectins in *C. vulgaris* only in phase I suggested that cell wall composition may change during algal cell development. HGAs represent 60% of the total pectins in the cell walls of land plants (Voragen *et al.,* 2009), and are involved in the control of cell expansion and adhesion (Formiga *et al.,* 2013), but little is known about their presence and function in microalgae.

In previous works, JIM5 and JIM7 detected HGAs with different degrees of esterification in green algal species, such as *Netrium digitus* (Eder & Lutz-Meindl 2010) and *Oedogonium bharuchae* (Estevez *et al.,* 2008), and in our study, in *C. vulgaris, P. boryanum, and C. microporum*. However, this last species was the only positive labeling by LM20, that corroborates the high methyl-esterified nature of the epitopes in its cell walls, suggesting that the synthesis of pectins in *C. microporum* occurs throughout its development. This peculiarity seems to approximate the cell walls of *C. microporum* to the higher plants.

The lateral chains of RGI, (1→4) β-D-galactan, and (1→5) α-L-arabinans were positively labeled by LM5 and LM6, respectively, only in cultures of *C. microporum* in phase II, which also indicated some temporal changes in cell wall composition, and a possible involvement in flexibility and porosity, as already proposed for plant cell walls (McCartney *et al.,* 2000; Verhertbruggen *et al.,* 2013; Zynkwinska *et al.,* 2005).

The cell walls of *Mougeotia* sp., *Spyrogira* sp., and *P. duplex* were not labeled, suggesting the absence of the epitopes of the pectins accessed by the MAbsAs *Spirogyra* sp. and *Mougeotia* sp. belong to the Zygnematophyceae class, the taxonomic group of algae more closely related to plants (Turmel *et al.,* 2006; Delwiche & Cooper 2015), some similarity in the chemical composition of cell walls were expected. Other species of charophytes, such as *Micrasterias denticulata, Penium margaritaceum* and *Chara corallina* have already been described to be positively labeled by JIM 5 and JIM7 (Lutz-Meindl & Brosch-Salomon 2000; Domozych *et al.,* 2007; Domozych *et al.,* 2010; Domozych *et al.,* 2011). It is possible that our *Spirogyra* sp. and *Mougeotia* sp. strains have pectins with a different chemical conformation, which could not be recognized by the antibodies used in our study.

The cell walls of *Pediastrum duplex* were not labeled by any of the MAbs for the epitopes of pectins, but it was the only algae species in which the epitopes of the β-(1→4)-manno-oligosaccharides were labeled by LM21. These hemicelluloses, such as the mannans labeled by LM21, are non-cellulosic polysaccharides involved with cellulose and pectins in the structure of plant cell walls (Kinnaert *et al.,* 2017), and are less studied among the microalgae. Also, the epitopes of xylans and xyloglucans were labeled only in *C. microporum,* and the labeling of the epitopes of (1-4)-β-D-xylosyl residues by LM10 in cell walls of *C. microporum* in both phases of growth (I and II), particularly suggest the presence of these hemicelluloses during the entire life cycle of this species.

Xyloglucans, the most common hemicelluloses found in plants (Marcus *et al.,* 2008), have been previously detected in the charophyte *Micrasterias denticulata* (Eder *et al.,* 2008) and, in our study, were labeled by LM15 only in *C. microporum* (Chlorophyceae). Hemicelluloses were not detected in the charophytes *Mougeotia* and *Spirogyra,* contradicting the expectations. We believe that the use of monoclonal

antibodies, instead of the polyclonal antibodies used by Eder *et al.,* (2008), may have restricted the detection of hemicelluloses. In fact, polyclonal antibodies can recognize multiple epitopes on any type of antigens, while MAbs show high specificity of epitope recognition. The mannans, considered the main hemicellulose in charophytes (Popper & Fry 2003), and commonly found in green algae (Popper & Tuohy 2010), have not been detected either in *Spirogyra* sp. and *Mougeotia* sp. in our study. LM21 could label mannan epitopes in *P. duplex*, (Chlorophyceae) in phase I, but not in phase II, which indicates that this polysaccharide does not seem to be of overall occurrence along the alga life cycle.

The present study described the variety of algal cell wall components among the six species studied, employing MAbs. The addition of new evidences on cell wall composition is important because cell surface interactions can determine culture growth and development (Gonçalves *et al.,* 2019). Challenge posed by the great diversity of algae relies on the lack of tools specifically applicable to study their cell walls. As a matter of fact, we took for granted the similarities on the composition of algae and plant cell walls, due to their evolutionary relationships, and we were able to detect few components in common between their cell walls. Indeed, the labeling of cellulose by CFW in the cell walls of the six microalgae was shown to be similar among them. Regarding pectins and hemicelluloses however, a mosaic of results was detected by the MAbs and their related epitopes. Pectins and hemicelluloses varied among species and growth phases and indicated *Coelastrum microporum* as the algal species most similar to plants in terms of polysaccharide composition and pectins. *Spirogyra* sp. and *Mougeotia* sp., despite representing the charophytes, i.e. the group evolutionarily closer to the plants, were not labeled for the majority of the carbohydrates tested, suggesting that despite the evolutionary proximity, these organisms may be different in terms of a more refined analysis of the carbohydrate composition of their cell walls. We concluded that among the

six algal species, *C. microporum* was the most similar to the plants in terms of cell wall composition, thus it could represent an interesting model for future researches focused on cell wall compounds common to plants and algae. We also believe that the development of specific epitopes for cell wall carbohydrates of algae, different from those produced for plants, could generate more reliable results. Additionally, regarding our comparative approach between algae and plants, we observed that the cell wall composition of algae is variable on a temporal scale, which can be related to functional aspects of the colonies.

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Capítulo 3: Composition of glycidic receptors in cell surface of green microalgae changes with cell aging

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Abstract

Lectin-carbohydrate interaction mediates important physiological processes when cell recognition is required. This interaction is very specific and lectins have been widely used as probes to detect carbohydrates in cell surfaces. Here, in the opposite way, we used three different glycodendrons to detect lectins on the cell surface of four microalgae species (*Chlorella vulgaris, Pseudopediastrum boryanum, Mougeotia* sp. and *Spirogyra* sp.). The glycodendrons named GD-Fuc, GD-Man and GD-GalNac have three residuals of L-fucose, D-mannose and *N*-acetylgalactosamine, respectively. It was also our goal to identify if there are variations in lectins recognition at different times of cultivation (3, 10, 19 and 26 days of growth) of the microalgae. *C. vulgaris* did not interact with GD-Fuc at any time, but showed interaction for GD-Man with 26 days of cultivation and for GD-GalNac with 3 days. *Spirogyra* sp. interacted with all the glycodendrons but with differences according to the cultivation times tested. *P. boryanum*, seems to have shown greater stability in the recognition of glycodendrons, since it recognized all of them in practically all the cultivation times tested. *Mougeotia* sp., on the other hand, showed no interaction with any of the glycodendrons at any time. These differences in expression suggest these organisms could have great versatility in recognition at different times in their life cycles. Knowledge about the composition of algal cell surface biomolecules, especially considering growth times and life cycles is still limited and this study solve some questions and expand the knowledge for exploration based in new tools.

Key words: cell-cell interaction, chlorophyte, lectins, glycodendrons.

1- Introduction

Cell-cell interactions are ubiquitous in nature (Ohan et al. 2019) and most of them are only possible due to the precise recognition involving the cells' surfaces. These interactions are involved and necessary for many physiological processes such as reproduction and infection (Shi et al. 2017), as well as ecological relationships such as parasitism (Basseri 2002), symbiosis (Wood-Charlson et al. 2006) and mutualistic associations (Díaz et al. 2016). For algae, especially the microscopic ones, there are other processes that require cellular recognition, such as colony formation (Ingram 1985) and adhesion to substrates (Palacio-López et al. 2019). The mechanisms involving cell-cell and cell-matrix interactions seem to be mediated by specific recognition among carbohydrates and lectins (Melgarejo et al. 2005).

Lectins are proteins of non-immune origin widely distributed in nature that bind to carbohydrates and sugar containing substances in a high specific and reversible way (Hamid et al. 2013; Santos et al. 2014). The binding between lectins from a cell surface and carbohydrates from other cell surface results in an agglutinated cell mass. This ability of lectins to agglutinate cells distinguishes them from other proteins that bind to carbohydrates, like sensor/transport proteins and antibodies (Santos et al. 2014); Rüdiger and Gabius 2011). Another particularity of lectins is their capacity to binding glycans without altering their covalent structure (Bondar et al. 2016). Due to all these biochemical and biological properties, lectins attract attention and, in general, are very used in researches that investigates characterization and changes in saccharides of cells surfaces (Praseptiangga 2015).

Lectins and carbohydrates can be used as tools to identify their complementary receptor in studies of elucidation of cell surface components (Gonçalves et al. 2019). In algae, lectins are already extensively used as probes for the identification of glycans on cell surfaces

(Hori et al. 1996, Aguilera and González-Gil 2001, Tien et al. 2005, Kim et al. 2007). However, the use of carbohydrates as probes to detect and identify cell surface lectins is still uncommon. In a recent work, Gonçalves et al. (2019) demonstrated that fluorescent dendritic molecules containing carbohydrates residuals (glycodendrons) could be used as probes to identify potential receptors (lectins) on the surface of microalgae. Glycodendrons were pointed as promising tools for studies on interactions involving cell surfaces, being advantageous due to their size (nanometer scale) and mainly because they can present several copies of the carbohydrate units on the periphery of these structures, which allows them to mimic membrane oligosaccharides. In addition, since glycodendrimers are monodisperse polymers have a defined structure, which allows a more accurate assessment of the interaction.

Surface carbohydrates form a set of information that can be considered the cell's identity and they allow the interaction with other cells and with the environment. This process is commonly mediated by proteins, like lectins. It interesting to note that this cell's identity can be not totally stable. There are few studies showing that the glycidic composition of cell surfaces is dynamic and can vary depending of the cell's development stage (Zheng et al. 2005). In case of lectins, we don't know any study in literature related to the variation of their composition on cell surface. However, at least a study (Wang et al. 2004) mentions that the Chlorophyta *Haematococcus* shows a highly dynamic cell surface in terms of proteic composition. Thus, we can suppose that the composition of lectins maybe is also dynamic, occurring a coordinated expression according to the needs of the cells during their life cycles.

The aim of this work was to verify the presence of lectins on cell surfaces of four species of green microalgae using three different glycodendrons as probes. In addition, we also

propose to identify if the expression of these lectins in cells varied according to the ages of the cultures.

2- Material and Methods

We used three different glycodendrons (GD-Man, GD-Fuc and GD-GalNAc) as probe to detect differences among cell surfaces to distinguish species and the cell aging. These glycodendrons are non-charged molecules that are formed by a dendritic structure containing a glycidic portion composed by residuals of D-mannose (in GD-Man), L-fucose (in GD-Fuc) or *N*-acetylgalactosamine (in GD-GalNAc). It was also synthesized an analogue control dendron containing diethylene glycol residuals instead the carbohydrate units. Diethylene glycol provides a soluble control molecule very similar to the other glycodendrons in terms of shape, no charge, size and solubility. All molecules used in this study were synthesized in analytical purity, according to a methodology previously described by Ribeiro-Viana (2012) .

The experiments were performed with four species belonging to three taxonomic classes: Chlorophyceae (105-*Pseudopediastrum boryanum*), Trebouxiophyceae (56-*Chlorella vulgaris)* and Zygnematophyceae (35-*Mougeotia* sp. and 7-*Spirogyra* sp.). The species were obtained from the Collection of Algae and Cyanobacteria of the Phycology Laboratory of the Federal University of Minas Gerais, Brazil. They were cultivated in CHU 10 medium at 20(±1) °C and 22 (±0,1) μmol photons m^{-2} s^{-1}, under a light: dark cycle of 12:12 h. The experiments were performed at four growing stages of the cultures: 3, 10, 19 and 26 days growing after last inoculation. All experiments and analyses were performed in triplicate, using living cells and no fixation process was applied. The species were exposed to glycodendrons and subsequently evaluated using confocal microscopy, according described by Gonçalves et al. (2019). Briefly, the cells exposed to glycodendrons were analyzed in a confocal microscope (Zeiss 5-Live). The fluorophores

on the slides were excited by a laser at 532 nm and the fluorescence was captured by a filter receiving waves from 550 to 615 nm. All the results presented here are representative of more than 90% of the individuals observed on the slides.

3- Results

Chlorella vulgaris was labeled by the glycodendrons GD-Man and GD-GalNac, but only for the cultures growing for 26 and 3 days of growth respectively (Figure 1). *Mougeotia* sp. showed no interaction with any of the glycodendrons tested in any of the growth times experienced (data not shown). All glycodendrons interacted with *Pseudopediastrum boryanum* growing during any period, except by the culture growing for 3 days with GD-GalNac, for which no fluorescence was detected by confocal microscopy (Figure 2). *Spirogyra* sp. was generally labeled after longer periods of culturing. This species was labeled by GD-Fuc after growing during 19 and 26 days, GD-Man for only 26 days of growth and by GD-GalNac with 3 and 26 days of growth (Figure 3). Unfortunately, it was not possible to verify the result of the interaction with GD-GalNac at the 19th day due to an artifact in the slide. A synthesis of all these results is presented in Table 1.

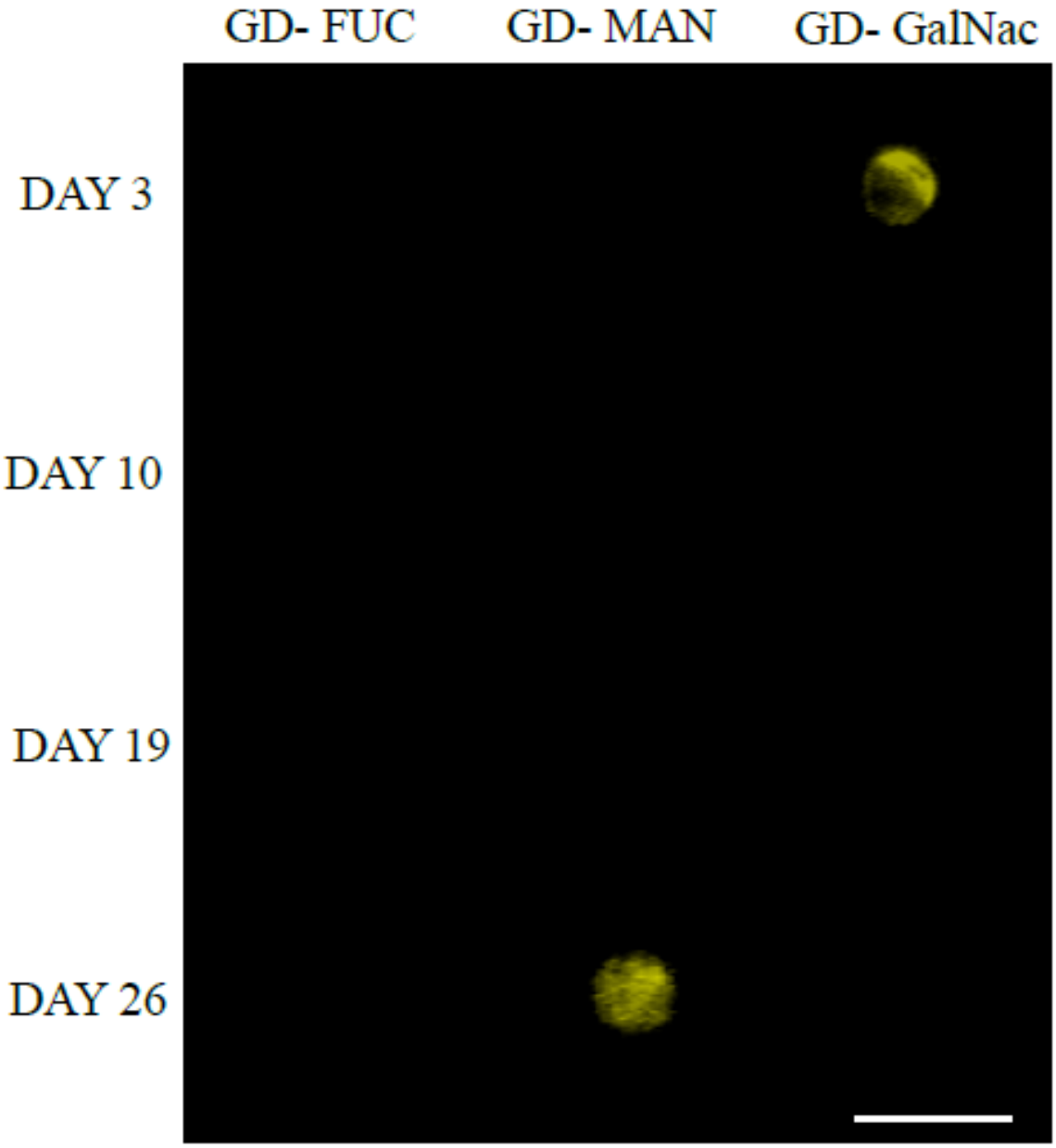

Fig. 1- Confocal images of *C. vulgaris* and the glycodendrons at the four stages of growth. Yellow: Fluorescence of the glycodendron. Scale bar: 10uM.

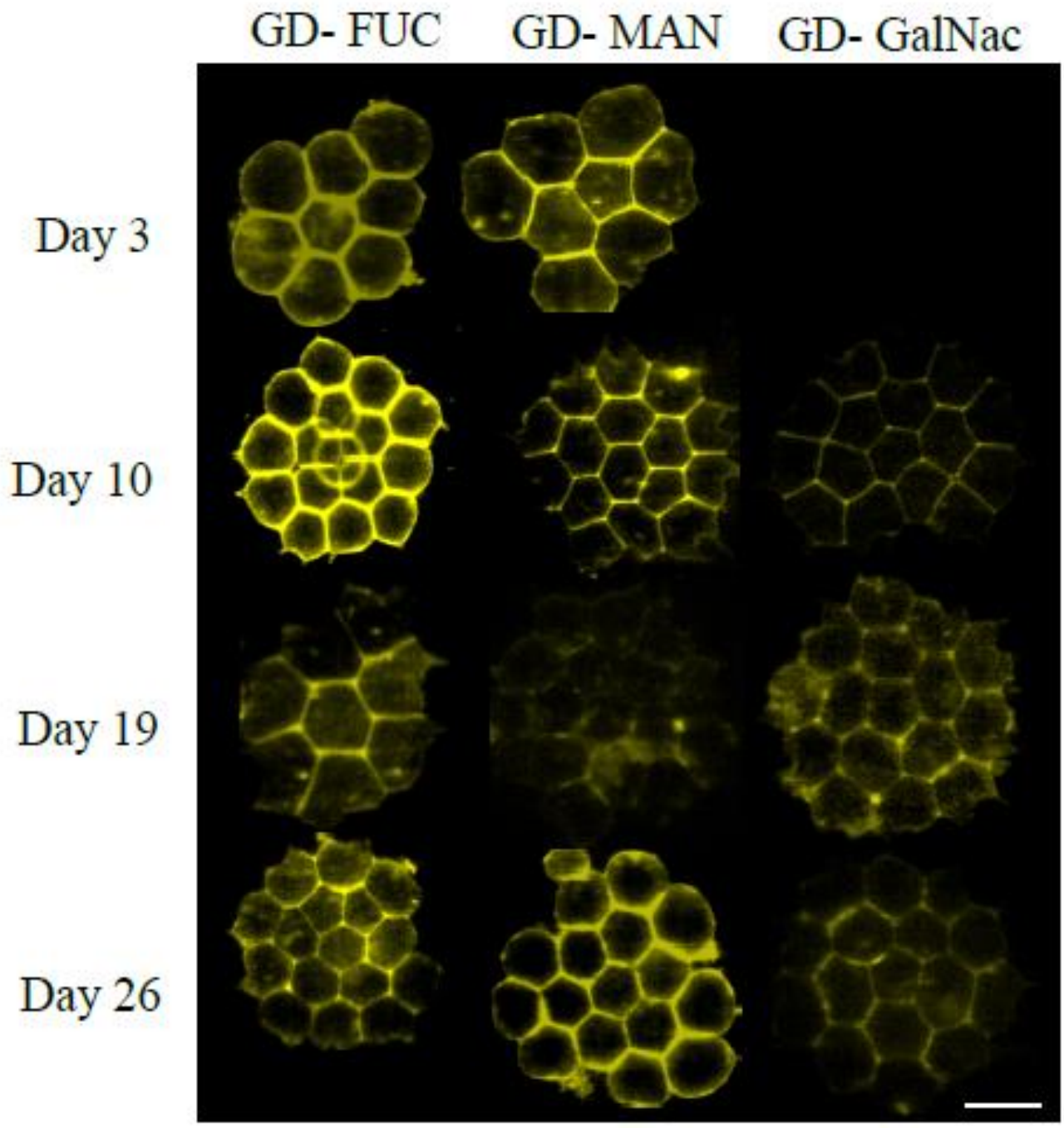

Fig. 2- Confocal images of *P. boryanum* and the glycodendrons at the four stages of growth. Yellow: Fluorescence of the glycodendron. Scale bar: 10uM.

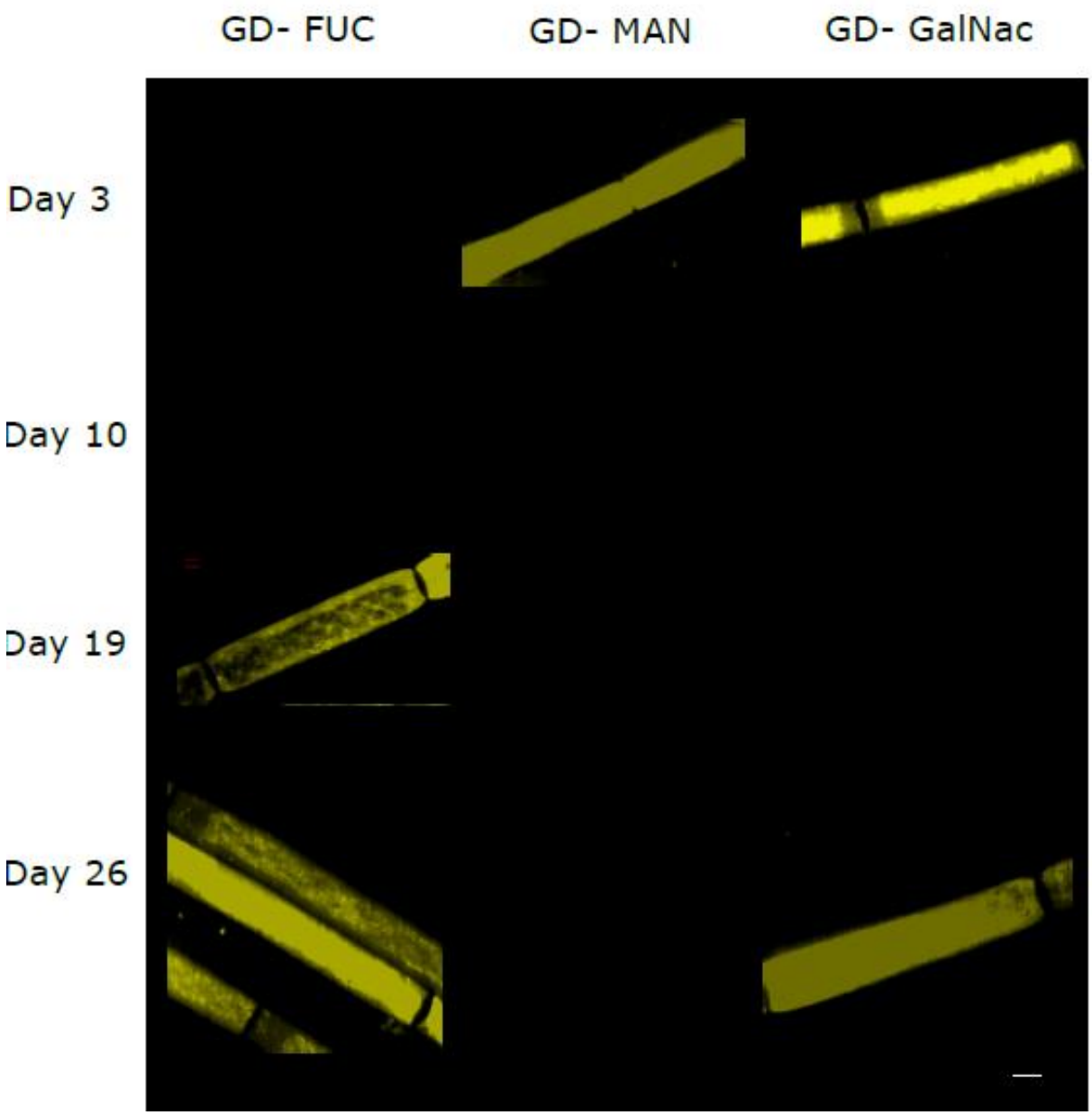

Fig. 3- Confocal images of *Spirogyra* sp. and the glycodendrons at the four stages of growth. Yellow: Fluorescence of the glycodendron. Scale bar: 10uM.

Quadro 1: Synthesis of the interactions between glycodendrimers and the algal species. (+) positive interaction, (-) no interaction. GalNHAc: N-acetylgalactosamine, Man: D-mannose, Fuc: L-fucose, GD glycodendron.

	GD-FUC				**GD-MAN**				**GD-GalNac**			
	Days of growth:											
	3	**10**	**19**	**26**	**3**	**10**	**19**	**26**	**3**	**10**	**19**	**26**
C. vulgaris	-	-	-	-	-	-	-	+	+	-	-	-
Mougeotia sp.	-	-	-	-	-	-	-	-	-	-	-	-
P. boryanum	+	+	+	+	+	+	+	+	-	+	+	+
Spirogyra sp.	-	-	+	+	-	-	-	+	+	-		+

4-Discussion

The use of carbohydrates (glycodendrons) as probes to detect lectins is a recent tool in the study of algal surface. Here we evaluate if there are temporal changes in the recognition of these probes on the cell surfaces of four green microalgae species, according to different stages of culture growth. Our results suggest that the composition of the glycidic receptors is species-specific and can vary in the same species along its growth. Since the interactions between lectins and carbohydrates are very specific and mediate important processes involving cell recognition (Figueirôa et al. 2017), the variation recorded here may determine temporal variations in the ability of the cell recognizing other cells or biological substrates.

Three among the four species studied here (exception was *Spirogyra* sp.) were also tested with the same glycodendrons in a recent work (see Gonçalves et al. 2019), but for only one growth phase. The results presented here reinforced the previous study, but add new

information by showing that all species can express different surface composition over time.

A new interaction was detected for *Chlorella vulgaris*. In the previous study this species did not interact with GD-Man (Gonçalves et al. 2019), but here it showed a positive interaction with this dendron. Interestingly, this interaction was observed for only a later growth stage in relation to the stages evaluated before (see Gonçalves et al. 2019). In contrast, the well evidenced labeling by GD-GalNac was not detected here. Despite the culture being the same, the medium was differed between the two studies, which may have some influence in the metabolism of this species and, consequently, the composition of their cell surface. Differences in composition and concentration of nutrients in the culture medium can affect the composition of cell surfaces molecules, like observed by Martel (2009). This author found more mannose glycoconjugates in cell surface of *Isochrysis galbana* cultured in a medium with limiting nitrogen concentrations. Kremp and Anderson (2004) also showed that dinoflagellates seems to show different cell surface carbohydrates composition and concentration depending on the cell nitrogen status.

Compared with the other species, *P. boryanum* showed a higher temporal stability, being labelled by all glycodendrons at all times tested, except by GD-GalNac in the third day of growth. For *P. boryanum*, more complex composition of lectins throughout all the cell life cycle could give it a capacity to recognize more diverse surfaces that the other algae may not do. Differently, for *Spirogyra* sp. the lectins for L-fucose and D-mannose became important only in the last stages of cell culture (19 and 26 days) and were not detected at the beginning of this experiment. The interaction with *N*-acetylgalactosamine occurred for only the shortest (day 3) and the longest (day 26) time of culturing *Spirogyra* sp. We expected to find evidences of the presence of this lectin in at least one of the four growth times tested, as a galactose-recognizing lectin has already been isolated from other

Spyrogira (Oliveira et al. 2017). The lectins present on the cell surfaces of chlorophyte members have complex specificity with carbohydrates (Singh et al. 2018) which increases the chances that the glycidic receptors detected by the glycodendrons in *Spirogyra* sp. are even lectins, since the high specificity of recognition is a major property of these proteins.

The "glycome" comprises the complete set of glycans and glycoconjugates produced by cells in reflection of specific conditions due to their development stage or in response to environment conditions (Varki et al. 2009). In dinoflagellates, for example, the cell surface glycoconjugates composition is altered throughout the cell cycle (Aguilera and González-Gil 2001) and can change depending on their physiological state (Kremp and Anderson 2004). The lectins, which are proteins that act as carbohydrate receptors, must also have their composition altered in some way, since the expression of proteins is generally a dynamic process and reflects a given cellular moment (Aslam et al. 2017).

Mougeotia sp. did not interact with any of the three glycodendrons tested in any of the four growth ages studied. This was also observed in our previous study (Gonçalves et al. 2019) and hence *Mougeotia* can be used as a negative control among the algae due its known inactivity for the tested dendrons. However, we avoid to affirm that this species does not have at all the lectins that could detect the carbohydrate tested here. If low expression was the typical condition here, it could be the cause of no visible fluorescence in confocal microscopy. But, we have to consider that the interaction between the lectins and dendrons containing carbohydrates residuals may be stronger and more stable when the number of copies of the interacting molecules is increased, which is known as the multivalent effect (Yilmaz and Becer 2015). The glycodendrons used here has a trimeric structure, with three residuals of each carbohydrate by molecule, so tests with glycodendrons with more copies also be tested in order to confirm the lack of these lectins as well as to perform genomics and proteomics analyses to the cells.

The genomic analyses will provide information about all coding genes and proteins existing in the cell or organism, as well as understanding of their functions (Fields et al. 1999), while the proteomics will allow us to describe all the proteins expressed in the cell, their products, modifications, interactions and mapping of signaling pathways (Balbuena et al. 2011). These techniques, however, are relatively expensive and algal genomes and proteomes poorly represented in the databases (Vadlapudi and Kaladhar 2012), which limit this kind of research.

There is a variety of lectins extracted from algae (Praseptiangga 2015), but little is known for many taxonomic groups and about all the roles of these proteins in the cell. Some studies suggest that they are involved in the interaction with other organisms, such as recognition of symbionts (Wood-Charlson et al. 2006), in predation relationships (Espinosa et al. 2010) and they could also be involved in the colony formation (Ingram 1985). Although it is known that the cell surface residuals of L-fucose, D-mannose and *N*-acetylgalactosamine are involved in cell recognition in others organisms (Ofek and Beachey 1978, Boettner et al. 2002, Spear et al. 2003), we known almost nothing about their function in algae. We could suppose they could have similar functions, but more studies are necessary to confirm that. In fact, L-fucose, D-mannose and *N*-acetylgalactosamine have already been described in cell surfaces of some species of other algal phyla, such as Dinophyta (Kremp and Anderson 2004), and some macroalgae species of Rhodophyta and Phaeophyta. In the cell surface of the brown macroalgae *Padina gymnospora*, L-fucose was detected by using a specific fluorescence-conjugated lectin (Salgado et al. 2005) and interactions between L-fucose and D-mannose and their respective lectins appear to mediate the gametes recognition in *Antithamnion nipponicum* (Rhodophyta) (Kim et al. 1996). Nevertheless, these so limited and restrict when

considering the great diversity of algal taxa and the higher possibilities of relationships among them, with other organisms and with other surfaces in the environment.

In conclusion, studies on the elucidation of components of cell surfaces provide important information for understanding the morphology and physiology of organisms and how they interact with each other and with the environment. However, little information is known about algae, probably due to the scarcity of studies that show the composition of biomolecules on the cell surface considering life cycles and possible temporal growth changes. We showed in this work, that the composition of the glycidic receptors (the lectins) of the four microalgae species tested appears to be species-specific and vary according to culture aging. The activity of lectins (ability to recognize and or agglutinate cells) can be altered due to physiological and abiotic factors (Babosha 2008), including growth phases (Singh et al. 2018). Thus, differences in lectin's activity may perhaps explain our results, but more refined studies, like proteome's analyses are required to confirm if the glycidic receptors are actually lectins and if their expression really changes throughout their life cycles.

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Capítulo 4: Binding proteins of D-mannose, L-fucose and *N*-acetylgalactosamine on *Coelastrum microporum* cell surface: Are they lectins? Detection with glycodendrons and genomic analysis

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Abstract

Cell surfaces have molecules that mediate important processes that involve cell recognition. Among these molecules are the lectins, proteins of non-immune origin that recognize carbohydrates in a highly specific way. In this work, we detected glycidic receptors of three carbohydrates (L-fucose, D-mannose and *N*-acetylgalactosamine) on the cell surface of *Coelastrum microporum*, a green alga from the Scenedesmaceae family, using carbohydrates labeling as probes, the glycodendrons. To verify if the presence of these glicydic receptors varies with cell age, we tested the interaction of glycodendrons with cultures at four different time ages: 3, 10, 19 and 26 days of growth. To refine our results, we carried out *C. microporum* genome sequencing and assembly, and searched for putative lectin-coding genes. *C. microporum* showed a positive interaction with the three carbohydrates tested in all cell ages tested, except for the first: 3 days of growth. These results suggested that *C. microporum* had all proteins receptors (the lectins) for the three tested carbohydrates and that these proteins appear to have their presentation aligned with the age of cultivation. The *C. microporum* genome showed a large number of protein-coding genes (more than 77,000) and among them we detected a gene for a lectin and more 137 genes for proteins that are related to carbohydrate binding, which have great chances of being lectins. Further studies, however, are needed to confirm the biological function of these proteins.

Key words: *C. microporum* genome, protein annotation, lectins, green algae, cell-cell interaction.

1- Introduction

Cell surfaces play important roles in organism physiology and have a wealth of information that determines how cells interact with their environment (Mahal and Bertozzi 1997). The molecules present in cell surfaces are the first interface of contact with the environment and other organisms. Thus, these molecules are protagonists in cell recognition events (Sung et al. 1985). Reproduction, infection by pathogens, adhesion to substrates, mutualistic interactions, among others processes require a precise cell recognition (Basseri 2002, Wood-Charlson et al. 2006, Díaz et al. 2016, Shi et al. 2017). Carbohydrates and proteins, especially lectins, are the main molecules on cell surfaces that mediate cell interactions (Melgarejo et al. 2005).

Lectins are proteins of non-immune origin that bind to carbohydrates and sugar containing substances in a high specific and reversible way (Hamid et al. 2013, Santos et al. 2014). Lectins bind to glycans without altering their covalent structure (Bondar et al. 2016). One of the consequences of this recognition is cell agglutination, which distinguishes lectins from other proteins that bind to carbohydrates, like sensor/transport proteins and antibodies (Rüdiger and Gabius 2011, Santos et al. 2014). Lectin-carbohydrate recognition represents a ligand-receptor interaction that is universal in living organisms (Bulgakov et al. 2004). There are reports of this interaction for many organisms, which include humans (Figueirôa et al. 2017), animals (Roberson and Armstrong 1980), fungi (Nayyar et al. 2017), bacteria (Flannery et al. 2015), plants (Bellande et al. 2017) and also algae (Ingram 1985).

Algae has been increasingly targeted as a sustainable source of alternative raw materials for the production of biofuels and compounds with high commercial value, which are used by the pharmaceutical, cosmetics and nutrition industry (Du et al. 2016). More specifically, there is a recent increasing interest in algal lectins due to their potential

for biomedical applications such as anti-HIV, antitumoral, antimicrobial, anti-inflammatory and antinociceptive activities (Singh et al. 2018).

With hundreds of species, Scenedesmaceae is one of the biggest families of Chlorophyceae. It is frequently dominant in freshwater lakes and rivers (Ishaq et al. 2016) and this abundance and species diversity make this family very interesting to be studied as a source of substances with commercial value. Some of the Scenedesmaceae, for example, are known to produce a huge variety of bioactive products with high added value, including their potential as a source of lectins (Silva et al. 2019).

Although there is an increasing search for new substances and more productive species and strains, there are relatively few studies focused on the algal cell surfaces, and this is proportionally more important when we consider their high taxonomic diversity. Some studies are focused on elucidating the physical-chemical properties of the algal cell coverings (Procházková et al. 2012, Ozkan and Berberoglu 2013, Xia et al. 2016, Hao et al. 2017), which influence their ability to adhere to substrates and to form biofilms. Other studies are dedicated to identifying what are the biomolecules present in the algal surfaces (Hori et al. 1996, Aguilera and González-Gil 2001, Tien et al. 2005, Kim et al. 2007). Both types of studies help us to understand how cells interact with other cells and with the environment, as well as a more detailed morphological knowledge of the organism.

In this work, based on the high specificity of interaction between carbohydrates and lectins, we used three different carbohydrates marked as probes (glycodendrons) to detect the presence of their respective receptor (the lectins) on cell surfaces of the Scenedesmaceae *Coelastrum microporum*. The use of carbohydrates as probes to detect lectins is still rare in the literature, especially regarding algae. In a recent study, Gonçalves and collaborators (2019) showed that glycodendrons are a promising and effective tool for this kind of research. In addition to their nanomeric size, glycodendrons are advantageous

because they can present several copies of the carbohydrate units on the periphery of these structures, which allows them to mimic membrane oligosaccharides.

Considering that the composition of surface molecules may not be static (Wang et al. 2004, Zheng et al. 2005) and vary according to the physiological and environmental conditions that the organism is in, we were also interested in to investigate if the glycidic receptors of *C. microporum* vary according to the culture age, and for this, we carry out experiments with cultures in four different ages. We also sequenced and analyzed the *Coelastrum microporum* genome to search for possible lectins and refine our results.

2- Material and methods

2.1- Culture

The *Coelastrum microporum* culture was obtained from the Collection of Algae and Cyanobacteria of the Phycology Laboratory of the Federal University of Minas Gerais, Brazil. This culture is maintained in CHU 10 medium at 20(±1) °C and 22(±0,1) μmol photons m^{-2} s^{-1}, under a light: dark cycle of 12:12 h. The experiments were performed at four growing ages: 3, 10, 19 and 26 days of growth. All experiments and analyses were performed in triplicate, using living cells, and with no fixation process.

2.2- Synthesis of the glycodendrons

Glycodendrons are non-charged molecules formed by a dendritic structure with the outermost glycidic portion composed by carbohydrates. Here, we use three glycodendrons named GD-Man, GD-Fuc and GD-GalNAc that have in their ends a glycidic portion composed of L-fucose, D-mannose and *N*-acetylgalactosamine, respectively. As a control, an analogue control dendron was synthesized, in which we use diethylene glycol instead of a carbohydrate unit. Diethylene glycol provides a soluble control molecule very similar in terms of shape, no charge, size and solubility to that of glycodendrons. These molecules

were synthesized in analytical purity, according to a methodology previously described by Ribeiro-Viana (2012).

2.3- Exposition of the species to the glycodendrons

The species was exposed to glycodendrons and subsequently evaluated using confocal microscopy, according described by Gonçalves et al. (2019). Briefly, glycodendrons and the control were dissolved to achieve a final concentration of 32 µmol/mL in the aqueous solutions. The culture samples were centrifuged by 5 minutes under 13,792.8 g (13,000 rpm) in a microcentrifuge (Eppendorf 5424) and supernatants were discarded. Pellets were resuspended in 25 µL of the aqueous solution containing the dendrimer to be tested. The microtubes were then kept by 1 hour at 24°C in a dark chamber and finally put on slides and evaluated under confocal confocal microscope (Zeiss 5-Live) of the Image Acquisition and Processing Center (CAPI-UFMG). The fluorophores on the slides were excited by a laser at 532 nm and the fluorescence was captured by a filter receiving waves from 550 to 615 nm. All the results presented here are representative of more than 90% of the individuals observed on the slides. We used the software LSM *IMAGE BROWSER* (Zeiss) for additional preparation of the images.

2.4- Cultivation and Genomic DNA Isolation

Coelastrum microporum was grown photoautotrophically to exponential phase in CHU 10 medium at 20 (±1) °C and 22 (±0,1) µmol photons m^{-2} s^{-1}, under a light: dark cycle of 12:12 h. Genomic DNA was extracted following the protocol adapted from Doyle and Doyle (1991). In resume, 200 mL of culture was filtered in fiberglass filter and this filter washed with 500uL mL of a 2% CTAB solution. We added liquid nitrogen into the flask containing samples and macerated cells to break their cell walls. Then, we extracted DNA with β-mercaptoethanol and chloroform solution: isoamyl-alcohol. We performed

several steps of DNA washing with ethanol and TE solution. Finally, we treated the DNA with RNAse and kept the DNA at -20 ^{o}C until use.

2.5- Genome Sequencing and Assembly

The *C. microporum* genome was sequenced using whole-genome shotgun approach. The TruSeq Nano DNA kit was used to prepare the fragments library (550 bp fragment length), and the 150 bp paired-end sequencing was carried out on Illumina NovaSeq platform. Quality control assessment and raw data filtering were performed using Fastqc (v. 0.11.5) (Andrews 2010) and Trimmomatic (v0.39) (Bolger et al. 2014), respectively. Reads with mean *Q* scores less than 15 considering a 4-mer sliding-window were eliminated. *De novo* genome assembly were performed with SPAdes (v3.9.0) using k-mer sizes ranging from 27 to 123.

2.6- Genome annotation

The prediction of protein-coding genes was performed with Augustus (v. 3.2.2) (Stanke and Morgenstern 2005) using the *Chlamydomonas reinhardtii* genes parameters. *Chlamydomonas reinhardtii* is one of the well-studied green algae in recent years (Kurotani et al. 2017). The protein functional prediction was carried out with PANNZER2 program (Törönen et al. 2018). This tool was one of the best (top 10) in the CAFA3 challenge and also had a friendly web-interface (Zhou et al. 2019). As a selection criterion to identify lectin-coding genes, we selected only proteins for wich the only or main function was "carbohydrate binding". Since PANNZER2 attributes a value for the robustness of results, we only selected proteins with scores above 0.4, which generally provide reliable results.

2.7- Comparison of chlorophyte genomes

We have collected data (genome size, GC content and number of predicted proteins) from some other Chlorophyta in the literature and compared them with the data obtained for *Coelastrum microporum* genome. Only nuclear and annotated genomes were considered, which resulted in only nine species to be compared. Many species have only their mitochondrial and plastid genomes sequenced, these were not selected.

3- Results

3.1- Detection of glycidic receptors

All results of interactions with glycodendrons are shown in Figure 1. *Coelastrum microporum* was not labeled by any glycodendrons at day 3 and was labeled by all glycodendrons (GD-Man, GD-Fuc, GD-GalNac) with 10 and 19 days of growth. At day 26, cultures were labeled by GD-Man and GD-Fuc, but not by GD-GalNac.

3.2- Assembly and functional annotation of *Coelastrum microporum* genome

The *C. microporum* strain studied here had a genome of 200 Mbp with 54% GC content (proportion of guanine and cytosine bases in the DNA molecule). Table 1 shows a comparison between the genome of *C. microporum* and other Chlorophyte. *C. microporum* has a much higher number of predicted proteins compared to the other species. The size of its genome was alsohigher compared with the other species, except by *Dunaliella salina*, which has a genome larger than 340Mb but almost half of the number of predicted proteins compared to *C. microporum. Scenedesmus obliquus (*currently called *Tetradesmus obliquus)*, which belongs to the same family as *C. microporum* also had a large genome with 172.4 Mb and a large number of predicted proteins, but much smaller than *C. microporum*.

Table 1- Comparison of the characteristics of the *C. microporum* genome with the genomes of other Chlorophyta.

Species	Genome size (Mb)	GC%	Nº of predict proteins	Reference
Coelastrum microporum	200	54	77149	This work.
Dunaliella salina	343.7	49.1	36851	Polle et al. 2000
Scenedesmus obliquus AS-6-11 (Currently, *Tetradesmus obliquus)*	172.3	52	31964	Chen et al. 2020
Ostreococcus tauri	12.6	59	8166	Derelle et al. 2006
Ostreococcus lucimarinus	13.2	58	7651	Palenik et al. 2007
Auxenochlorella protothecoides	22.9	63	7039	Gao et al. 2014
Volvox carteri	138	56	14520	Prochnik et al. 2010
Chlamydomonas reinhardtii	121	64	15143	Merchant et al. 2007
Chlorella vulgaris	40	61	10724	Cecchin et al. 2019

The prediction made by Augustus showed 77,149 protein-coding genes in *Coelastrum microporum* genome, and 137 of those were related to recognition of carbohydrates according to PANNZER2 and one more was detected as a lectinAs our study was focused on interaction via the cell surface, only proteins located in that cell region or those in which the region was not predicted were considered. These 137 proteins were located in cell membrane (59), extracellular region (15) or have their locations not identified (63).

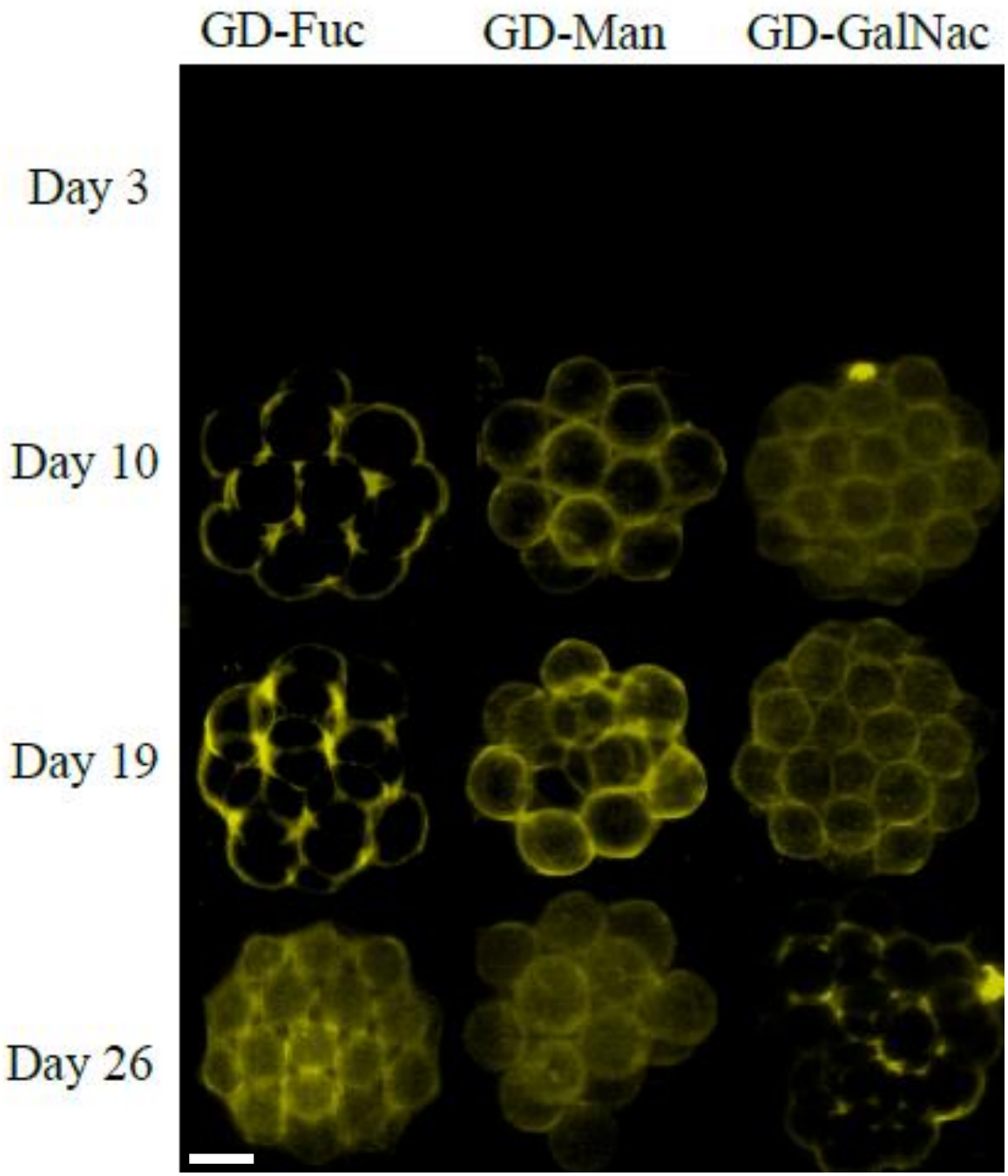

Figura 1- Confocal images of *C. microporum* and their interactions with the glycodendrons in 4 diferents growth ages. Yellow color: glycodendron fluorescence. Scale bar: 10uM.

4- Discussion

In this work, we produced more information that allowed to investigate if *Coelastrum microporum* glycidic receptors are probably lectins. The first reason to consider that, is the highly specificity of lectin-carbohydrate interaction. Although the results presented here corroborate those obtained in a previous study (Gonçalves et al. 2019), with *C. microporum* interacting with the same glycodendrons, the interactions were only detectable after 10 days of culture growth, with no labeling at day 3. These results suggested that the cell surface molecules capable to recognize carbohydrates may be differently synthesized according to the *C. microporum* culture aging. In fact, organisms surface biomolecules composition seems to be dynamics, showing variations that are determined by physiological factors (Zheng et al. 2005).

Some microalgae show a carbohydrate composition in their cell surface that can vary according to its physiological status (Kremp and Anderson 2004) and is also dependent on the availability of nitrogen (Martel 2009). For benthic diatoms, the composition of extracellular polymeric substances (EPSs), which is composed of carbohydrates, lipids and proteins, also can vary according to cell's physiological state and abiotic conditions such as light availability (Smith and Underwood 2000, De Brouwer and Stal 2002). In relation to lectins, this type of information is still very rare for microalgae. However, if we consider proteins in general, the chlorophyte *Haematococcus* has a highly dynamic proteic composition of its cell surface (Wang et al. 2004).

Lectins in algae have been increasingly described (Singh et al. 2018) and those that recognize mannose, fucose and *N*-acetylgalactosamine appear to be common among organisms. D-mannose, L-fucose and *N*-acetylgalactosamine are common carbohydrates in nature and participate in various recognition processes involving other organisms (Ofek

and Beachey 1978, Boettner et al. 2002, Spear et al. 2003). These carbohydrates were also detected in some recognition processes that involve algae. In *Antithamnion nipponicum* (Rhodophyta), L-fucose and D-mannose and their respective lectins appear to mediate the gametes recognition (Kim et al. 1996). In other microalgae, these carbohydrates (D-mannose, L-fucose and *N*-acetylgalactosamine) and their respective lectins seem to be responsible for the recognition of these microalgae by their predators (bivalves) (Espinosa et al. 2010). In *Tetraselmis*, despite an unknown function, mannose and *N*-acetylgalactosamine were detected by lectins on the surface of the flagellum of different strains (Becker et al. 1995) and in algae of the genus *Codium* (Chlorophyta), some lectins with specificity for GalNAc have already been described (Praseptiangga 2015). In addition, a very recent study show that a mannose-binding lectin is present in *Tetradesmus obliquus*, a Scenedesmaceae, such as *C. microporum* (Chen et al. 2020). Thus, although that are few studies focused on this subject, it was not a surprise our finds related to the presence of receptors that recognize these carbohydrates in *C. microporum*.

In order to better understand our results, we sequenced and annotated the *Coelastrum microporum* genome. Whole genomic algae sequences allowed the prediction of the encoded proteome, which allows an analysis of the potential metabolic capacities of the cell (Bowler and Allen 2007). *Coelastrum microporum* presented a genome of ~ 200 Mbp, which was large when compared to other algae species, such as *Chlamydomonas reinhardtii* (110-111 Mb), *Eudorina* sp (184.0 Mb.) *Chlamydomonas schloesseri* (130.2 Mb) *Edaphochlamys debaryana* (142.1 Mb) (Craig et al. 2020) and *Chloropicon primus*, with 17.4 Mb (Lemieux et al. 2019). The GC content, as expected, had a considerable value (54%), since green algae usually tend to have GC contents greater than most eukaryotes (30-40%) and rarely below 50% (Weiss et al. 2010). GC content is one of the most important qualitative aspects of the genome architecture, playing a critical role in

gene and genome regulation and determines the physical properties of DNA molecules (Šmarda et al. 2014). In prokaryotes, the GC content is well studied and widely used in the taxonomy (Šmarda et al. 2014) and combined with genome size, serves as a simple tool for basic genome characterization and taxa delimitation. In eukaryotic genomes, the general content of GC has a narrower variation, decreasing in variation with the incidence of multicellularity and an increase in structural and functional complexity (Šmarda et al. 2012). GC content also seems to be related to the amount of genes found in DNA (Bernardi et al. 1988). In fact, *C. microporum* showed a large number of genes.

The number of protein-coding genes found in *C. microporum* was very high, being over 77,000. It was an expressive number when compared to other algae (table1). *Chlamydomonas*, for comparison, has 15,143 proteins-coding genes (Merchant). A large amount of protein has already been reported for *C. microporum*, which presented 21% of protein of its total biomass (Carneiro et al. 2019), a high value when compared which other green algae such as *Chlorella ovalis* (10-11%) and *Dunaliella primolecta* (12-13%) (Slocombe et al. 2013). However, considering function, in general, more than half of the proteins encoded by the algae genomes are of unknown function and their functional capabilities have yet to be discovered (Blaby-Hass and Merchant 2019). In fact, the protein annotation obtained by PANNZER2 showed a large number of uncharacterized proteins or proteins without any information (data not shown). This may be due in part to the lack of characterized sequences of algae origin in public databases (Kurotani et al. 2017). Despite that, PANNZER2 predicted one protein as lectin and many others with great potential to also be lectins (137 in total). Considering that carbohydrate-binding proteins is widely distributed in freshwater microalgae (Chu et al. 2004), we have a good chance of actually detecting lectins in *C. microporum*.

The identification of lectins in microalgae, besides helping to understand the physiology and morphology of organisms, generate knowledge about lectins repertoire existing in algae, which have interesting biotechnological applications. Algal lectins have potential for a variety of biomedical applications (Singh et al. 2015, 2018) like potential for use in preventing HIV transmission (Li et al. 2008) and also against the hepatitis C virus (HCV) (Takebe et al. 2013). Red algae and cyanobacteria lectins are better known, but green algae lectins are also being described and their potential uses are being investigated (Oliveira et al. 2017, Singh et al. 2018). Compared to lectins characterized from animal and terrestrial plant sources, little is known about the biochemical and structural properties of macroalgal lectins (Harnedy and Fitzgerald 2011). About microalgae, although considerable progress has been made in understanding the distribution and biochemical characteristics of some lectins, much less is known in comparison with terrestrial plant lectins, which limits their uses (Silva et al. 2019). Thus, the elucidation of *C. microporum* lectins would also contribute to knowledge in this field.

In summary, this work showed that the green algae *Coelastrum microporum* has glycidic receptors for three different carbohydrates (D-mannose, L-fucose and *N*-acetylgalactosamine) and according to the functional annotation of its genome, these receptors can be lectins. However, more refined studies, such as proteomic analysis to identify and characterize these proteins, are still required.

5- References

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Considerações finais

Nesse trabalho foram geradas informações sobre coberturas e superfícies celulares de microalgas. As paredes celulares de seis espécies de microalgas verdes, pertencentes aos filos Chlorophyta e Charophyta foram estudadas. Moléculas receptoras de carboidratos foram detectados na superfície de cinco delas e *Coelastrum microporum*, teve ainda seu genoma sequenciado e anotado.

As microalgas possuem um diversificado repertório de coberturas celulares. Apesar disso, notamos que havia uma limitação de conhecimento sobre o assunto, principalmente considerando a grande diversidade do grupo. Poucas espécies são bem estudadas, sendo frequentemente feitas generalizações, que podem ser equivocadas. Assim, vimos a necessidade de fazer um levantamento minucioso sobre as informações disponíveis a respeito de coberturas celulares algais, o que resultou na revisão bibliográfica apresentada no capítulo 1. Espera-se que a publicação dessa revisão seja útil para consulta rápida por ficologistas, assim como foi fundamental para nortear os experimentos desse projeto. Vimos que as coberturas celulares algais são variadas em termos de composição e arquitetura, tanto entre os grandes grupos (Okuda 2002), quanto dentro de um mesmo táxon (Domozych et al. 2012).

De fato, as espécies aqui testadas mostraram uma composição de parede celular variada quanto aos tipos de pectinas e hemiceluloses. Essa variação, que ocorreu não somente entre espécies, mas também em uma mesma espécie com diferentes tempos de cultivo, sugere que existe uma dinâmica dos componentes da parede celular associada ao estado fisiológico das células. Em plantas, as pectinas da parede celular passam por processos de de-esterificação bem conhecidos (Voiniciuc et al. 2018) e, apesar de não esclarecido para algas, o metabolismo de pectinas descrito para *Penium margaritaceum* é semelhante ao descrito para plantas (Domozych et al. 2014). No presente trabalho, os

resultados da imunocitoquímica sugeriram que *C. microporum* também possui uma dinâmica de pectinas semelhante à descrita para plantas. Considerando a grande similaridade na composição e dinâmica de parede celular apresentadas, sugerimos essa espécie como um possível organismo modelo para estudos de células vegetais.

Assim como a composição das paredes celulares, os receptores glicídicos para D-manose, L-fucose e *N*-acetilgalactosamina detectados nas superfícies celulares das espécies também variaram de acordo com a idade das células, sugerindo uma expressão coordenada desses receptores com o estado fisiológico do organismo. Os carboidratos presentes nas superfícies das células podem ter sua expressão diferenciada durante o crescimento celular (Zheng et al. 2005) e, portanto, uma expressão diferenciada de proteínas receptoras de carboidratos também pode ser esperada, já que a interação entre carboidratos e proteínas (especialmente as lectinas) são chave para muitos processos que envolvem reconhecimento celular (Melgarejo et al. 2005, Reina and Rojo 2013).

As lectinas são proteínas ubíquas na natureza e conhecidas por sua capacidade de interagir com carboidratos de forma altamente específica (Santos et al. 2014). Por causa dessa especificidade, lectinas são consideradas sondas confiáveis e muito úteis para detecção de carboidratos em superfícies celulares. A via contrária (uso de carboidratos como sondas, como os glicodendrons por exemplo) ainda é raramente explorada, mas como mostrado recentemente por Gonçalves e colaboradores (2019), também é uma ferramenta promissora para esse tipo de estudo. Os glicodendrons são moléculas que apresentam várias cópias de um carboidrato, o que mimetiza os glicídios presentes na natureza (Reina and Rojo 2013), conferindo maior robustez para as interações e os resultados. Utilizando os glicodendrons, detectamos receptores nas superfícies das microalgas testadas, os quais provavelmente são lectinas receptoras dos carboidratos testados: D-manose, L-fucose e *N*-acetilgalactosamina.

Os glicodendrons permitem uma segura detecção de receptores glicídicos nas superfícies celulares, mas, no entanto, não permite sua caracterização. O sequenciamento e anotação do genoma de *C. microporum* permitiu a busca por lectinas e foi encontrado um número considerável de proteínas (137) com tais características. No entanto, estudos complementares, como análises proteômicas, ainda são necessários para comprovação e caracterização dessas moléculas.

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