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Protoplast Technology in Brown Algae (Phaeophyceae): Studies on Protoplast Isolation, Culture and Regeneration of 7 Brown Algal Species

Graduate School of Chosun University

Department of Integrative Biological Science

Jose Giovanni Jesus Avila Peltroche

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갈조류(Phaeophyceae)의 원형질체 기술: 갈조 7 종의 원형질체

분리, 배양 및 재생에 관한 연구

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글로벌바이오융합학과

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지도교수 조 태 오

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Jose Giovanni Jesus Avila Peltroche

Approved:

Committee chair   _____
Prof. Gwang Won Cho
Chosun University

Committee   _____
Prof. Young Sik Kim
Gun San University

Committee   _____
Prof. Wung Gi Shin
Chung Nam University

Committee   _____
Prof. Sang Gi Song
Chosun University

Committee   _____
Prof. Tae Oh Cho
Chosun University

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Chosun University Graduate School

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ABSTRACT[*]

Protoplast Technology in Brown Algae (Phaeophyceae): Studies on Protoplast Isolation, Culture and Regeneration of 7 Brown Algal Species

Jose Giovanni Jesus Avila Peltroche
Advisor: Prof. Tae Oh Cho, Ph.D.
Department of Integrative Biological Science
Graduate School of Chosun University

Protoplast technology uses protoplasts (e.g. cells whose cell wall has been removed by enzymatic digestion) as powerful experimental material for *in vitro* manipulations and crop improvement. This technology encompasses two main components: 1) protoplast isolation; and 2) protoplast culture and regeneration. Brown algae are a group of, mostly marine, photosynthetic organisms that are used in food, animal feed, traditional medicine, alginate industry, cosmetics and for pharmaceutical applications. Despite their high economic importance, protoplast technology in brown algae lags far behind that of other multicellular algae (e.g. green and red algae) and higher plants. Also, most protocols rely on crude extracts or non-commercial enzymes for producing protoplasts, which are expensive, time consuming and/or low reproducible. Thus, protocols with commercial enzymes are needed for properly establishing protoplast technology in brown algae. In this study, I selected 7 brown algal species (*Dictyopteris pacifica*, *Ecklonia cava*, *Hecatonema terminale*, *Petalonia fascia*, *Scytosiphon lomentaria*, *Sphacelaria fusca* and *Undaria pinnatifida*) and developed protoplast isolation protocols for them using only commercial enzymes. Among these species, the economic brown alga *U. pinnatifida* and other 3 (*H. terminale*, *P. fascia* and *Sp. fusca*) were selected for protoplast culture. In *U. pinnatifida*, I further explored the effect of

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light-emitting diodes (LED) on protoplast regeneration from the microscopic gametophytes and macroscopic sporophytes. Finally, I tested the potential of protoplast-derived aposporous filaments (PDAFs) for clonal propagation of *U. pinnatifida* sporophyte. In all the species, high amount of protoplasts were obtained using a simple mixture of commercial enzymes (cellulase RS and alginate lyase). Protoplasts yields ranged from 10^4-10^5 protoplasts g^{-1} fresh weight (FW) in the filamentous forms *H. terminale* and *Sp. fusca*, to 10^6-10^7 protoplasts g^{-1} FW in more complex brown algae (*D. pacifica*, *E. cava*, *P. fascia, S. lomentaria* and *U. pinnatifida*). *Dictyopteris pacifica*, *E. cava*, *H. terminale* and *Sp. fusca* represented new reports for protoplast production. The most important factors during isolation were growth, chelation pre-treatment, pH and osmolarity. Successful regeneration was achieved, for first time, in *H. terminale*, *P. fascia* and *Sp. fusca*. In *U. pinnatifida*, an improved method for protoplast culture and regeneration was developed. Critical conditions during this step were regeneration medium, initial protoplast density, antibiotics, light exposure, starting time of osmolarity reduction, and temperature. LED experiments in *U. pinnatifida* showed that dichromatic light (red plus blue, 1:2) enhanced protoplast regeneration and growth from filamentous gametophytes, and incremented the formation of normal sporophytes from PDAFs. In green LED, PDAFs could be propagated without formation of sporophytes while keeping a high potential for producing them upon dichromatic light exposure. Subculturing PDAFs with subsequent 6 weeks of re-growth could sustain sporophyte production over time. Regenerated sporophytes were diploid and showed identical genotype with the mother PDAF culture. This suggests that PDAFs could be used for sustained clonal propagation of *U. pinnatifida* sporophyte, opening a new possibility of protoplasts uses in brown algae.

초록[*]

갈조류(Phaeophyceae)의 원형질체 기술: 갈조 7 종의 원형질체 분리, 배양 및 재생에 관한 연구

Jose Giovanni Jesus Avila Peltroche

지도교수 : 조태오 교수님

글로벌바이오융합학과

조선대학교 대학원

원형질체 기술은 원형질체(세포벽을 제거시킨 세포)를 *in vitro* 조작이나 작물개선을 위한 유용한 실험재료로 사용한다. 이 기술은 크게 2 가지 즉 1) 원형질체 분리, 2) 원형질체 배양 및 재생으로 구성되어 있다. 갈조류는 식품, 동물사료, 전통의학, 해조류 산업, 화장품 및 제약 분양에서 널리 사용되는 해양 광합성 조류이다. 이런 경제적 중요성에도 불구하고, 갈조류의 원형질체 기술은 다른 다세포조류(녹조류, 홍조류) 및 고등식물에 비해 개발이 늦어지고 있다. 또한 알려진 방법들은 비상업적 효소나 미정제추출물을 사용하여 비싸고 시간이 오래 걸리거나 그 생산량 또한 적다. 따라서, 갈조류에 원형질체 기술을 확립하려면, 상업적 효소를 사용한 원형질체 생산량이 높은 방법이 필요하다. 본 연구에서 7 종(*Dictyopteris pacifica, Ecklonia cava, Hecatonema*

terminale, Petalonia fascia, Scytosiphon lomentaria, Sphacelaria fusca, Undaria pinnatifida)의 갈조류를 대상으로 상업적인 효소들을 사용한 각종의 원형질체 분리방법을 개발했다. 원형질체 분리방법이 성공한 이들 종들 중 경제적으로 중요한 *U. pinnatifida* 외 3 종(*H. terminale, P. fascia, Sp. fusca*)을 대상으로 원형질체 배양을 진행하였다. *Undaria pinnatifida* 의 배우체와 포자체로부터 분리한 원형질체를 대상으로 생에서 LED 의 효과를 연구하였다. 또한 *U. pinnatifida* 포자체의 클론 증식으로 나온 PDAFs (protoplast-derived aposporous filaments)의 재생 가능성을 연구하였다. 갈조류 7 종에서 상업적 효소(cellulase RS and alginate lyase)의 간단한 혼합으로 확보한 원형질체 수율은 사상체 형태인 *H. terminale* 과 *Sp. fusca 에서*는 10^4-10^5 protoplasts g^{-1} fresh weight (FW) 이고, 좀더 복잡한 구조를 가진 갈조류 (*D. pacifica*, *E. cava*, *P. fascia*, *S. lomentaria*, *U. pinnatifida*)에서는 10^6-10^7 protoplasts g^{-1} FW 이었다. 이들 중 *D. pacifica*, *E. cava*, *H. terminale* 과 *S. fusca* 의 원형질체를 분리는 본 연구를 통해 처음으로 보고되는 것이다. 원형질 분리에 있어서 가장 중요한 요소는 growth, chelation 전 처리, pH 과 삼투압이었다. 또한 본연구를 통해 처음으로 *H. terminale*, *Sp. fusca* 과 *P. fascia* 에서 성공적인 원형질체의 재생이 이루어졌다. 그리고 *U. pinnatifida* 의 원형질체 배양 및 재생에서는 좀더 개선된 방법들이 개발되었고 이 단계에서 중요한 조건들은 재생 배지, 초기 원형질체 밀도, 항생제, 빛 노출, 삼투압 감소 시작 시간 및 온도로 파악되었다. *Undaria pinnatifida* 의 LED 실험은 두 색의 조합(red plus blue, 1:2)이 사상형의 배우자체에서 원형질체 재생 및 성장을 강화할 뿐만 아니라, 포자체의 원형질체로부터 발달한 PDAF 가 정상적인 포자체로 재생되는 비율을 증가시켰다. 녹색 LED 는 PDAFs 가 포자체로 발달을

억제하고, 두 색의 조합(red plus blue, 1:2)은 포자체로 발달을 촉진시키는 것으로 나타났다. PDAFs 는 6 주 성장 후 계대배양을 지속하면, 포자체로 발달을 유지할 수 있었다. PDAF 로부터 재생된 포자체는 이배체였으며, PDAF 의 모체와 동일한 유전자형을 보였다. 이 결과는 원형질체에서부터 유래한 PDAF 가 *U. pinnatifida* 의 지속적인 클론증식에 사용될 있는 가능성을 보여주었으며, 갈조류에서 원형질체를 활용한 대량생산의 새로운 길을 열어줄 것이라 기대된다.

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I. INTRODUCTION

1. Brown algae

Brown algae are a diverse group of multicellular photosynthetic organisms that comprise approximately 2000 species worldwide. They produce laminaran as the storage polysaccharide, and have chlorophyll *a* and *c* as the major photosynthetic pigments. Their main accessory pigment is fucoxanthin, which gives the characteristic brown color to this group of algae (Kawai & Henry 2016). Their cell walls are mainly composed of alginates and fucoidans, and a small amount of cellulose (1-8%; Cronshaw et al. 1958; Kloareg & Quatrano 1988). Most of brown algal species are found in marine environments, where they are usually referred as brown seaweeds; however, some few species are obligate freshwater inhabitants, such as *Heribaudiella fluviatilis* and *Sphacelaria lacustris*. Brown algae are distributed in polar, cold temperate, warm temperate and subtropical/tropical regions (Bringloe et al. 2020). High species diversity is found in cold temperate waters, with Fucales (e.g. *Ascophyllum*, *Fucus* spp., *Hormosira banskii*) dominating the rocky intertidal and kelps (species of Laminariales) forming dense communities in the subtidal and lower intertidal zones (Kawai & Henry 2016).

All brown algae belong to the class Phaeophyceae (Ochrophyta), which diverged from its closest sister group, Schizocladiophyceae, approximately 260 million years ago in the Permian Period (Kawai & Henry 2016). This class is one of the five groups of eukaryotes that acquired complex multicellularity, being the other ones red algae (Rhodophyta), green algae (Chloroplastidia including land plants), animals (Metazoan), and fungi (Fungi; Bogaert et al. 2013). Phaeophyceae comprises 19 orders that present a wide variety of forms and, mostly, a diplohaplontic life cycle, where the gametophyte (haploid) and sporophyte (diploid) are multicellular (Fig. 1). Complex forms (parenchymatous) have emerged multiple times during the evolution of brown algae (e.g. Laminariales, Fucales, Dictyotales). Similarly, simple forms (uniseriate branched filaments) can be found in basal (Discosporangiales) and more recent groups (Ectocarpales and Asterocladales). Diplontic life cycle, where only the diploid generation is multicellular, is only present in two orders: Fucales and Ascoseirales (Bringloe et al. 2020).

Brown algae offer a wider phylogenetic perspective of the processes that control development and have led to multicellularity. Fucoid species, such as *Fucus* and *Silvetia*, and filamentous forms, such as *Ectocarpus* and *Sphacelaria*, have been used as models for exploring mechanisms like embryogenesis, polarity and asymmetric cell division (Bogaert et al. 2013). As primary

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producers, brown algae are essential for coastal environments, especially in cold and temperate waters. Ecosystems like kelp forests support complex trophic chains that provide a wide range of ecosystem goods and services, which have been valorized in 500,000-1,000,000 USD per year per kilometer of coastline (Wernberg et al. 2018).

A
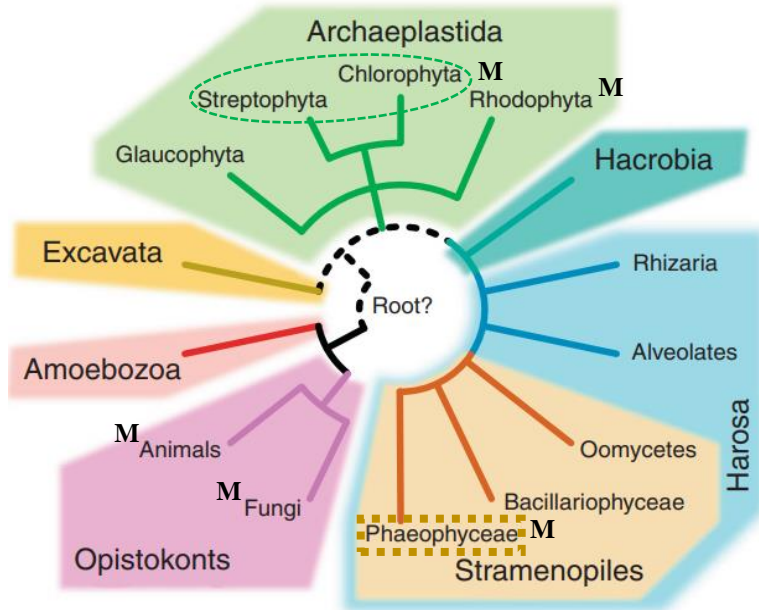
B
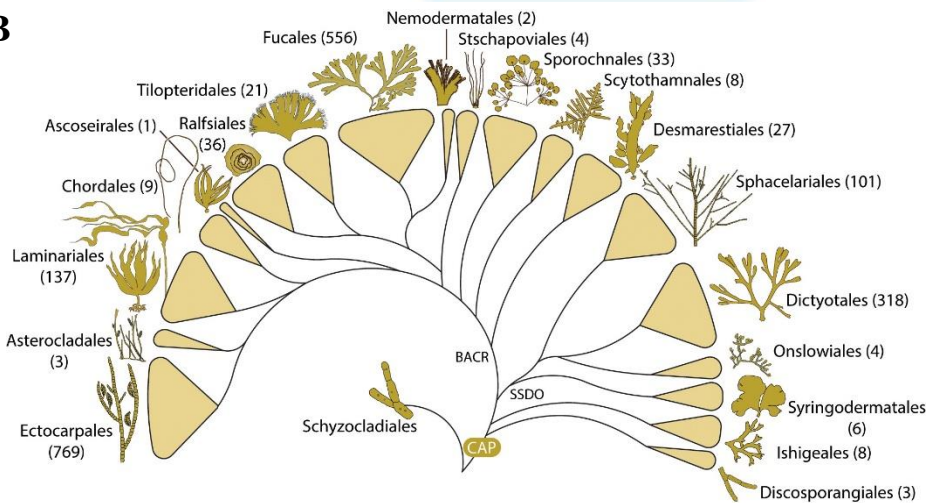
Fig. 1. Phylogeny of brown algae. (A) Brown algae (Phaeophyceae; dotted rectangle) within eukaryotes highlighting groups with complex multicellularity (M). Chlorophyta and Streptophyta are considered as one group, Chloroplastidia (dotted ellipse) (from Charrier et al. 2012). (B) Phylogeny of brown algal orders. The number of species per order appear in brackets. CPA, common ancestor of Phaeophyceae; SSDO: Sphacelariales, Syringodermatales, Dictyotales, Onslowiales clade; BACR: brown algal crown radiation (from Bringloe et al. 2020).

Uses of brown algae

Food

Brown algae are an important component of Asian cuisine, especially in countries like China, Korea and Japan. For instance, *Undaria pinnatifida* is used for the preparation of *miyukkuk*, a famous Korean soup made by cooking this seaweed in a clam stock. Other example is the kelp *Saccharina japonica*, whose aqueous extract is a key element in Japanese *dashi* (soup stock) and can be also consumed as a tea (Mouritsen et al. 2018). Both species, together with *Sargassum fusiforme*, are among the most important farmed seaweeds worldwide in terms of biomass (FAO 2020). In other regions, brown algae are also present in human diet but in a lesser extent compared to Asia. *Durvillaea antarctica* and *Hormosira banksii*, among other types of seaweeds, are part of the traditional Maori diet in New Zealand. *Alaria esculenta* was eaten as thick pudding with milk or cream in Iceland, where other brown algae, such as *Laminaria digitata*, *L. hyperborea* and *Ascophyllum nodosum*, have been consumed in times of starvation (Hallsson 1964; Mouritsen et al. 2018). The use of brown algae as human food relies on their low caloric content but high amounts of vitamins, minerals and dietary fibers, which give them a great nutritional value (Leandro et al. 2019).

Animal feed

Brown algae are also used in animal feed in Europe and Asia. In 1960s, Norway was the pioneer on producing an additive to animal feed based on dried and milled brown seaweeds (Kılınç et al. 2013). *Ecklonia cava* is commercially farmed to supply a summer feed for the abalone industry in Korea. Other kelp species, such as *Undaria* and *Saccharina,* are also used for this purpose. In fact, their utilization as abalone feeds has been increasing in the last years, with a 60% of their production destined to the abalone industry in 2012 (Kim et al. 2017).

Traditional medicine

Traditional medicine in Asia includes brown algae in various treatments. They have been commonly used for goiter, the thyroid enlargement resulting from a severe iodine deficiency, due to their high iodine content (1500-8000 ppm in dried kelps; Dharmananda 2002). In Chinese traditional medicine, 11 species of *Sargassum* have been employed for treating oedeme due to

retention of phlegm and morbid fluids, acute esophagitis, chronic bronchitis, dysuria, among other conditions (Liu et al. 2012). *Saccharina japonica* constitutes an important medical ingredient in Japanese, Korean and Chinese traditional medicine. It is used for treating thrombosis, gall disease, hard lump, edema, thyroid tumor, tuberculosis and beriberi (Sanjeewa & Jeon 2018).

Alginate industry

Alginates are the major component extracted from brown algae. Their ability to form gels and stabilize emulsions in the presence of certain metal cations, such as calcium (Ca^{2+}), make them useful in food, textile printing, papermaking and pharmaceutical industries (Peteiro 2018). However, more profitable uses, such as in biomedical applications and novel therapies (e.g. gene therapy), have emerged during the last years (Lee & Mooney 2012; Fernando et al. 2019) Commercial alginates are mainly produced from the genera *Laminaria*, *Saccharina*, *Lessonia*, *Macrocystis*, *Durvillaea*, *Ecklonia* and *Ascophyllum*. These species are usually harvested, manually or mechanically, from wild populations. *D. potatorum* and *M. pyrifera* present the highest level of alginates, with up to 55% and 45% of the dry weight. In 2009, 95,000 tonnes of brown seaweeds (dry weight) were harvested, with *Lessonia* and *Laminaria* accounting for 65% of global production, followed by *Saccharina* with 21%. In the same year, the alginate market reported an estimated value of US$ 318 million worldwide (Bixler & Porse 2011; Peteiro 2018).

Cosmetic and pharmaceutical applications

The inclusion of brown algae (parts or extracts) in cosmetic products is due to the wide range of properties they present. Antioxidant, anti-aging, moisturizing, and skin softening and elasticity are among the most common properties reported. Cosmetic products include creams, facial masks, balms, lotions, oils and shampoos, which are produced from *Alaria esculenta, Fucus vesiculosus, Sargassum fusiforme, Saccharina latissima, S. japonica, Macrocystis pyrifera, Undaria pinnatifida,* among other brown seaweed species (Leandro et al. 2019). Substances derived from brown algae have shown interesting biological activities with promising pharmaceutical applications. Fucoxanthin (pigment) from *S. japonica* has shown anti-UVB and anti-melanogenic activities (Thomas & Kim 2013), while phlorotannins from *Ascophyllum nodosum* have the potential for treating diabetes (Zhang et al. 2008). In fact, there is a patent

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containing *A. nodosum* and *F. vesiculosus* extracts, combined with green seaweed extracts, for the treatment of diabetes type 2 and its complications (Daniels 2020).

Other uses

Brown algae are the main source of commercial plant biostimulants, such as Kelpap from *Ecklonia maxima* or Algifert from *Ascophyllum nodosum* (Sharma et al. 2013). Kelps can be used for bioremediation of excess nutrients, like nitrogen or phosphorus. Also, dissolved metals in contaminated waters can be bio-accumulated by brown seaweeds, like cadmium in *Sargassum* species and *Laminaria digitata* (Neveux et al. 2018).

2. Protoplast technology

1.1. Definition

Protoplast technology is a well-known approach in plant tissue culture that provides powerful experimental material for *in vitro* manipulations and crop improvement bypassing sexual reproduction. It dates back to the end of the 19th century, when Klercker (1892) extruded the living cell contents from sliced leaves of the water plant *Stratiotes aloides*. Since then, protoplast technology has become an important complement to traditional breeding (Davey et al. 2005a, b). Recently, there has been a renewed interested on it due to its usefulness in genome-editing and gene silencing techniques (Burris et al. 2016). Protoplasts are cells whose cell walls have been removed by mechanical but mostly enzymatic methods. Although they are usually produced from plant cells, protoplasts can also be obtained from bacteria (Kami et al. 2019) and fungi (Turgeon et al. 2010). Spheroplast is another term often used in protoplast research, especially in bacteria and fungi, and it refers to cells having some cell wall material on them (Cove 1979). While some studies require the complete removal of the cell wall (e.g. cell polarity studies), others (e.g. genetic studies) can use spheroplasts instead (Zaban et al. 2012, Takahashi et al. 2016).

Protoplast technology relies on a series of technical operations that can be divided in two main components: 1) protoplast isolation; and 2) protoplast culture and regeneration. During isolation, protoplasts are released from their cell walls using one or, usually, a combination of cell wall lytic enzymes. Osmotically favorable conditions are necessary for stabilizing the membrane and avoiding damage or lysis of the spherical protoplasts. During protoplast culture and regeneration, protoplasts are allowed to resynthesize their cell walls, divide and regenerate under suitable culture conditions (Cove 1979). Protoplasts are considered, at least theoretically, as totipotent cells, which means that they can re-enter the cell cycle, go through repeated mitotic divisions and regenerate a whole new organism (Eeckhaut et al. 2013), a process often called whole plant regeneration in plant tissue culture. The above-mentioned components are fundamental for the application of protoplast technology in basic and applied studies.

1.2. Uses

The importance of developing protocols for protoplast isolation, culture and regeneration relies on the multiple applications protoplasts have. Their lack of cell walls and potential totipotent capacity make them ideal for cellular, physiological and genetic studies, especially those ones related to membrane composition, cell wall formation, genetic transformation, somatic hybridization, and dedifferentiation. In order to better explore the different applications of protoplast technology, the classification of Davey et al. (2005a) is used in this section with some modifications, including more recent studies. Although the uses are mostly focused on plant cells, studies on bacterial and fungal cells are also included.

Somatic hybridization to generate novel plants

The fusion of protoplasts from different parental origins is one of the most important uses of protoplast technology. The goal of this technique is to obtain somatic hybrids when there is sexual incompatibility among parental organisms which carry certain characteristics (Cove 1979). Desired traits included disease resistance, abiotic stress resistance, quality characters and cytoplasmic male sterility. The resulting cell hybrid contains the nucleus and cytoplasm of both parents fused, although cybrids (cells with the nucleus of one parent but the cytoplasm of both) might also occur. Depending on the taxonomic distance of the parentals, fusions can be classified as interspecific, intergeneric or interkingdom (Chawla 2009). Although protoplast fusion can occur spontaneously in plants and fungi, to date, two main methods for somatic hybridization have been stablished: 1) polyethylene glycol (PEG)-induced fusion; and 2) electrofusion. In the former, PEG polymers are added to a protoplast mixture causing a massive cell clumping and, eventually, fusing the cells when the membranes are closed enough to contact each other. Factors like pH, calcium concentration and purity of PEG affect the fusion rate in this method. The later procedure involves the use of a high frequency AC field for bringing protoplasts in close contact, and the application, for short time, of a single high-voltage DC pulse for protoplast fusion (Gaynor & Ravindar 1985). The PEG method is the most widely used in plant protoplasts due to its high-frequency heterokaryon (cells with two or more genetically different nuclei) formation (mostly binucleate), low cytotoxicity, and low cost (Chawla 2009). Somatic hybridization has been successfully applied in important plant cultivars, such as potato (Tiwari et al. 2018), citrus

(Dambier et al. 2011) and wheat (Liu & Xia 2014), fungi (Park & Wellings 2011), microalgae (Abomohra et al. 2016) and seaweeds (Gupta et al. 2015).

Transformation of protoplasts

In organisms such as fungi and plants, cell walls represent a barrier for the introduction of genetic material. Due to the fluid mosaic characteristics of the cell membrane, foreign DNA can be introduced in cells through chemical and/or physical methods (Davey et al. 2005b). In this sense, cell wall-less cells (i.e. protoplasts) are ideal for gene transfer and expression, either stable, when the gene is integrated into the nuclear or plastid genome; or transient, when the gene is expressed for only a short period and subsequently lost in the next generation (Chawla 2009). Methods for direct DNA transfer in protoplasts can be divided in: 1) physical methods, which include electroporation, microinjection, lipofection and sonication; and 2) chemical methods, which include PEG-mediated transformation (Keshavareddy et al. 2018). To date, electroporation and/or PEG are the approaches normally used to induce DNA uptake into protoplasts, both from fungal (Rodriguez-Iglesias & Schmoll 2015) or plant origin (Shen et al. 2014). Protoplast transformation has also been reported for microalgae (Kumar et al. 2018) and seaweeds (Reddy et al. 2008). Recently, genome-editing tools, such as the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system, has been applied in protoplasts for testing mutagenesis efficiency rapidly (Lin et al. 2018). The easy screening of the efficiency of gene-silencing and genome editing targets has increased the interest of developing protoplasts systems in plants (Burris et al. 2016).

Protoclonal variation

The phenotypic diversity displayed by regenerated individuals from protoplasts is called protoclonal variation. Although many of the variations affect negatively the growth and performance of the regenerated organisms, positive changes can be detected when there is enough material for screening the different phenotypes (Grosser & Omar 2011). This approach represents the simplest form of genetic manipulation, and does not require DNA recombinant techniques, prior knowledge of the genetic basis of specific traits, complicated protocols or specialized apparatus (Davey et al. 2005b). Positive protoclonal variation has been mainly studied in plants. For example, Grosser et al. (2007) showed that protoplast-derived populations

from sweet oranges exhibited higher levels of useful variation, resulting in several commercial clones, including a seedless protoclone.

Miscellaneous uses

Protoplasts have become a biological tool for a wide range of studies due to their lack of cell membrane, "single-cell" nature and high sensitive to culture conditions. In addition the abovementioned uses, cellular and subcellular processes, effects of hormones and stressors, plant-viral interactions, among other factors, can be explored in protoplasts bypassing the complexity of tissues and organs (Davey et al. 2005b; Tagawa et al. 2019; Pasternak et al. 2020). Among the applications described in Table 1, single-cell transcriptome using protoplasts have shown an enormous potential for profiling developmental processes in plants (Shulse et al. 2019), and it can be naturally extended to other multicellular organisms with cell walls like seaweeds. Also, seedling production from protoplasts shows a big potential for sustainable and large scale production of marine macroalgae (Gupta et al. 2018).

Table 1. Examples of recent uses of protoplasts from plants, fungi, bacteria and algae.

Application	Organism	Reference
Production of useful metabolites by using plant protoplasts with artificial cell walls.	Several plant species (e.g. *Wasabi japonica*, *Catharantus roseus*, *Nicotiana tabacum*, *Coffea arabica*)	Aoyagi 2011
High-throughput single-cell transcriptome for analysing profiles in different cell populations.	*Arabidopsis thaliana*	Shulse et al. 2019
Expansion of fungal host range of mycoviruses for biocontrolling fungal diseases and studying of virus-host interactions	Phytopathogenic fungi (*Diaporthe* sp., *Cryphonectria parasitica*, *Valsa ceratosperma*, *Glomerella cingulata*)	Kanematsu et al. 2010
Protein synthesis and regeneration of giant protoplasts as evidence of bacterial adaptability in extreme conditions	*Escherichia coli*	Tabata et al. 2019
Relationship between cell shape and intracellular organization in live bacteria	*Escherichia coli* and *Bacillus subtilis*	Renner et al. 2013

Table 1. Continued.

Application	Organism	Reference
Structural polysaccharide deposition on the plasma membrane and cortical microtubules	*Betula platyphylla* var. *japonica*	Tagawa et al. 2019
Seedling production for marine macroalgal aquaculture	Green (*Ulva lactuca* and *Monostroma nitidum*) and red seaweeds (*Porphyra okhaensis*)	Dipakkore et al. 2005; Gupta et al. 2018
Creation of self-sustained cell hybrid for insulin production and energy via photosynthesis	Green microalgae *Parachlorella kessleri* fused with rat insulinoma cell line	Heller et al. 2014

1.3. Protoplast technology in brown algae

Protoplast research in marine macroalgae dates back to 1979, when Millner et al. isolated protoplasts from the green seaweed *Ulva intestinalis* using a combination of driselase and pectinase. Since then, enzymatic methods became predominant in seaweed protoplast research, although mechanical methods are still used for coenocytic forms (Klochkova et al. 2016). To date, protoplast isolation and regeneration has been accomplished in 109 seaweed species (Fisher & Gibor 1987; Gross 1990; Polne-Fuller & Gibor 1990; Amano and Noda 1992; Dai et al. 1993; Notoya et al. 1993; Chen & Shyu 1994a,b; Matsumura 1998; Kaladharan et al. 2000; Buschmann et al. 2001; Reddy et al. 2008; Yeong et al. 2008; Gupta et al. 2011; Bodian et al. 2013; Huddy et al. 2013, 2015; Chen et al. 2018). Among them, 31 species correspond to brown marine macroalgae (Fig. 2, Table 2). In this section, the status of protoplast technology in brown seaweeds is reviewed and updated. Emphasis is given to protoplast isolation and regeneration studies, as well to uses of protoplasts in brown algal research.

Protoplast research in brown algae

In 1984, Saga and Sakai reported for first time the protoplast isolation from a brown algal species, the kelp *Saccharina japonica*, using a crude enzyme solution from the sea urchin *Strongylocentrotus intermedius*. A detailed review of the literature from 1984 to 2019, revealed that the bulk of protoplast studies (>50%) were published from 1987 to 1997, which also coincided with an increment of works related to protoplasts from other seaweeds. The number of publications diminished and remained almost constant (1 or less per year) from 1998 to 2012. There has not been publications primarily focused on protoplast isolation and regeneration from brown seaweeds from 2012 onward. In contrast, studies on protoplasts from other seaweeds persisted during this period (Fig. 3).

Most of protoplast studies have been focused on kelps due to their commercial importance. *Undaria pinnatifida* is the most researched species, followed by *Macrocystis pyrifera*, *Saccharina japonica* and *Laminaria digitata*. Less attention has been put to other kelp species, such as *Ecklonia radiata* or *S. longissima.* Little work has been done in *Sargassum*, with exception of *S. muticum,* and *Fucus* species despite their ecological and economic relevance. The number of studies dealing with filamentous species are low (1 or 2 per species; Fig. 2).

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However, the model organism *Ectocarpus* is the only one, among brown seaweeds, with a well-established protocol for protoplast isolation and regeneration.

Protoplast isolation has been performed using enzyme mixtures consisting of, at least, one non-commercial enzyme (mainly from the marine herbivores *Haliotis* or *Aplysia*) or crude extract (mainly limpet or abalone acetone powder); and commercial enzymes (mainly cellulase Onozuka R-10 or RS, and macerozyme R-10). There are no works using only currently available commercial enzymes. One non-enzymatic method has been reported with promising results (Kevekordes et al. 1993); however, no further information about its application has been found (Table 2). The highest protoplast yields (10^8 protoplasts g^{-1} fresh weight) have been reported for *Ectocarpus siliculosus*, *Macrocystis pyrifera*, *Petalonia binghamiae* and *P. fascia*; while the lowest ones (up to 10^3 protoplasts g^{-1} fresh weight), for *Padina arborescens* and *Sphacelaria* sp.

Protoplast regeneration has not been tested in most complex forms (e.g. *Sargassum* species) or it has resulted on the formation of callus-like structures (*Dicytopteris* or *Laminaria digitata*) or microcolonies (*Macrocystis pyrifera*). Whole plant regeneration has been accomplished in several filamentous forms (e.g. *Sphacelaria* sp., *Ectocarpus*, gametophytes from Laminariales) and two kelp species (*Undaria pinnatifida* and *Saccharina japonica*; Table 2).
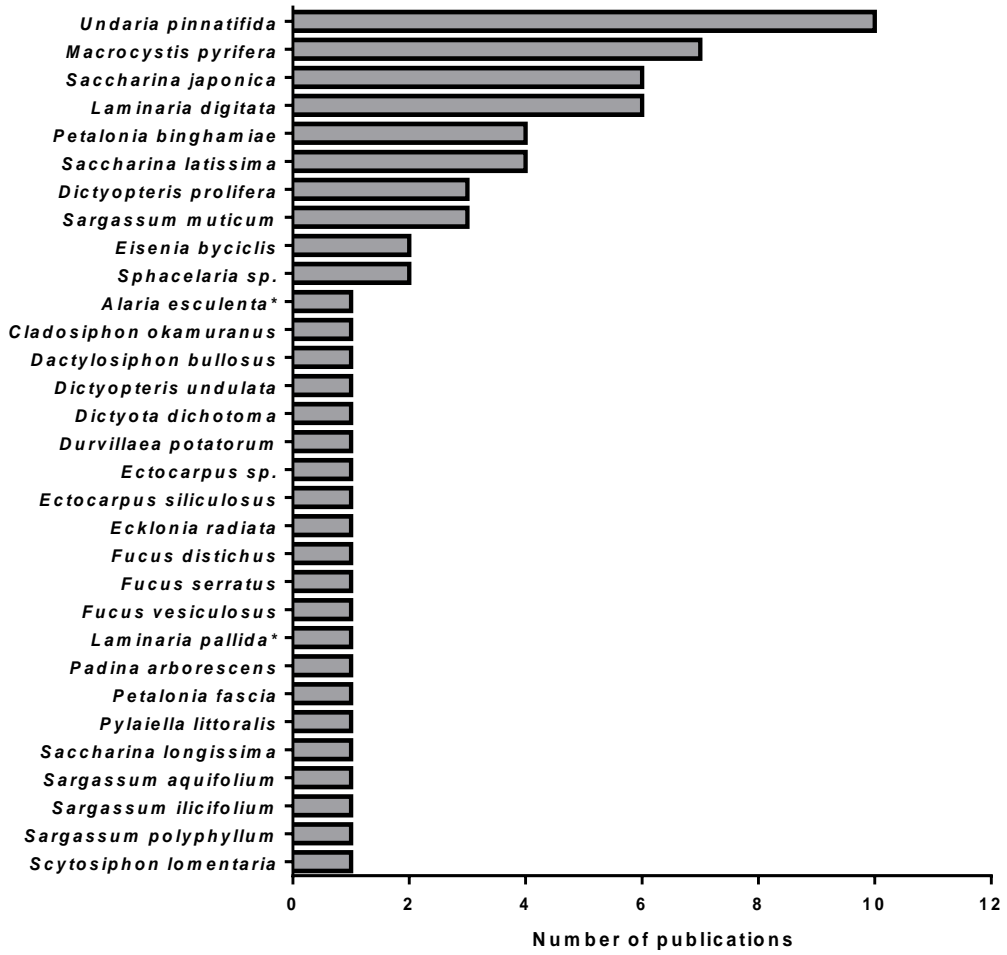
Fig. 2. Number of publications on protoplast isolation and regeneration from brown algae by species. *Only microscopic gametophyte
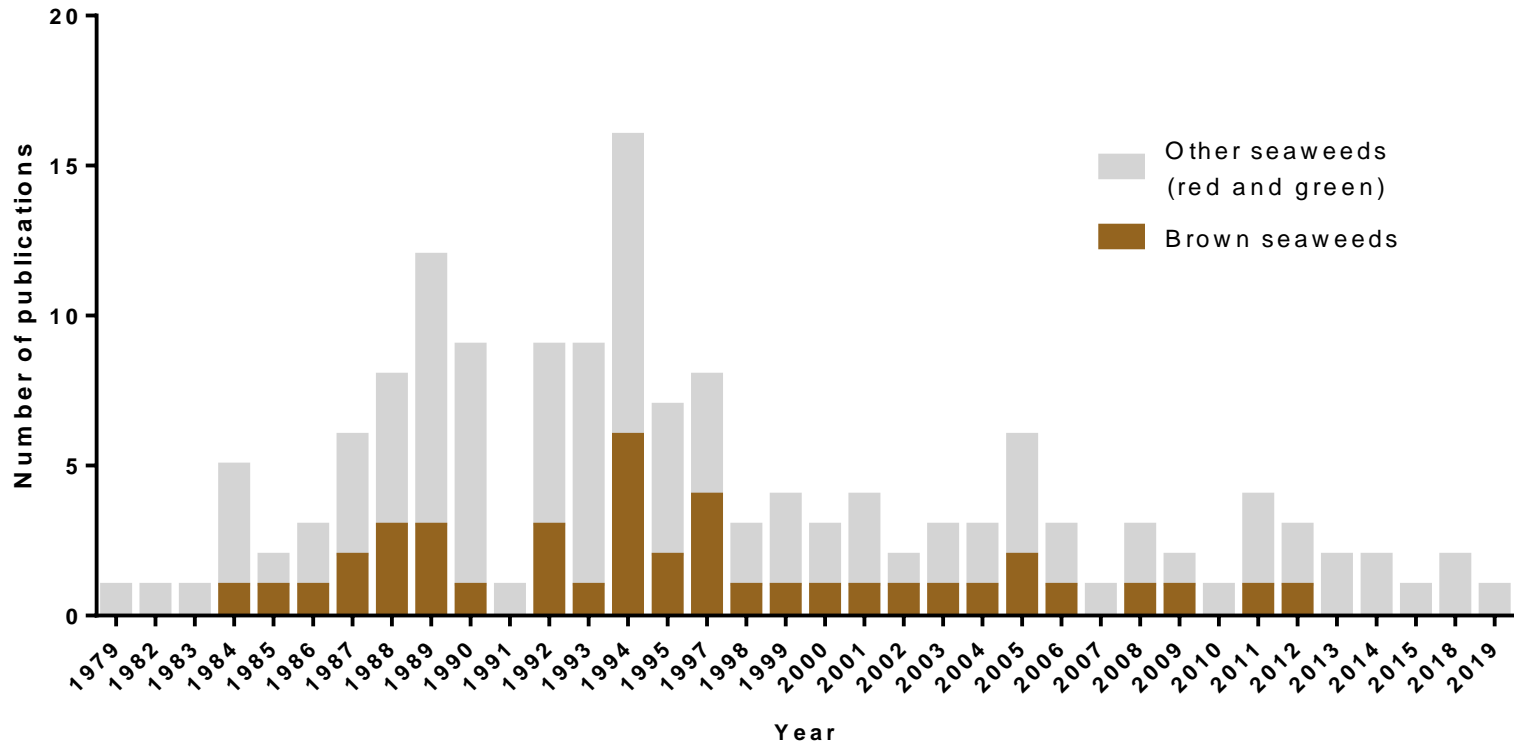
Fig. 3. Number of publications on protoplast isolation and regeneration from brown (brown bar chart) and other (gray bar chart) seaweeds by year between 1979 and 2019.

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Application of protoplasts in brown algal research

Protoplasts from brown algal species have been used in various studies since the mid-90s (Fujimura et al. 1994). Production of alginate and volatile compounds have been reported from protoplasts of three species (*Dictyopteris prolifera*, *Laminaria digitata* and *Saccharina latissima*), opening the possibility to produce commercially useful substances from cell cultures, in a similar way to what has been done in plants (Fujimura et al. 1994; Rodde & Larsen 1997). Virus-host interactions have been only explored in the model organism *Ectocarpus siliculosus* (Kuhlenkamp & Müller 1994), while physiological and cellular studies, especially those ones involving the cytoskeleton, have been performed in the filamentous brown algae *Sphacelaria* (Rusig et al. 1994), the kelp *S. latissima* (Benet et al. 1994) and the gametophytes of the giant kelp *Macrocystis pyrifera* (Varvarigos et al. 2004, 2005). Expressed sequence tag analysis has been applied in protoplasts from *L. digitata,* revealing a higher expression of stress genes in these cells compared to intact thalli. Also, the transcripts have shown that these genes mainly code for proteins involved in cell protection against oxygen radicals (Roeder et al. 2005). Regenerated sporophytes from protoplasts of *Saccharina japonica* and *Undaria pinnatifida* have exhibited normal growth upon transplantation into the sea, becoming fertile later on. These studies show the feasibility of using protoplasts for clonal kelp aquaculture (Matsumura et al. 2000, 2001). Recently, protoplasts from *E. siliculosus* have helped to unravel the non-cell autonomous mechanism behind its life cycle transition (Arun et al. 2013); and an algal protoplast digest product from *E. siliculosus* var. *subulatus* (freshwater strain) have allowed a better understanding of the cultivable microbiome associated to *Ectocarpus* (Kleinjan et al. 2017).

Table 2. Brown algal species from which protoplast isolation and regeneration have been accomplished.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Alaria esculenta (gametophyte)	10^5-10^6	Cellulase (CELF) Alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*	WPR	Benet et al. 1997
Dactylosiphon bullosus	10^5-10^6	Hepatopancreas extract from *Trochus maculatus* Cellulase Onozuka RS Macerozyme R-10 β-1,4-mannan, porphylan, and β-1,3-xylan-degrading enzymes	NT	Yamaguchi et al. 1989

Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Dictyopteris prolifera	3.3 x 10^7	Cellulase Onozuka R-10 Macerozyme R-10 Driselase Bigalase M Sumizyme X Crude enzyme solution from *Crassostrea gigas*	Callus-like structure	Fujimura et al. 1995
D. undulata	8.2 x 10^6	Cellulase Onozuka R-10 Macerozyme R-10 Driselase Hemicellulase Pectolyase Y-23 Pectinase Crude enzyme solution from *Crassostrea gigas*, *Haliotis gigantea* and *Batillus cornutus*	Callus-like structure	Kajiwara et al. 1988

Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Dictyota dichotoma	8.8×10^6	Cellulase Onozuka R-10 Macerozyme R-10 Driselase Hemicellulase Pectolyase Y-23 Pectinase Crude enzyme solution from *Crassostrea gigas*, *Haliotis gigantea* and *Batillus cornutus*	Callus-like structure	Kajiwara et al. 1988
Durvillaea potatorum	6×10^7	Non-enzymatic method. Protoplasts were isolated by removing wall-bound calcium with EGTA and substituting it with sodium from isolation medium	NT	Kevekordes et al. 1993

Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Ecklonia radiata	5 x 10^7	Non-enzymatic method. Protoplasts were isolated by removing wall-bound calcium with EGTA and substituting it with sodium from isolation medium	NT	Kevekordes et al. 1993
Ectocarpus sp.	NS	Cellulase Alginate lyase from *Haliotis tuberculata*	WPR	Coelho et al. 2012
Ectocarpus siliculosus	Up to 1 x 10^8	Cellulase Onozuka R-10 Macerozyme R-10 Alginate lyase from *Aplysia vaccaria*	WPR	Kuhlenkamp & Müller 1994
Eisenia byciclis	2.33 x 10^7	Cellulase Onozuka R-10 Sea hare (*Aplysia juliana*) extract (buccal juice)	NT	Wakabayashi et al. 1999

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Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Fucus distichus (zygotes)	More than 95% of the cell population	Cellulases Alginate lyases from abalone acetone powder and *Aplysia punctata*	Multicellular embryos	Kloareg & Quatrano (1987)
F. serratus	5.8×10^6	Cellulase Onozuka R-10 Macerozyme R-10 Crude extract of gland gut of *Aplysia vaccaria*	NT	Mussio & Rusig 2006
F. vesiculosus	1.8×10^6	Cellulase Onozuka R-10 Macerozyme R-10 Crude extract of gland gut of *Aplysia vaccaria*	NT	Mussio & Rusig 2006
Laminaria digitata (sporophyte)	10^6	Cellulase (CELF) Alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*	Rhizoid-bearing callus-like structures, and abnormal bladelets	Benet et al. 1997

Table 2. Continued.

Species[†]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
L. digitata (gametophyte)	10^6	Cellulase (CELF) Alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*	WPR	Benet et al. 1997
L. pallida (gametophyte)	10^5-10^6	Cellulase (CELF) Alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*	WPR	Benet et al. 1997
Macrocystis pyrifera (sporophyte)	10^7-10^8	Cellulase (CELF) Alginate lyases from abalone acetone powder and *Aplysia vaccaria*	Microcalli	Kloareg et al. 1989
M. pyrifera (gametophyte)	2×10^7	Cellulase Onozuka R-10 Macerozyme R-10 Driselase Abalone acetone powder	WPR	Varvarigos et al. 2004

Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Padina arborescens	10^0-10^3	Hepatopancreas extract from *Trochus maculatus* Cellulase Onozuka RS Macerozyme R-10 β-1,4-mannan, porphylan, and β-1,3-xylan-degrading enzymes	NT	Yamaguchi et al. 1989
Petalonia binghamiae	10^8	Cellulase Onozuka RS Macerozyme R-10 Limpet acetone powder	WPR	Chen & Shyu 1994a,b
P. fascia	10^8	Cellulase Onozuka RS Macerozyme R-10 Limpet acetone powder	Survival analysis	Chen & Shyu 1994a
Pylaiella littoralis	0-9.3 x 10^5	Cellulase Onozuka R-10 Macerozyme R-10 Alginate lyase from *Aplysia vaccaria*	WPR	Mejjad et al. 1992

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Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Saccharina japonica	3.2×10^7	Cellulase Onozuka RS Abalone acetone powder	WPR	Matsumura et al. 2000
S. latissima	10^6-10^7	Cellulase (CELF) Alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*	Small laminae (up to c. 5 mm)	Benet et al. 1997
S. longissima	8.83×10^6	Cellulase Onozuka RS Abalone acetone powder	First cell divisions	Matsumura 1998
Sargassum aquifolium	NS	Cellulase Onozuka RS Limpet acetone powder	NT	Fisher & Gibor 1987
S. ilicifolium	10^5	Cellulase Onozuka RS Macerozyme R-10 Limpet acetone powder	NT	Chen & Shyu 1994a
S. muticum	10^7	Enzymes from *S. muticum*-fed amoeba *Trichosphaerium*	NT	Polne-Fuller et al. 1990

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Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
S. polyphyllum	NS	Cellulase Onozuka RS Limpet acetone powder	NT	Fisher & Gibor 1987
Scytosiphon lomentaria	10^4-10^5	Hepatopancreas extract from *Trochus maculatus* Cellulase Onozuka RS Macerozyme R-10 β-1,4-mannan, porphylan, and β-1,3-xylan-degrading enzymes	NT	Yamaguchi et al. 1989
Sphacelaria sp.	4 x 10^3	Cellulysine Pectolyase Y23 Alginate lyases from *Aplysia punctata*, *Haliotis tuberculata*, or *Patella vulgata*	WPR	Ducreux & Kloareg 1988

Table 2. Continued.

Species[+]	Protoplast yield (protoplasts g^{-1} fresh weight)	Enzyme mixture	Regeneration status	Reference*
Undaria pinnatifida (sporophyte)	1.92 x 10^7	Cellulase Onozuka RS Abalone acetone powder	WPR	Matsumura et al. 2001
U. pinnatifida (gametophyte)	1.5-2.5 x 10^7	Cellulase from *Sporotrichum dimorphosporum* Alginate lyases from *Haliotis tuberculata* and *Aplysia vaccaria*	WPR	Zha & Kloareg 1996

[+]Data was not available for *Cladosiphon okamuranus*. *In case of species appearing in multiple publications, only those works with the highest protoplast yield or more advanced status of regeneration were selected. EGTA, Ethylene glycol tetraacetic acid; NS, not specified; NT, not tested; WPR,

3. Factors affecting protoplast isolation, culture and regeneration

The success of a protoplast technology relies on establishing optimal conditions for obtaining, culturing and regenerating protoplasts. Several factors affect different points of these processes and they need to be tested in order to set up successful protocols. The list of the major factors that affect protoplast isolation, culture and regeneration are displayed in Fig. 4. In this section, a detail explanation of each factor is given based on studies in plant cells (land plants and algae). When available, studies in fungal and bacterial cells are also included.

1.4. Factors affecting protoplast isolation

Physiological state of tissue and cell material

Explants (clean and/or sterilized small pieces of tissue) from actively growing zones of the plant, or young tissue are usually recommended for obtaining high yields of protoplasts due to the increased digestibility of their cell walls. In the model organism, *Arabidopsis thaliana*, the mesophyll tissue is usually chosen because it allows the isolation of relatively uniform cells in high quantities (Chawla 2019). Protoplasts from kelps (Laminariales, Phaeophyceae) are isolated in high numbers when using meristematic explants (Benet et al. 1997); and some species of *Gracilaria* (Rhodophyta) give better protoplast yields when they are cultivated at high growth rate (Björk et al. 1990). Callus tissue and cell suspension cultures are also good sources of protoplasts. These should be used in the early log phase of growth (Strauss & Potrykus 1980; Chawla 2019). In the yeast *Saccharomyces*, actively dividing cells from the mid to late log phase are recommended for obtaining high amounts of protoplasts (Shahin 1972).

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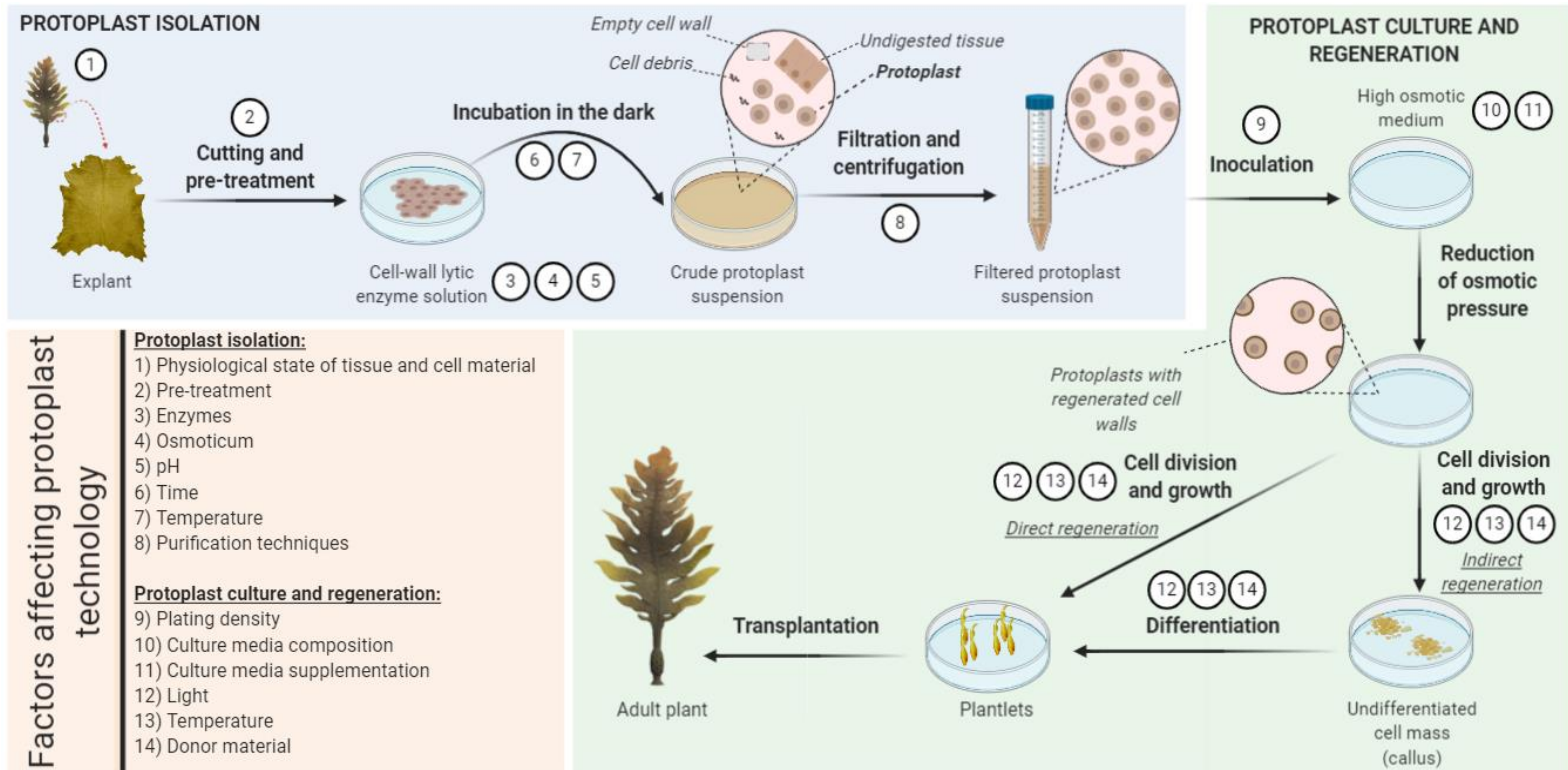
Fig. 4. A schematic view of protoplast isolation, culture and regeneration in brown algae showing the main factors that affect each step.

Pre-treatment

Before incubation in cell wall lytic enzymes, the explants can be treated in different ways for easing the protoplast release. Incubation in a high osmotic solution induces plasmolysis, which allows the cell membrane to detach from the cell wall (Collin & Edwards 1998); avoids accumulation of contaminants during enzymatic digestion (Butler et al. 1990); and helps to maintain cell integrity during isolation (Compton et al. 2000). In *Arabidopsis thaliana* leafs, exposure of mesophyll cells by removing the lower epidermal layer with 3M Magic tape ("Tape-*Arabidopsis* Sandwich" method) have improved the protoplast isolation protocol for this model organism (Wu et al. 2009). Pre-treatment with mercapto-compounds, such as β-mercaptoethanol, usually makes yeast cells more susceptible to lytic enzymes (Ezeronye & Okerentugba 2001). In red and brown seaweeds, the cohesion of their cell walls are maintained by cations such as K^+, Mg^{2+} and Ca^{2+}. Pre-incubation with cation chelators disrupts this cohesion, making the cell wall easier to digest, and improving protoplast yields (Butler et al. 1989; Kloareg et al. 1989; Le Gall et al. 1990; Lafontaine et al. 2011).

Enzymes

The type of enzymes used for cell wall digestion is one of the most important factors during protoplast release. The enzyme mixtures will depend on the composition of the cell wall and their concentrations must be tested to avoid cell damages from contaminants in enzymatic preparations. In plants, the commercial cellulase Onozuka R-10 (from the molds of *Trichoderma reesei* and *T. viride*) and macerozyme R-10 (from *Rhizopus* fungus) are used for degrading cellulose, hemicellulose and pectin, the three primary components of plant cell walls (Chawla 2019). Both enzymes are also widely used for degrading the cell walls of green, red and brown seaweeds, in combination with other commercial or non-commercial enzymes. These ones include, agarase, pectolyase and abalone acetone powder, in red seaweeds (Dipakkore et al. 2005; Gupta et al. 2011; Huddy et al. 2013); and alginate lyases or crude extracts from marine herbivores, in brown seaweeds (Fisher and Gibor 1987; Ducreux and Kloareg 1988; Mejjad et al. 1992; Chen and Shyu 1994a; Benet et al. 1997). Reddy et al. (2006) pointed out that macerozyme R-10 could be excluded from the enzyme preparation if the pectin or its derivates are not part of the algal cell wall. Proteinase K and commercial cellulases from *Trichoderma* sp.

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or *Aspergillus niger* have been used in protoplast isolation from green microalgae and dinoflagellates (Berrios et al. 2015; Abomohra et al. 2016; Levin et al. 2017). Although the cell wall composition of microalgae varies among phylogenetic groups and strains, cellulose is the main component in Cyanophyta, Chlorophyta, Xantophyta and the dinoflagellate *Symbiodinium* (Baldan et al. 2001; Domozych et al. 2012, Levin et al. 2017). Glucanex, a commercial enzyme preparation from *Trichoderma harzianum*, is used to degrade cell wall of filamentous fungi and for spheroplast production from yeast. This preparation contains β-glucanase, cellulase, protease, and chitinase activities, which can digest the main components of fungal cell walls (Rodriguez-Iglesias & Schmoll 2015). Lisozyme, which degraded the peptidoglycan in the bacterial cell wall, is used for generating protoplasts from Gram-negative and positive bacteria. It is expected that this enzyme cannot convert, in most cases, bacterial cells into protoplasts *sensu stricto.* This is because the other cell wall components cannot be effectively degraded (Martin 1963). However, true protoplasts have been confirmed in several ways from the Gram-positive bacteria *Bacillus megaterium* (Weibull 1953; Weibull & Bergström 1958).

Osmoticum

After cell wall digestion, protoplasts are prone to burst if they are not in a solution with the appropriate osmotic pressure. Solutes used as osmoticum can be classified as ionic (salts) or nonionic (sugars), depending on their chemical nature (Bhojwani & Dantu 2013). In plants, red and green algae, mannitol, a metabolic inert sugar, is used as osmotic stabilizer in the range of 450-800 mM (Dipakkore et al. 2005; Liu et al. 2006; Lafontaine et al. 2011; Bhojwani & Dantu 2013; Gupta & Reddy 2018). In brown algae, the ionic osmoticum, NaCl, improves the protoplasts yields from *Laminaria* (Butler et al. 1989; Benet et al. 1997)*,* and is currently used in the protoplast isolation protocol for the model organism *Ectocarpus* (Coelho et al. 2012). In fungi and bacteria, ionic and nonionic osmotica have been used in protoplasts studies (Martin 1963; Rodriguez-Iglesias & Schmoll 2015). These ones include the salts, NaCl, KCl and $MgSO_4$ (ionic); or sucrose, mannitol and polyethyleneglycol (nonionic).

pH

The ideal pH value for protoplast isolation is determined by the enzymatic combination as the activity of the enzymes is pH dependent (Bhojwani & Razdan 1996). Usually, pH values in the range of 4.7-6.5, and 5.0-6.0 are recommended for plant (Dipakkore et al. 2005; Reddy et al. 2008; Bhojwani & Dantu 2013; Huddy et al. 2015) and fungal cells (Chou & Tzen 2015; Ren et al. 2018; Wu & Chou 2019), respectively.

Time

Incubation time varies depending on the species, strains or physiological state of the explant (Barcelo & Lazzeri 1995; Mukherjee & Creamer 2013; Machmudi et al. 2019). The optimal values must be determined after trials (Chawla 2019). Long incubation times can damage protoplast membrane reducing protoplast yield and viability due to the impurities commercial available enzymes contain (Hosoe et al. 2019; Sangra et al. 2019). Too short times can lead to an incomplete cell wall digestion and poor protoplast release (Burris et al. 2016). Incubation periods can range from few minutes (in the Gram-positive bacteria *Bacillus megaterium*; Martin 1963) or less than one hour (in the yeast *Saccharomyces cerevisiae;* Tomo et al. 2013), to 15 (in the ornamental plant *Etlingera elatior*; Silva Júnior et al. 2012) or 24 hours (in the brown seaweed *Sargassum muticum*; Fisher & Gibor 1987).

Temperature

Incubation temperature is closely related to the activities of the enzymes and to the temperature at which the species naturally grows (Davis 1985; Huddy et al. 2013). Samples are usually incubated at 20-30 °C (Bhojwani & Dantu 2013; Rodriguez-Iglesias & Schmoll 2015; Kärkönen et al. 2020). Lower temperatures can affect protoplasts membrane stability, whereas higher temperatures can reduce viability and cause agglutination of cell organelles compromising their suitability for metabolic uses (Kovac & Subik 1970; Rao & Prakash 1995).

Purification techniques

After cell wall digestion, the protoplast preparation contains cell debris, undigested tissue and empty cell walls. In order to remove the contaminants, the suspension is filtered using nylon of

steel sieve of pore size 30-100 µm (Bhojwani & Dantu 2013) and washed by centrifugation with washing medium using a swing-bucket centrifuge (Cao et al. 2016). This last step is repeated twice or thrice for removing traces of enzymes (Kumari 2019). Protoplast can be further purified using a discontinuous gradient for reducing the contaminating enzymes, damaged protoplasts, organelles and cell debris (Hughes et al. 1978). Gradients of Ficoll, Percoll or OptiPrep are preferred over sucrose flotation because they are metabolically inert (Huddy et al. 2015; Chawla 2019).

1.5. Factors affecting protoplast culture and regeneration

Plating density

The amount of protoplasts in the culture medium is critical for cell wall regeneration and cell division. For example, Sangra et al. (2019) reported an optimal plating density of 1-2 x 10^4 protoplasts mL^{-1} in alfalfa (*Medicago sativa*) cultivar Regen-SY, while Jin et al. (2020) found that an initial concentration of 5 x 10^3 protoplasts mL^{-1} gave the maximum regeneration rate in the fungus *Hirsutella sinensis*. Ideal protoplast densities range from 5×10^3 to 1×10^5 protoplasts mL^{-1} in higher plants, and in green and red seaweeds (Evans & Bravo 1983; Reddy et al. 2006; Yeong et al. 2008; Chen et al. 2018). Fast depletion of nutrients or toxins secreted by cells undergoing necrosis can explained the decreased regeneration ability in cultures with high plating density (Davey et al. 2005a; Yeong et al. 2008).

Culture media composition

Protoplasts are initially cultured in the dark or low light intensity in a culture medium with a suitable osmoticum, which is often the same used during isolation (Chawla 2019). Nutritional requirements can vary depending on the species and cultivars. In higher plants, Murashige and Skoog (MS) and Gambor's B5 media, with some modifications, are commonly used (Kärkönen et al. 2020). Other protoplast culture media include KM medium (Kao & Michayluk 1975) for the American elm (*Ulmus Americana*; Jones et al. 2012) and alfalfa (*Medicago sativa*; Sangra et al. 2019), and Nitsch's medium for gravepine (*Vitis vinifera*; Bertini et al. 2019). Calcium, which stabilizes the cell membrane, is usually 2-4 times higher than in the original formulations.

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Glucose is included as a carbon source. The presence of kinetin and auxin is vital for protoplast culture in higher plants. A low kinetin/auxin ratio is suitable for cell division, while high ratio is required for regeneration (Kumari 2019). As the protoplasts start to resynthesize their cell walls, osmotic pressure can be reduced by adding osmotic-free medium. Maintaining protoplasts in high osmotic medium can inhibit their growth (Chawla 2019). In seaweeds, protoplasts are cultured in sterilized natural seawater (Benet et al. 1997; Gupta et al. 2018) or enriched seawater medium (MES or PES medium; Coelho et al. 2012; Wang et al. 2014; Huddy et al. 2015). In some cases, osmoticum is excluded from the medium (e.g. *Ulva* species) without affecting the regeneration ability (Reddy et al. 2006). Small amounts of calcium the media (2-5 mM) have shown positive effects on protoplast viability in brown algae (Benet et al. 1997). Glucose and sucrose have been used as sources of carbon in protoplast culture from the brown filamentous algae *Sphacelaria* (Ducreux & Kloareg 1988); however, they do not seem to play a major role in protoplast culture from seaweed, as their inclusion has shown negative effects on protoplast survival in kelps (Benet et al. 1997), and they are not included in culture media for red seaweed protoplasts (Dipakkore et al. 2005; Huddy et al 2015). Unlike higher plants, the addition of phytohormones is not necessary for cell division and regeneration. The continued presence of osmoticum (>0.4 M) in the culture medium can hamper cell division and further growth (Reddy et al. 2008). As in higher plants, osmotic pressure can be reduced by adding omostic-free medium. It is worth to note that seawater enrichment is not always necessary for a successful regeneration (Benet et al. 1997). In fungi, protoplast culture media and osmotica are variable. Yeast malt potato dextrose agar (YMPDA) with 0.8 M sucrose has been used for protoplast regeneration from the spores of the biocontrol fungus *Pseudozyma flocculosa* (Cheng & Bélanger 2000). Czapek medium with 0.6 M NaCl has shown the best regeneration rate in the taxol-producing fungus *Ozonium* sp. (Zhou et al. 2008). A protoplast regeneration medium with slow-acting nutrient components and 0.6 M mannitol has been successfully used for protoplast regeneration of *Hirsutella sinensis* fungus (Jin et al. 2020). According to Lalithakumari (1996), inorganic salts are more effective for fungi while sugar and sugar alcohols are more advisable for higher plants and yeasts. Calcium does not seem to be an important component in protoplast culture medium from fungal cells. In wood-degrading fungi, glucose levels of 1-2% maximize protoplast regeneration (Chen & Jeffrey 1993).

Apart from liquid culture, other culture techniques have been described for higher plants, such as semi-solid culture (with agar, agarose or alginate as the gelling agent), hanging droplet method and feeder layer, each one with their own advantages (Chawla 2019). For example, reduction of polyphenols (inhibitors of cell growth), and easy manipulation and monitoring of individual cell colonies are benefits of agar culture (Davey et al. 2005b). Semi-solid technique has also been reported for the green microalgae *Botryococcus braunii* (Berrios et al. 2015) and the biocontrol fungus *Pseudozyma flocculosa* (Cheng & Bélanger 2000). Seaweed protoplasts have been cultivated in alginate beads with subsequent formation of microcalli (in *Macrocystis pyrifera*; Kloareg et al. 1989) or new thalli (in *Ulva intestinalis*; Rusig & Cosson 2001). Green and red seaweed protoplasts can attach to nylon threads and nori nets, respectively, in liquid cultures, showing their potential for being used as seed stock in aquaculture (Dipakkore et al. 2005; Reddy et al. 2006)

Culture media supplementation

Besides nutrients, salts and osmotica, culture media can be supplemented with other components that can promote cell survival, division and/or regeneration. Phytosulfokine can increase the plating efficiency of protoplasts from *B. vulgaris* probably due to its antioxidant properties and nurse cell effect (Grzebelus et al. 2012). Nonionic surfactants, such as Pluronic® F-68, can enhance mitotic division of plant protoplast-derived cells by promoting the uptake of nutrients, growth factors, and oxygen (Davey et al. 2005a). Antibiotics can also stimulate cell division. For example, cefatoxine promoted mitotic division and cell colony formation of protoplasts from seedling leaves of the woody plant passionfruit (*Passiflora edulis*) at concentration of 250 µg mL^{-1} (d'Utra Vaz et al. 1993). In algae, antibiotics are used for control bacterial growth, especially when sugars, such as sucrose, are used as carbon source and osmoticum. Kanamycin is used in protoplast culture of the dinoflagellate *Symbiodinium* (Levin et al. 2017); and antibiotic mixtures consisting of penicillin G, streptomycin and chloramphenicol, or streptomycin and erythromycin, are used when culturing protoplast from the brown seaweeds *Ectocarpus* and *Macrocystis pyrifera* female gametophyte (Varvarigos et al. 2004; Coelho et al. 2012). Polyamines play a key role in plant cell morphogenesis. Majewska-Sawka et al. (1997) and Jazdzewska et al. (2000) showed that two polyamines (putrescine and spermine) stimulated cell division in leaf protoplasts of cytoplasmic male sterile and male fertile diploid sugarbeet (*Beta*

vulgaris). Polyamines might also regulate morphogenesis and developmental process in seaweeds. Although there are no reports on the effects of these compounds in seaweed protoplast culture, this system might be ideal for studying polyamines uptake and its role in seaweed developmental processes (Kumar et al. 2015). Some artificial oxygen carriers (e.g. perfluorocarbon liquids and hemoglobin solutions) have been added to protoplast culture media in order to sustain adequate levels of oxygen, which is crucial to maintain protoplast viability and mitotic cell division (Davey et al. 2005a).

Light

In photosynthetic organisms, light is an important factor during protoplast culture. As previously mentioned, protoplast are initially cultured in the dark or dim light. Initiation of cell division does not require light and high intensities applied from the beginning of the culture can inhibit protoplast growth. When light is needed, cultures can be provided with constant illumination or photoperiodic light (Ochatt & Power 1992; Chawla 2019). Nassour and Dorion (2002) reported that protoplast cultures from micropropagated plants of *Pelargonium* x *hortorum* 'Alain' grew better under 14:10-h light/dark photoperiod (light intensity of 120 µmol m^{-2} s^{-1}) than in continuous darkness. The same photoperiod, with a light intensity of 50 µmol m^{-2} s^{-1}, was also effective for protoplast culture of the medicinal plant *Phellodendron amurense* (Azad et al. 2006). Photoperiodic light regimen is also needed in wild pear, *Pyrus communis* var. *pyraster* (Ochatt & Caso 1986). Light intensity is crucial for plant regeneration from protoplast-derived callus, as their optimal values range from dark to 40.5 µmol m^{-2} s^{-1} depending on the species (Ochatt & Power 1992). In seaweeds, light intensity has shown little effect on protoplast regeneration from *Ulva* and *Pyropia* (Reddy & Fujita 1991, Gall et al. 1993), while high illumination has induced photoinhibition in protoplast from the brown algae *Saccharina latissima* (Benet et al. 1994). Color light also plays an important role in cell division and regeneration. White light is recommended for inducing shoot formation from protoplasts in land plants (Compton et al. 2000). Blue and red lights have shown to increase the number of asymmetric cells on protoplasts of the moss *Physcomitrella* (Jenkins and Cove 1983). To date, the effect of color light on seaweed protoplast cultures has not been assessed.

Temperature

Protoplast cultures are usually incubated in a temperature range of 25-30 °C, with most of land plant species being kept at 25 °C. This factor does not seem to be a critical for plant regeneration from protoplast-derived callus; however, there are some species (e.g. *Atropa belladonna* and *Lycopersicon peruvianum*) where temperature is critical for cell division (Ochatt & Power 1992). In green seaweeds, maximum regeneration rate has been reported at 20 °C and 25 °C, while protoplast cultures at 30 °C have failed to develop normal thalli (Reddy & Fujita 1991). In the kelp *Undaria pinnatifida*, direct regeneration has been observed in cultures at 5 °C, while the formation of callus-like masses or gametophyte-like filaments have been reported in cultures at 10 °C or 15 °C, and 15 °C or 18 °C, respectively (Matsumura et al. 2001). Temperatures used in microalgae, red seaweeds and fungi usually match with those ones used for culturing the donor material (Waaland et al. 1990; Yan & Wang 1993; Zhao et al. 2004; Huddy et al. 2015; Levin et al. 2017; Jin et al. 2020).

Donor material

Protoplast regeneration can be largely affected by the genotype of the donor material, type of explant and its culture conditions (Eeckhaut et al. 2013). In *Arabidopsis thaliana*, the percentage of root formation in protoplast cultures has shown variations depending on the genotype used (Damm & Willmitzer 1988). Also, growth conditions of the donor plants and their developmental stage have influenced the formation of microcalli (Masson & Paszkowski 1992). In the red seaweed, *Gracilariopsis lemaneiformis*, juvenile plants are only obtained from tip segments, which also show the highest growth rate and branch formation (Wang et al. 2014). In the brown seaweed, *Saccharina latissima*, protoplasts from the meristematic area of the blade have shown the highest plating efficiency (Benet et al. 1997). In fungi, the use actively growing mycelia (in exponential phase) from the raised fruiting body of pathogenic species *Ganoderma boninense* is fundamental for achieving successful protoplast regeneration (Govender et al. 2016).

4. Problems and aims of the project

Protoplast technology offers a wide range of applications for bacteria, fungi, algae and plants (Martin 1963; Davey et al. 2005a,b; Reddy et al. 2008; Rodriguez-Iglesias & Schmoll 2015;

Echeverri et al. 2019). Since cellular and physiological studies, until genetic transformation, and genome-editing and gene silencing technologies, the use of protoplasts has experienced a re-emergence during the last years (Burris et al. 2016). Protoplast studies in seaweeds have made major progresses in red (Dipakkore et al. 2005; Wang et al. 2014; Huddy et al. 2015; Chen et al. 2018) and, especially, green seaweeds, the latter having reliable protocols for protoplast isolation, culture and regeneration (Gupta et al. 2018; Reddy & Gupta 2018). Although investigations in brown seaweed protoplasts are not scarce, most of the studies have not been sustained during the last few years compared to other seaweeds (Fig. 2). In addition, most protocols rely on non-commercial enzymes or crude extracts for protoplast isolation, which make them expensive, time consuming and low reproducible (Gupta et al. 2011; Inoue et al. 2011). This hampers the possibility of using brown seaweed protoplasts for the wide range of applications protoplast technology provides.

The aims of our investigation were (1) to establish protocols for protoplast isolation, in large amounts, culture and regeneration from seven brown algal species (*Dictyopteris pacifica*, *Ecklonia cava Hecatonema terminale*, *Petalonia fascia*, *Scytosiphon lomentaria*, *Sphacelaria fusca* and *Undaria pinnatifida*) using commercial available enzymes and under optimal conditions; (2) to further improve protoplast regeneration from gametophytes and sporophytes of the economic brown alga *U. pinnatifida* using color light-emitting diodes (LEDs); and (3) to explore the ability of protoplast-derived aposporous filaments (PDAFs) for clonal propagation of *U. pinnatifida* sporophytes.

The chapter 1 of the part 1 provides the first report of protoplast isolation and regeneration from cell-filament suspension cultures of *Hecatonema terminale* (Ectocarpales) using commercial available enzymes.

The chapter 2 of the part 1 optimizes protoplast isolation conditions from the gametophytes of the economic brown seaweed *Undaria pinnatifida* (Laminariales) using response surface methodology, a multivariate approach for developing, improving and optimizing processes.

The chapter 3 of the part 1 proposes a protocol for obtaining protoplasts from all cell types of the filamentous brown algae, *Sphacelaria fusca* (Sphacelariales), which are able to undergo

whole plant regeneration. This is the first report of protoplast isolation and regeneration from *S. fusca*

The chapter 4 of the part 1 provides an improved method for isolating and regenerating protoplast from the sporophyte of the economic brown seaweed *Undaria pinnatifida* (Laminariales) based on commercial available enzymes.

The chapter 5 of the part 1 explores the optimal factors for protoplast isolation from the potential economic brown seaweed, *Petalonia fascia* (Ectocarpales), and describes, for first time, its whole plant regeneration process.

The chapter 6 of the part 1 provides the best commercial lytic enzymes and conditions for protoplast production from three economic brown seaweeds, *Scytosiphon lomentaria* (Ectocarpales), *Dictyopteris pacifica* (Dicytotales) and *Ecklonia cava* (Laminariales). This is the first report of protoplast isolation from *D. pacifica* and *E. cava*.

The chapter 7 of part 1 summarize the main findings regarding optimal conditions for protoplast isolation, culture and regeneration in the brown algal species evaluated.

The part 2 explores the effect of color light, using LEDs, on protoplast division, regeneration and growth from gametophytes and sporophyte of the economic brown seaweed *Undaria pinnatifida*.

The part 3 explores the effects of LED lighting, initial culture density, subculturing and cryopreservation on the ability of PDAFs for clonal propagation of the economic brown seaweed *Undaria pinnatifida*.

II. GENERAL MATERIALS AND METHODS

1. Biological material

A total of seven brown algal species collected from the coasts of Korea were used for protoplast experiments. Cultures were established from six out the seven species. Only field material from *Ecklonia cava* was used for protoplast isolation. The species were chosen based on their anatomical complexity, from filamentous to parenchymatous forms, and importance in basic and applied research (Table 3). Stock cultures were maintained in 100×40 mm Petri dishes containing PES medium under constant illumination at 10 °C, with light intensity 1-5 μmol photons $m^{-2} s^{-1}$ of white fluorescent light. Isolation techniques and growth conditions for each species are explained in detail in every chapter.

2. Culture media and experimental solutions

Provasoli enriched seawater (PES) medium

Stocks and actively growing cultures were maintained in PES medium (Provasoli 1968; Table 4). All components were prepared separately in autoclaved distilled water, and later stored at 4°C in glass amber bottles. To prepare 1 L of PES stock solution, 8 mL of component 1, 3, 4 and 5; 80 mL of component 2; and 200 mL of component 6 and 7 were mixed. The volume was then brought to 1 L with distilled water. The solution was filter-sterilized with a 0.22-μm 500-mL bottle top vaccum filter (Corning, Germany) and stored at 4 °C in 100-mL Erlenmeyer flasks. To prepare the PES medium, 20 mL of the PES stock solution were added to 980 mL of sterilized natural seawater. For growth experiments in *Undaria pinnatifida* gametophytes, a modified PES medium (m-PES), which did not contain Fe^{3+} supplementation, was used in order to effectively inhibit (Suzuki et al. 1995).

Table 3. Brown algal species used in protoplast experiments.

Species	Morphology, anatomy and growth*	Importance	Type of sample	Collection site	Date
Hecatonema terminale	Branched filaments with haplostichous growth by diffuse meristem (Womersley 1987)	Protoplast studies (Mejjad et al. 1992)	Culture	Chuja Island, Jeju	June 26, 2013
Undaria pinnatifida (male and female gametophytes)	Branched filaments with haplostichous growth by diffuse meristem (Womersley 1987)	Aquaculture, cosmetics and production of bioactive compounds (Mori et al. 2004; Wu et al. 2004; SEPPIC 2020)	Culture. Seven strains were used for protoplast isolation.	South and southeast coasts of Korea	2013 and 2016
Sphacelaria fusca	Branched filaments with polystichous growth by an apical cell (Cho 2010)	Cell polarization studies (Rusig et al. 2001)	Culture	Jindo Island, Jeollanam-do	July 27, 2017

Table 3. Continued.

Species	Morphology, anatomy and growth*	Importance	Type of sample	Collection site	Date
U. pinnatifida (sporophyte)	Single, flat, large, and pinnate blade with stipe and holdfast, and with parenchymatous growth by intercalary meristem (Cho 2010)	Food, cosmetics and pharmaceutical applications (Yamanaka & Akiyama 1993; Wang et al. 2018)	Culture. Young sporophytes were obtained from crossing different strains of male and female gametophytes	NA	NA
Petalonia fascia	Small blades with polystichous growth by diffuse meristem (Boo 2010)	Biomedical applications (Kim & Jung 2019)	Culture	Geoje Island, Gyeongsangnam-do	March 16, 2018
Scytosiphon lomentaria	Terete and hollow fronds with polystichous growth by diffuse meristem (Boo 2010)	Food and production of bioactive compounds (Zhuang et al. 2014)	Culture	Gijang county, Busan	February 19, 2020

Table 3. Continued.

Species	Morphology, anatomy and growth*	Importance	Type of sample	Collection site	Date
Dictyopteris pacifica	Branched and complanate thallus with polystichous growth by apical cell row (Cho 2010)	Production of bioactive compounds (Zatelli et al. 2018)	Culture	Uljin county, Gyeongsangbuk-do	May 1, 2018
Ecklonia cava	Single, flat and large blade with stipe and holdfast, and with parenchymatous growth by intercalary meristem (Cho 2010)	Food, phycocolloid extraction, and biomedical applications (Kim & Jung 2019; Hwang & Park 2020)	Field. Young sporophyte	Namhae Island, Gyeongsangnam-do	May 18, 2018

*Growth descriptions were according Charrier et al. (2012). NA, not applicable.

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Table 4. Components for Provasoli enriched seawater (PES) stock solution

Component		Concentration (mM)
① Na$_2$ β-glycerophosphate·5H$_2$O		163
② NaNO$_3$		412
③ Cyanocobalamin (vitamin B$_{12}$)		0.007
④ Thiamine·HCl (vitamin B$_1$)		1.482
⑤ Biotin (vitamin H)		0.020
⑥ Iron-EDTA solution	(NH$_4$)$_2$Fe(SO$_4$)$_2$·6H$_2$O	1.790
	Na$_2$EDTA·2H$_2$O	1.773
⑦ Trace metals solution	H$_3$BO$_3$	18.114
	FeCl$_3$	0.296
	MnSO$_4$·H$_2$O	0.71
	ZnSO$_4$·7H$_2$O	0.076
	CoSO$_4$·7H$_2$O	0.171
	Na$_2$EDTA·2H$_2$O	2.686

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All algal manipulations and media preparations were done under aseptic conditions in a HB-402 clean bench (Hanbaek Scientific Co., South Korea) using pre-sterilized glass or plastic wares.

Solutions for protoplast experiments

For protoplast isolation, samples were treated with chelation solution (Table 5) before enzymatic digestion in order to improve protoplast production. All the components were mixed in autoclaved distilled water and the pH was adjusted to 5.5. The solution was stored at 4°C in glass bottles. Enzymatic solution was prepared in a similar way, adjusted to a pH of 6.3, and stored at 4°C in glass bottles (Table 6). The pH values of the enzymatic solution were later optimized in subsequent experiments. Both solutions were filter-sterilized with 0.22-μm syringe filters (Sartorius, Germany) just before being used in protoplast isolation. The components of chelation and enzymatic solution are according Coelho et al. (2012)

For protoplast culture and regeneration, the culture media and antibiotic solutions tested are presented in the chapter 1 and chapter 4 of the part 1, respectively.

Table 5. Components for chelation solution.

Component	Concentration (mM)
NaCl	665
$MgCl_2 \cdot 6H_2O$	30
$MgSO_4$	30
KCl	20
EGTA-Na_4	20

Table 6. Components of enzymatic solution.

Component	Concentration (mM)
NaCl	400
$MgCl_2 \cdot 6H_2O$	130
$MgSO_4$	22
KCl	160
$CaCl_2 \cdot 2H_2O$	2
MES	10

3. Protocols for protoplast isolation, culture and regeneration

Protoplast isolation was performed by the protocols of Benet et al. (1997) and Coelho et al. (2012) with some modifications. Approximately 100-300 mg plants from 1-L round flasks (for filamentous forms) or explants of 4-6 mm^2 (for more complex forms) were incubated in a 0.22-µm filter–sterilized enzymatic solution (Table 6) containing different combinations of enzymes at 15 °C with shaking at 70 rpm for 15 h in the dark. Protoplasts were filtered by using a 25-µm nylon mesh to remove undigested filaments and concentrated by centrifugation at 100×g for 10 min. Chelation pre-treatment was conducted with a calcium-chelating solution (Table 5) for 20 min prior to enzymatic digestion (Coelho et al. 2012). Protoplasts were washed three times with enzymatic solution by centrifugation for 10 min at 100×g. Alternatively, protoplasts were washed twice with enzymatic solution and laid on a 0.9 M sucrose solution. After centrifugation for 10 min at 100×g, the purified protoplasts appeared as a brown band between the sucrose and enzymatic solution phases. They were harvested and washed once with enzymatic solution.

Cells were dispensed into regeneration medium and cultured at 20°C in the dark and at low protoplast density. After 1-4 days in the dark, osmotic pressure was reduced slowly by adding PES medium (1/5 the volume of the initial regeneration medium). Cultures were exposed to 1-2 µmol photons m^{-2} s^{-1} 14:10-h light/dark photoperiod. Osmotic pressure was further reduced during the next 2 days by adding PES (2/5 the volume of the initial regeneration medium each day). Cultures were finally exposed to 10-40 µmol photons m^{-2} s^{-1} under the same photoperiod. The medium was renewed every week thereafter.

The protocols were adjusted in subsequent experiments to optimize protoplast production and regeneration for each brown algal species tested. A detail explanation of optimal parameters and further modifications of the protocols can be found in every chapter of part 1 and 2.

4. Factors tested during protoplast experiments

The factors tested during protoplast isolation, culture and regeneration (part 1 and 2), along with their response variables, are listed in Table 7. All the protoplast isolation conditions, excepting "origin of explants", were evaluated in *Hecatonema terminale* and *Undaria pinnatifida* gametophytes because of their ease of cultivation and manipulation (Forbord et al. 2018). Additionally, the availability of cell-filament suspension cultures provided a constant source of

tissue and rapidly growing cells (Doelling & Pikaard 1993; Rorrer & Cheney 2004; Wang et al. 2015). The most important factors identified during these experiments were then tested in the rest of species. Protoplast yields was the main response variable during this stage. They were estimated by using a haemocytometer and expressed as protoplasts g^{-1} fresh weight (FW).

The viability of protoplasts was assessed by the red chlorophyll autofluorescence (Pouneva 1997) under a Leica DMi8 inverted microscope (DMi8; Leica, Germany) fitted with a Leica EL6000 external light source for fluorescence excitation and equipped with a 470/40 nm emission filter and a 515 nm suppression filter. The removal (true protoplasts) of cell walls was confirmed by staining the protoplasts with 0.01% calcofluor white M2R (SigmaAldrich, USA; (Bhojwani & Dantu 2013) and by examining under a Leica DMi8 inverted microscope equipped with a 360/40 nm emission filter and a 425 nm suppression filter. Average protoplast sizes were calculated by using ImageJ 1.46r software (Abràmoff et al. 2004) based on 100 cell measurements per repetition. Photomicrographs were taken using a Leica inverted microscope equipped with a Leica DFC450C camera.

Factors affecting protoplast culture and regeneration were investigated only in four species: *Hecatonema terminale*, *Undaria pinnatifida* (both gametophytes and sporophyte), *Sphacelaria fusca* and *Petalonia fascia.* Calcofluor white was included in regeneration medium at a final concentration of 10 µg mL^{-1} for describing cell wall formation. Cell survival was assessed through red chlorophyll autofluorescence and morphological observations. Regeneration was evaluated in detail at different stages in *U. pinnatifida* gametophytes and sporophyte, and *P. fascia*. A modified definition of the term "final plating efficiency" (FPE, Ochatt and Power 1992) was used for assessing the response of cultured protoplasts. Final growth was also estimated in protoplast cultures from *U. pinnatifida* gametophytes under LEDs.

As a result of protoplast experiments in the sporophyte of *Undaria pinnatifida*, PDAFs were obtained and maintained in stock cultures. For testing the ability of PDAFs for clonal propagation of *U. pinnatifida* sporophyte (part 3), four factors were analyzed: 1) LED lighting, 2) initial culture density, 3) subculturing, and 4) cryopreservation. The main response variable was the number of sporophytes produced per treatment. In the case of cryopreservation, post-thawed viability was assessed using erythrosine staining (Kuwano et al. 2004). The ploidy level and genotypes of PDAFs and individual sporophytes were determined using 4′,6-diamidino-2-

phenylindole (DAPI) staining (Zitta et al. 2012) and *U. pinnatifida* microsatellite markers (Daguin et al. 2005; Shan & Pang 2009), respectively.

For statistical analyses, the significance threshold was set at $p = 0.01$ in order to reduce the true Type I error rate (at least 7%, but typically close to 15%) (Sellke et al. 2001). All statistical tests were performed by using Minitab 17.1 (State College, PA, USA) or R v.3.5.1 (R Core Team 2016). All graphs were created in Graphpad Prism 6.0 (GraphPad Software, USA) or Minitab.

The specific methodologies applied regarding analyses conducted, experimental design and statistics are explained in detail in every chapter.

Table 7. Factors and response variables assessed during protoplast isolation, culture and regeneration.

Stage	Factor	Response variable	Species
Protoplast isolation	Growth phase of culture	Protoplast yield (protoplasts g^{-1} FW)	*Hecatonema terminale*, *Undaria pinnatifida* gametophytes
	Origin of explants	Protoplast yield (protoplasts g^{-1} FW)	*U. pinnatifida* sporophyte
	Pre-treatment	Protoplast yield (protoplasts g^{-1} FW)	All
	Enzymes	Protoplast yield (protoplasts g^{-1} FW)	All
	pH	Protoplast yield (protoplasts g^{-1} FW)	*H. terminale*, *U. pinnatifida* gametophytes
	Temperature	Protoplast yield (protoplasts g^{-1} FW)	*H. terminale*, *U. pinnatifida* gametophytes
	Incubation time	Protoplast yield (protoplasts g^{-1} FW)	*H. terminale*, *U. pinnatifida* gametophytes and sporophyte, *Petalonia fascia*
	Osmolarity	Protoplast yield (protoplasts g^{-1} FW)	All

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Table 7. Continued.

Stage	Factor	Response variable	Species
Protoplast culture and regeneration	Plating density	Final plating efficiency (%)	*H. terminale*
	Regeneration medium	Final plating efficiency (%) or morphological observations	*H. terminale*, *U. pinnatifida* sporophyte
	Antibiotic mix	Cell survival (%) or final plating efficiency (%)	*U. pinnatifida* sporophyte, *P. fascia*
	Temperature	Cell survival (%), dividing cells (%, in different stages) and/or final plating efficiency (%)	*U. pinnatifida* sporophyte, *P. fascia*
	Light exposure and start time of osmolarity reduction	Morphological observations and final plating efficiency (%)	*Sphacelaria fusca*
	Light spectra and intensity (using LEDs)	Cell survival (%), protoplast development in different stages, and growth.	*U. pinnatifida* gametophytes and sporophyte

III. RESULTS AND DISCUSSION

PART 1. ESTABLISHMENT OF PROTOCOLS FOR PROTOPLAST ISOLATION AND REGENERATION FROM BROWN ALGAE USING COMMERCIAL AVAILABLE ENZYMES

CHAPTER 1. Protoplast isolation and regeneration from *Hecatonema terminale* (Ectocarpales, Phaeophyceae) using a simple mixture of commercial enzymes*

1. Introduction

Protoplasts are naked living plant cells lacking a cell wall; these cells are potentially totipotent and represent an important biological tool for genetic improvement, tissue culture, and physiological studies (Reddy et al. 2008; Baweja et al. 2009). Their utility in genome-editing and gene silencing technologies has led to a reemergence of protoplast systems over the past few years (Burris et al. 2016). The development of this type of systems is based on the establishment of reproducible protocols for protoplast isolation (Bhojwani & Razdan 1996).

In brown algae, protoplast isolation has been reported in 31 species (Fig. 2, Table 2), mainly commercial and anatomically complex species such as *Undaria pinnatifida* (Xiaoke et al. 2003) and *Saccharina japonica* (Inoue et al. 2011). In these studies, the complex cell walls were digested using alginate lyases or crude extracts from either marine bacteria or the digestive systems of herbivorous marine invertebrates together with commercial cellulases (Reddy et al. 1994, 2008). However, these alginate lyases are not commercially available, which makes the isolation process expensive and time consuming because they have to be produced. In addition, the activities of the crude extracts fluctuate over time (Cocking 1972; Fitzsimons & Weyers 1985; Kloareg et al. 1989), resulting in low or no reproducibility of the results. Thus, protoplast isolation protocols using commercial enzymes are fundamental for the development of protoplast systems in brown algae.

Hecatonema terminale (Kützing) Kylin is a widespread filamentous and heterotrichous brown seaweed characterized by more or less compact basal discs with radiating branched uniseriate filaments, true phaeophycean hairs, occasionally intercalary longitudinal divisions, and

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plurilocular sporangia (Womersley 1987). The reasons for choosing this species for this study were (1) there are no previous reports on protoplast isolation; (2) its primitive anatomy which is ideal for protoplast research (Mejjad et al. 1992); and (3) the availability of cell-filament suspension cultures that provide a constant source of tissue and rapidly growing cells (Doelling & Pikaard 1993; Wang et al. 2015).

In this study, we tested and selected commercial lytic enzymes for protoplast production from cell-filament suspension cultures of *Hecatonema terminale* as a first step in the development of an ideal protoplast system in brown algae. We also evaluated the effect of growth on protoplast yields and optimized protoplast isolation conditions (temperature, incubation time, pH and osmolarity) In addition, we described the regeneration processes of protoplasts isolated with the best enzymatic mixture and optimal parameters.

2.   Materials and methods

Hecatonema terminale was collected by scuba diving at 1 m depth off of Chuja Island, Jeju, Korea, on June 26, 2013. Filaments of *H. terminale* were cultured in 100×40 mm Petri dishes containing PES medium under a 14:10-h light/dark photoperiod with a light intensity of 40 µmol photons $m^{-2} s^{-1}$ at 20 °C. The medium was renewed every 3 weeks. After 3–4 months in culture, plants were accumulated and transferred into 500-mL flat-bottomed round flasks filled with PES medium under aeration with a light intensity of 40–72 µmol photons $m^{-2} s^{-1}$ under the same temperature and photoperiod. The air was sterilized using 0.22-µm surfactant-free cellulose acetate (SFCA) syringe filters (Corning, Germany). One month later, plants were finally transferred to 1-L flat-bottomed round flasks and cultured under the same conditions. The medium was renewed every 2 weeks. Clone spheres were broken up monthly using an Ultra–Turrax homogenizer (T25, Ika–Works Inc., USA) in order to maintain homogenous cell-filament suspension cultures.

Identification of the culture strain

Cultures maintained in 60 mm × 15 mm Petri dishes without agitation were used for morphological characterization. Photomicrographs were taken using a Leica inverted microscope (DMi8; Leica, Germany) equipped with a Leica DFC450C camera. Taxonomic identification was performed according to Clayton (1974) and Womersley (1987) and confirmed molecularly. Genomic DNA extraction, PCR amplification, DNA purification, and sequencing were performed according to Bustamante et al. (2016) using cultured samples. The plastid *rbc*L and mitochondrial COI genes were amplified using the primer combinations described by Kogame et al. (1999) and Lane et al. (2007). The amplified gene sequences were compared to the GenBank nucleotide database using the BLAST program (Altschul et al. 1997).

Protoplast isolation and purification

The commercially available cell wall lytic enzymes used for this study included cellulase Onozuka RS and R-10, macerozyme R-10 (Yakult Co. Ltd., Japan), and alginate lyase (Sigma-Aldrich, USA). Different enzyme combinations were evaluated and the optimal enzyme mix was selected for the highest protoplast yield (Table 8). Various concentrations for each enzyme within optimal mixture were also tested.

Protoplast isolation was performed as described in "General Materials and Methods" using 100–300 mg plants from 1-L round flasks. We investigated the effect of chelation pre-treatment for each enzyme mixture. Protoplast isolation was repeated three times in each treatment.

Table 8. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Hecatonema terminale.*

Commercial enzymes	Composition of enzyme mixtures					
	A	B	C	D	E	F
Cellulase RS (%)	2	-	1	1	2	2
Cellulase R-10 (%)	-	2	1	1	-	-
Macerozyme R-10 (%)	-	-	-	-	2	2
Alginate lyase (U mL^{-1})	3	3	-	3	3	-

60

Growth experiments

H. terminale was cultured in 100-mL Erlenmeyer flaks containing PES medium plus antibiotic mix (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin and 5 mg L^{-1} chloramphenicol) at an inoculation density of 2 g FW L^{-1} under aeration and following the same conditions of isolation and culture process. Five (Holdt et al. 2013) and three biological replicates (Gupta et al. 2011) were used for dry weight calculation and protoplast isolation, respectively. Flasks were taken randomly the first, second day and then each two days for a total period of 14 days. For dry weight calculation, all the biomass was filtered under vacuum using a pre-dried and pre-weighed filter paper (n°20, 150 mm diameter; Hyundai, Korea). The filter paper was then washed thoroughly with distilled water during 30 seconds, dried at 80°C for 48 h and then reweighed. For protoplast isolation, the best enzymatic mix was used.

The duration of the exponential phase was determined using the semi-logarithmic plot of dry weight (x) as function of time (t). The specific growth rate (μ) during the exponential phase was calculated according Holdt et al. (2013) using the following equation:

$$\ln(x) = \mu \cdot (t - t_0) + \ln(x_0)$$

Optimization of protoplast isolation conditions

In order to determinate the optimal conditions for protoplast production, *H. terminale* was incubated for various periods (3, 6, 9, 12 and 15 h), pH (5.8, 6.3, 7 and 7.5), temperatures (15, 20 and 25 °C), and concentrations of alginate lyase (2, 3 and 4 U/ml) and cellulase RS (0, 1, 2 and 3%) at 70 rpm in the dark. These experiments were performed in three biological replicates using the best enzymatic mix and a one-factor-at-a-time design. In addition, the effect of driselase inclusion and osmolarity on protoplast yield was tested. The osmolarity of the enzymatic solution was evaluated in two levels: normal osmolarity (1× = 1570 mOsm L^{-1} H$_2$O) and increased osmolarity (1.6× = 2512 mOsm L^{-1} H$_2$O). Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. These two factors were evaluated in a 2x2 factorial design with four repetitions in each four treatment combinations.

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as described in "General Materials and Methods". The protoplast viability was further confirmed by the exclusion of 0.05% Evans Blue. Also, a bursting assay was carried out according to Björk et al. (1990), to verify the absence of cell walls.

Protoplasts regeneration experiments

Protoplast culture and regeneration was performed as described in "General Materials and Methods". Protoplasts were dispensed into 1 mL of regeneration medium in 24-well tissue culture test plates. To investigate the optimal protoplast density and regeneration medium for protoplast regeneration, the 16 combinations from four initial protoplast densities (2.4×10^3, 9×10^3, 7×10^4, and 1×10^5 protoplasts mL^{-1}) and four regeneration media were tested at 20 °C in the dark (Table 9). After 2 days in the dark, osmotic pressure was reduced slowly using PES medium and cultures were gradually exposed to a final light intensity of 40 μmol photons m^{-2} s^{-1} white LED light (DyneBio Co., Korea), 14:10-h light/dark photoperiod at 20 °C. The medium was renewed every week. The response of cultured protoplasts was assessed using a modified definition of the term "final plating efficiency" (FPE, Ochatt & Power 1992) that is based on distinct developmental stages post first mitotic division. In this study, FPE was defined as the percentage of the originally plated protoplast (P_0) that had proliferated into uniseriate filaments with at least one branch (P_{fb}), which is the basic anatomic architecture of this species and occurs, for *H. terminale*, at least after 13 days of culture. FPE (%) was calculated using the following equation:

$$FPE(\%) = \frac{P_{fb}}{P_0} \times 100$$

Table 9. Regeneration media used in protoplast cultures of *Hecatonema terminale*. Components in bold are the osmotica of each medium. Osmolarities were calculated as 1570 mOsm L^{-1} H$_2$O for RM1, RM2, and RM3 and 1300 mOsm L^{-1} H$_2$O for RM4. Calcofluor was included at a final concentration of 10 μg mL^{-1}.

Regeneration medium	Component	Reference
RM1	PES with 285 mM **NaCl** and 5 mM CaCl$_2$	Mejjad et al. 1992
RM2	PES with 285 mM **NaCl**, 0.7 mM sucrose, and 1 mM glucose	Ducreux and Kloareg 1988
RM3	PES with 570 mM **sorbitol**	Cheng and Shyu 1994a
RM4	Seawater with 50mM **MgCl$_2$** and 75 mM **KCl**	Benet et al. 1997

Statistical analysis

Normality and homoscedasticity were examined by using the Shapiro–Wilk and Levene tests, respectively, prior to conducting parametric tests. Two-way analysis of variance (ANOVA) was used for the comparison of protoplast yield under different enzyme mixtures and chelation pre-treatment, and under driselase inclusion and osmolarity. One-way ANOVA was performed to examine the effect of growth and various isolation conditions on protoplast yields. Tukey's post-hoc test was used when the results of ANOVA were significant. Orthogonal comparison between protoplast yields from exponential and stationary phase was performed. Effect sizes (Sullivan & Feinn 2012) were presented for ANOVA analysis as ω^2, and for orthogonal comparison as r (Field 2009) when significant results were obtained.

3. Results

Strain identification

The vegetative characteristics of *Hecatonema terminale* are shown in Fig. 5. Cultures (with or without aeration) did not present reproductive structures during the study. Suspension cultures consisted of small clumps approximately 1 mm in diameter and branched free-living filaments. Our morphological identification of *H. terminale* was confirmed molecularly. In molecular analyses, a 1245-bp portion of the 1476-bp rbcL gene was sequenced for the strain (MH500017) of *H. terminale*. The rbcL sequence of our strain was 99% identical to *Hecatonema* sp. 86 (AF207802.1). Our COI–5P gene sequence (MH500016) was 99% identical to all *H. terminale* strains reported by Peters et al. (2015).
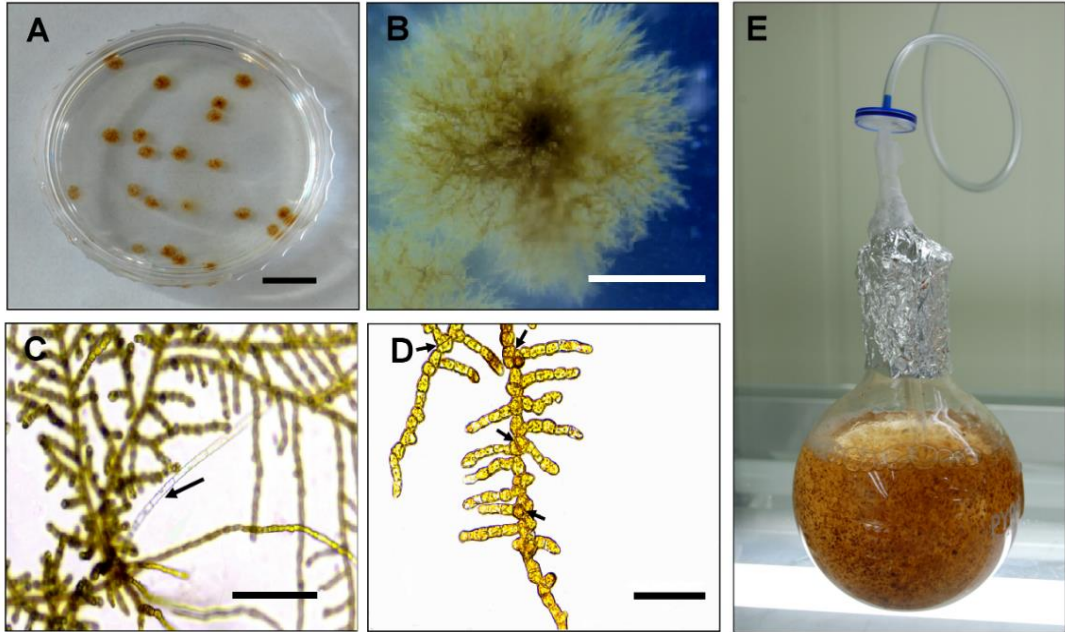
Fig. 5. Thallus of *Hecatonema terminale*. (A) and (B) Cultures without aeration. (C) Thallus with phaeophycean hair (arrow). (D) Filament with longitudinal divisions (arrows). (E) A 2-week-old suspension culture in 1-L flat-bottomed round flasks with aeration. The scale in (A) is 1 cm; the scale in (B) is 1 mm; the scale in (C) is 200 μm; the scale in (D) is 50 μm.

Protoplast isolation using enzymes

Our various mixtures of four enzymes (cellulase RS, cellulase R-10, macerozyme R-10, and alginate lyase) showed that Mixture A (cellulase RS and alginate lyase) with chelation pre-treatment produced the highest number of protoplasts ($3.52 \pm 0.23 \times 10^5$ protoplasts g^{-1} FW), followed by Mixture D (cellulase RS, cellulase R-10, and alginate lyase) with chelation pre-treatment ($2.75 \pm 0.15 \times 10^5$ protoplasts g^{-1} FW), Mixture E (cellulase RS, macerozyme R-10, and alginate lyase) with chelation pre-treatment ($1.20 \pm 0.06 \times 10^5$ protoplasts g^{-1} FW), and Mixture A (cellulase RS and alginate lyase) without chelation pre-treatment ($1.03 \pm 0.05 \times 10^5$ protoplasts g^{-1} FW) (Table 11).

Chelation pre-treatment showed high effects on all kinds of enzyme mixtures (Tables 10 and 11). Addition of a calcium-chelating solution prior to enzymatic digestion significantly increased protoplast amount in mixtures A and D by 2.4- and 2.0-fold, respectively. Interestingly, chelation pretreatment resulted in lower release of protoplasts from enzyme mixtures that did not contain alginate lyase (Table 11).

Table 10. Results of two-way ANOVA evaluating the effect of different enzyme mixtures and chelation pre-treatment (20 mM EGTA) on *Hecatonema terminale* protoplast yield.

Effects	SS	*df*	MS	*F*	*p*	ω^2
Enzyme mixture	26.01	5	5.20	233.03	< .001	0.63
Chelating pre-treatment	7.91	1	7.91	354.36	< .001	0.19
Interaction	7.18	5	1.43	64.28	< .001	0.17

SS, sum of squares; *df*, degrees of freedom; MS, mean square; *F*, F statistic; *p*, significance level; ω^2, omega squared (effect size)

Table 11. Protoplast yields of *Hecatonema terminale* obtained from different enzyme combinations with or without chelation pre-treatment (20 mM EGTA). Uppercase superscript letters indicate significant differences among pre-treatments for each enzyme mixture and lowercase superscript letters among enzyme mixtures ($p < 0.01$). Values are presented as mean $\pm$ SD (n = 3).

Enzyme mixture	Protoplast yield ($\times 10^5$ protoplasts g^{-1} fresh wt.)	
	With chelating pre-treatment	**Without chelating pre-treatment**
A	$3.52 \pm 0.23^{a;A}$	$1.03 \pm 0.05^{a;B}$
B	$0.53 \pm 0.18^{b,c;A}$	$0.10 \pm 0.04^{b;A}$
C	0.27 ± 0.10^{c}	NP
D	$2.75 \pm 0.15^{d;A}$	$0.91 \pm 0.23^{a;B}$
E	$1.20 \pm 0.06^{e;A}$	$0.80 \pm 0.07^{a;A}$
F	0.19 ± 0.16^{c}	NP

NP, no protoplasts

Effect of growth on protoplast yield

The enzyme mix A with chelating pre-treatment was used for this experiment. *H. terminale* presented an exponential phase of 4 days, a specific growth rate of 0.26 days^{-1}, and a 1.78-fold biomass increment (Fig. 6). Stationary phase was reached after 5 days with a final biomass of 4.92 g L^{-1}. Lag and death phase were not observed during the experiment. According to ANOVA analysis, the days of culture had a large effect on protoplast yield ($p < 0.001$, $\omega^2 = 0.72$). The highest ($6.75 \pm 1.21 \times 10^5$ protoplasts g^{-1} FW) and lowest ($2.89 \pm 0.45 \times 10^5$ protoplasts g^{-1} FW) amount of protoplast were reported in the day 2 and 8, respectively, with a linear decrease during this period (Figure 2; $p < 0.001$, $\omega^2 = 0.74$). Protoplast yield remained constant since the day 8 until the end of the experiment ($p = 0.996\text{-}1$). Further experiments showed that cultures maintained for one month or more than one year yielded $2.95 \pm 1.28 \times 10^5$ and $8.30 \pm 2.73 \times 10^3$ protoplast g^{-1} FW, respectively. Protoplast yield in the exponential phase were 1.94 times higher ($6.16 \pm 0.84 \times 10^5$ protoplasts g^{-1} FW) than in the stationary phase ($3.17 \pm 0.21 \times 10^5$ protoplasts g^{-1} FW; $p < 0.001$, $r = 0.89$). Viability was between 99-100% (Evans Blue) and around 98% (red chlorophyll autofluorescence) during the experiment.
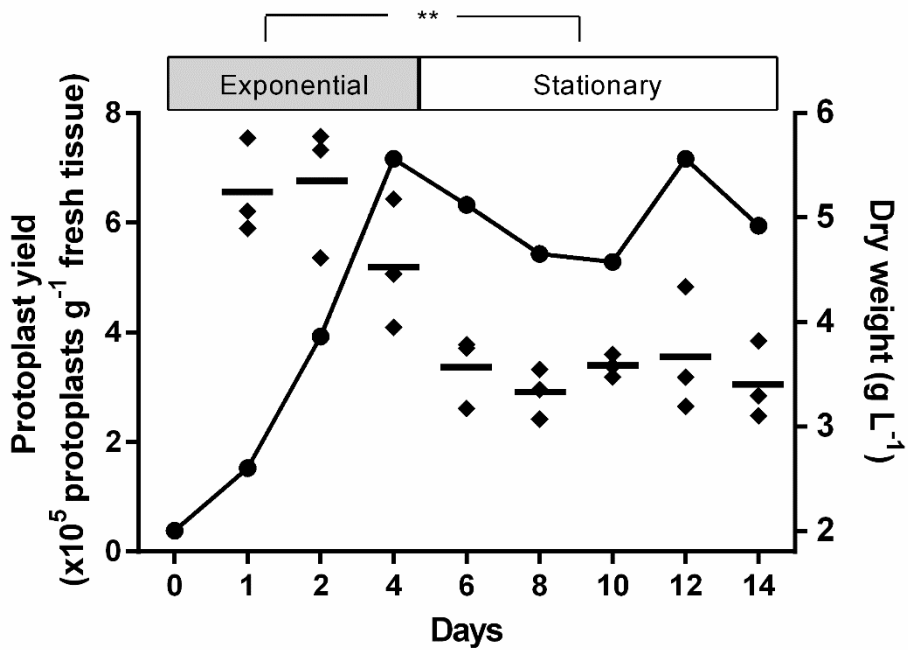
Fig. 6. Growth curve (●) and protoplast yield (♦) from *Hecatonema terminale* suspension cultures during 14 days. Double asterisk (**) indicates highly significant difference between protoplast yields from exponential and stationary phases ($p < 0.001$). Independent data points and averages (horizontal lines) are shown for protoplast yield (n = 3). Only mean values are presented for dry weight (n = 5).

Optimum protoplast isolation conditions

Incubation times tested did not affect protoplast yield ($p = 0.827$); however, it showed a slight increase from 3 ($3.66 \pm 1.09 \times 10^5$ protoplasts g^{-1} FW) to 6 hours ($4.36 \pm 0.44 \times 10^5$ protoplast g^{-1} FW). An incubation time of 6 hours was chosen for all further protoplast isolations since it presented the maximum values for protoplast yield (Fig. 7A). True protoplasts were released in higher amounts as soon as 3 hours after incubation time and during all the experiments (98-99%).

Among the pH values tested, significant differences in protoplast yield were not detected ($p = 0.929$; Fig. 7B). The most suitable temperature was found to be 25°C giving a yield of $20.89 \pm 2.09 \times 10^5$ protoplasts g^{-1} FW ($p < 0.001$, $\omega^2 = 0.93$), which represented an increase of 0.85 and 0.92-fold compared to 15°C and 20°C, respectively (Fig. 7C). A 25°C was selected as the optimum incubation temperature for further protoplast isolations.

Protoplast yields were not affected by alginate lyase ($p = 0.419$; Fig. 7D) and cellulase RS concentrations ($p = 0.114$; Fig. 7E); however, exclusion of cellulase RS from enzyme mixture A did not allow protoplast release. The inclusion of driselase did not improve protoplast yields ($p = 0.141$), while osmolarity increase had a negative effect on protoplast production ($p = 0.006$; Fig. 7F).

Cellulose degradation started to occur from 3 h after treatment of enzyme mixtures (Fig. 8A-C). Although some cell walls were not degraded completely, protoplasts were released through apical or one–sided holes in the cell wall (Fig. 9B). Protoplasts were spherical shape with several discoid chloroplasts (Fig. 9A, C). They were 11.6 ± 2.5 μm in diameter. True protoplast percentages were 98–100% with calcofluor white staining and the bursting assay (Figs. 8C and 9C), while spheroplasts (cells with partially removed cell walls) were 2% (Fig. 9D). The viability of freshly isolated protoplasts was 99–100% with Evans Blue staining and approximately 98% with red chlorophyll autofluorescence.
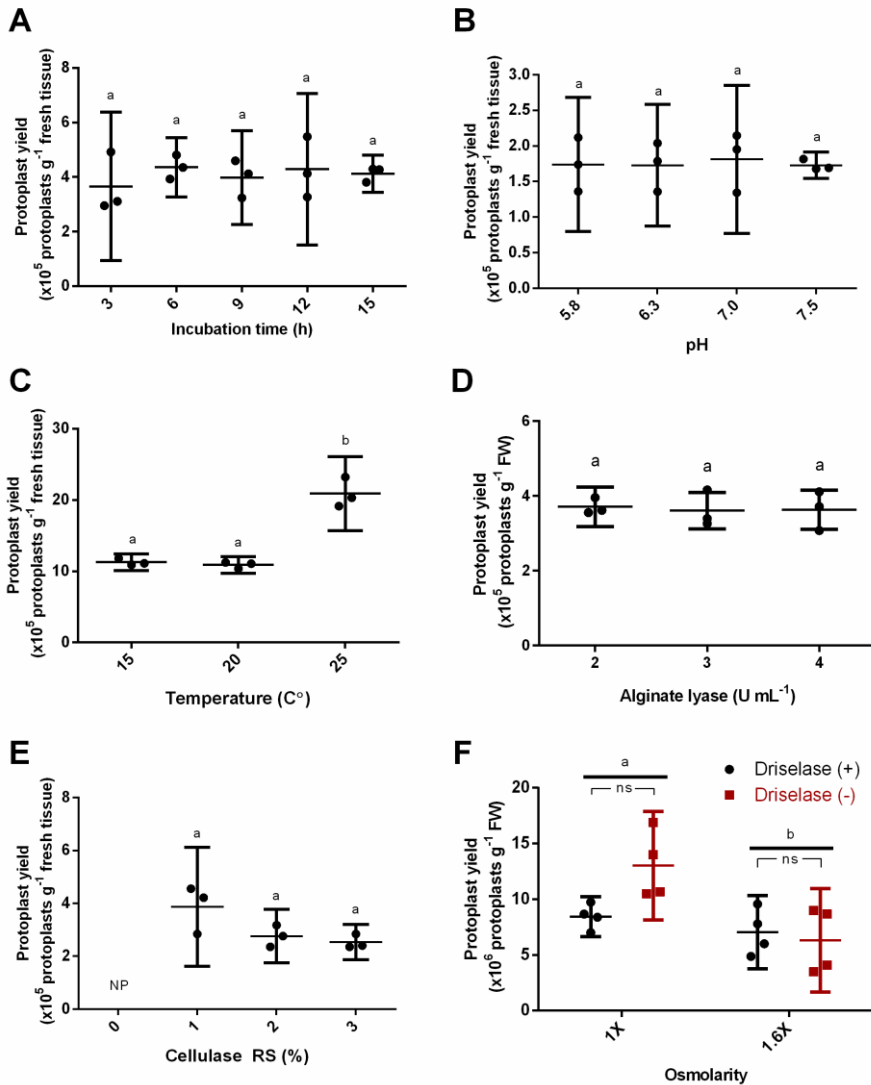
Fig. 7. Effect of different isolation conditions on protoplast yield from *Hecatonema terminale* suspension cultures. (A) Incubation time. (B) pH of enzymatic solution. (C) Incubation temperature. (D) Different concentrations of alginate lyase, in combination with 2% cellulase RS. (E) Different concentrations of cellulose RS, in combination with 3 U mL^{-1} alginate lyase. (F) Effect of osmolarity and driselase inclusion. Independent data points and averages (horizontal lines) are shown (n ≥ 3). Error bars represent 95% confidence intervals. Different letters indicate highly significant differences between means ($p < 0.001$). NP, no protoplasts; ns, no significant difference ($p > 0.01$).
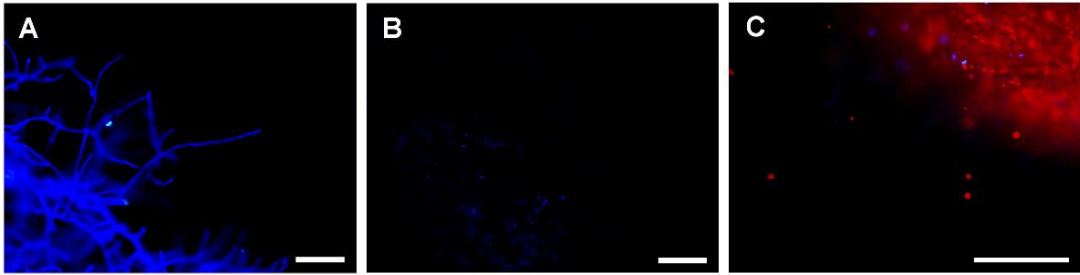
Fig. 8. Protoplast isolation from *Hecatonema terminale* after 3 h of incubation. (A) Thallus with cell wall (blue fluorescence) prior to enzymatic digestion. (B) Thallus with cellulose degradation after 3 h of enzymatic digestion. (C) True protoplasts (spherical cells with red autofluorescence) released from thalli. The scales in (A), (B), and (C) are 200 μm.
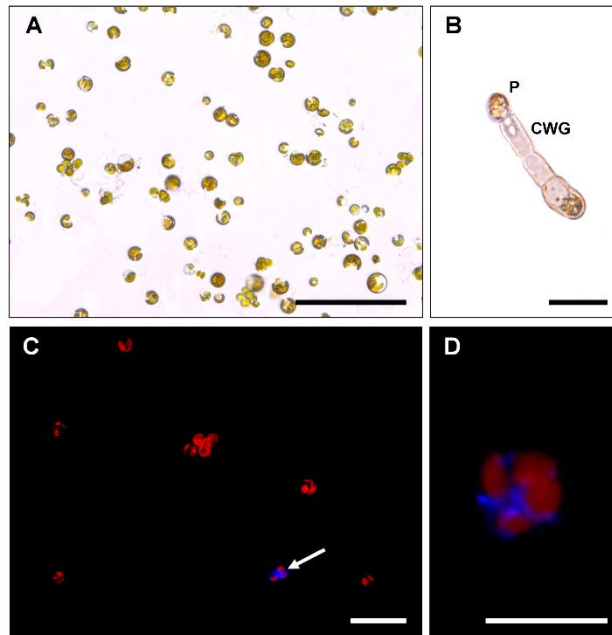
Fig. 9. Protoplast isolation from *Hecatonema terminale*. (A) Freshly isolated protoplasts. (B) Protoplast release. (C) True protoplasts (red autofluorescence) and a spheroplast (arrow). (D) Closer view of a spheroplast. P protoplast, CWG cell wall ghost. The scale in (A) is 100 μm; the scale in (B) is 40 μm; the scale in (C) is 50 μm; the scale in (D) is 20 μm.

Protoplast cell wall formation

After 3 h of culture in 10 μg mL^{-1} Calcofluor white, cell wall formation started with a blue fluorescence spot (positive staining) in one pole of the cell (Fig. 10A-C). After 72 h, additional blue fluorescence spots were detected on 90% protoplasts. Blue fluorescence spots spread across the protoplast surface and covered the whole cell (Fig. 10D-F). After 96 h of culture, 81% of protoplasts with positive staining were regenerated by their cell wall formation.

Protoplast regeneration

After cell wall formation, protoplasts underwent cell division in all combinations of initial protoplast densities and regeneration media. After 13 days of culture, protoplasts in RM1 with lowest initial protoplast density (2.4×10^3 protoplasts mL^{-1}) showed the highest value (74%) of FPE. However, protoplasts in RM3 were poorly developed by the formation of short unbranched filaments (Fig. 11).

Protoplast produced a bud in one pole of the cell prior to first asymmetric cell division (Fig. 12A, B). After 13 days of culture, buds developed into prostrate uniseriate filaments with one (11%) or more (54%) primary branches (Fig. 12C, D). After 17 days of culture, secondary and tertiary branches were produced. The well-defined heterotrichous thalli with phaeophycean hairs were clearly distinguished after 22 days of culture (Fig. 12E, F). *Mikrosyphar*-like plants also developed from protoplasts but in low percentage (9%) (Fig. 12G).
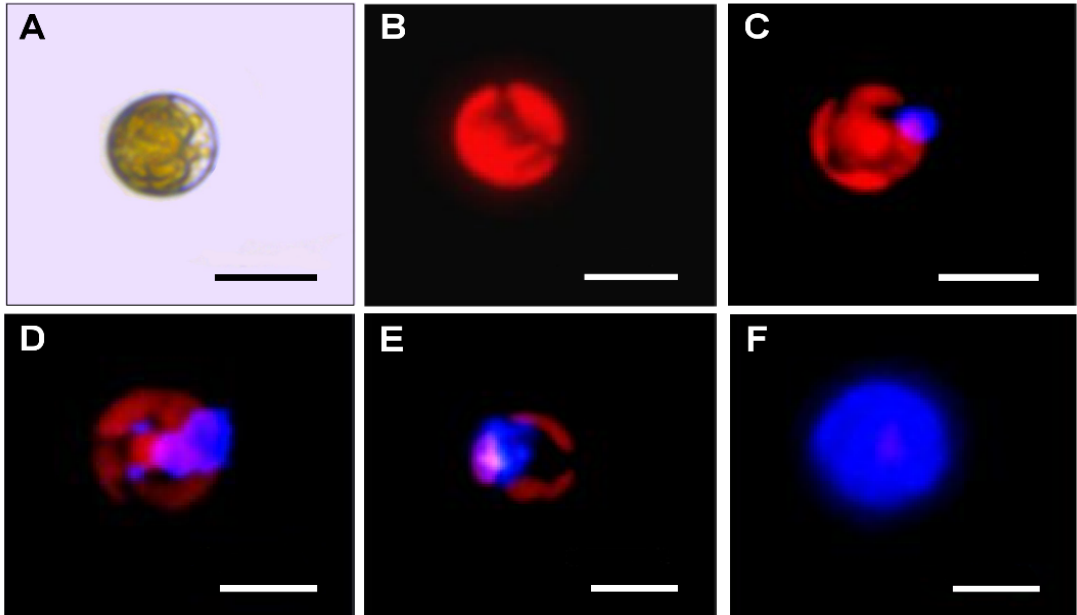
Fig. 10. Cell wall formation of protoplasts from *Hecatonema terminale* cultured in RM1 at 2.4 $\times$ 10^3 protoplasts mL^{-1}. (A) Light microscope image of a freshly isolated protoplast. (B) Fluorescence image of protoplast at initial stage. (C) Fluorescence image of cell wall formation after 3 h of culture. (D) Fluorescence image of cell wall formation after 6 h of culture. (E) Fluorescence image of cell wall formation after 12–48 h of culture. (F) Fluorescence image of cell wall formation after 72 h of culture. Areas showing bright blue fluorescence indicate cellulose deposition. The red autofluorescence of the chlorophyll reveals areas without cell wall. The scale in (A) is 20 μm; the scales in (B), (C), (D), (E) and (F) are 10 μm.
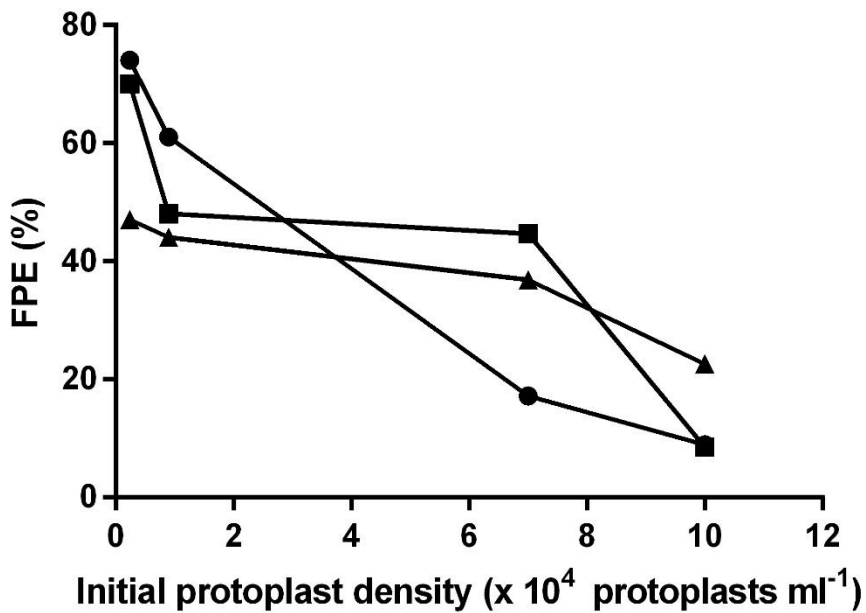
Fig. 11. Final platting efficiency (FPE) of protoplasts from *Hecatonema terminale* cultured at four initial protoplast densities (2.4×10^3 , 9×10^3 , 7×10^4, and 1×10^5 protoplasts mL^{-1}) and in RM1 (●), RM2 (■), and RM4 (▲). RM3 was excluded because of poor protoplast development. RM, regeneration medium.
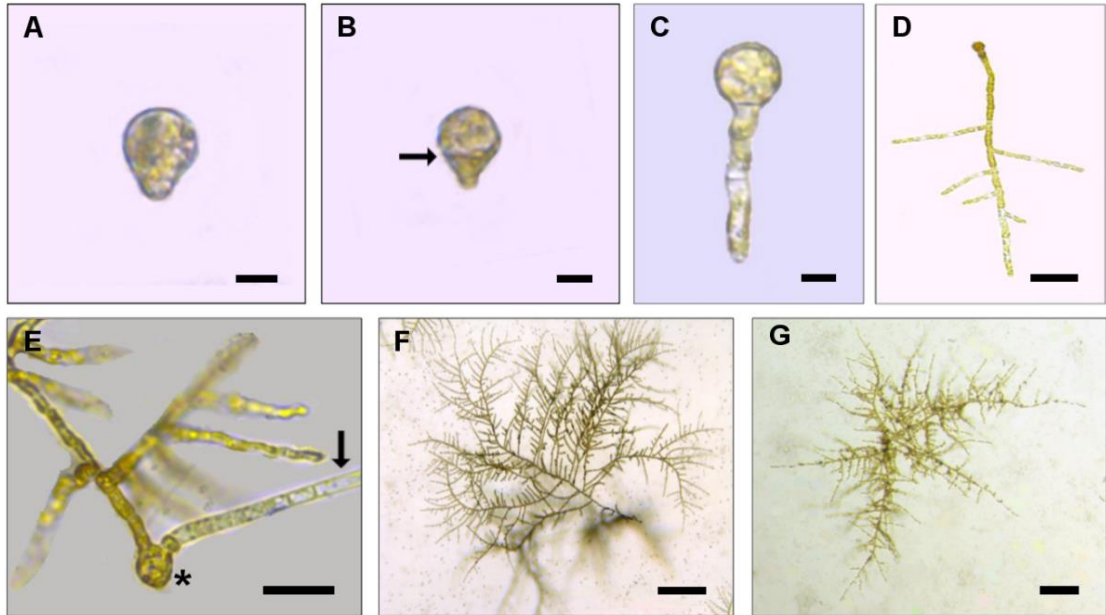
Fig. 12. Regeneration stages of protoplasts from *Hecatonema terminale*. (A) Bud in one pole of the cell prior to first cell division. (B) First asymmetric cell division. The arrow indicates the division plane. (C) 3-celled stage. (D) Branched filament after 13 days of culture. (E) A phaeophycean hair (arrow) arising from the initial protoplast (asterisk) in a regenerated plant. (F) Whole plant regeneration after 22 days of culture. (G) *Mikrosyphar*-like plant developing from a protoplast at 17 days of culture. The scales in (A), (B), and (C) are 10 μm. The scale in (D) is 60 μm. The scale in (E) is 20 μm. The scale in (F) is 400 μm. The scale in (G) is 100 μm.

4. Discussion

The cell walls of brown algae are comprised mainly of alginate and fucoidans and a small amount (1–8%) of cellulose. Although they do not produce xylans, they synthesize fuco-glucurono-xylans, which have been proposed to cross-link cellulose fibers and alginate gels (Cronshaw et al. 1958; Kloareg & Quatrano 1988). Filamentous brown algae tend to have simple cell wall compositions as low or no presence of sulfated fucans in Ectocarpales; (e.g., Kloareg & Quatrano 1988) and the use of simple enzyme combinations on them suggests low alginate content (Chen & Shyu 1994a). In this study, the highest protoplast yield for *Hecatonema terminale* was obtained using a simple mix of commercial cellulase RS (1%) and alginate lyase (3 U mL^{-1}). Although we are using the mixtures of commercial enzymes, our protoplast yield from *H. terminale* is superior to the amount of protoplasts reported for *Sphacelaria* sp. (Ducreux & Kloareg 1988) and in the range of values obtained for *Pylaiella littoralis* (Mejjad et al. 1992). These differences could be due to interspecific variation of cell wall composition and the type of enzyme mixtures used.

Cellulases RS and R-10 are the most common commercial enzymes used for isolating protoplasts from brown algae. In our study, cellulase RS was more effective than cellulase R-10, showing a 9-fold increase in protoplast yield. Removing cellulase RS from the best enzyme mixture (mixture A) yielded no protoplasts. The xylanase and cellulase activity (measured as filter-decomposing-activity) in the RS preparation is 5- and 2-fold higher than in the cellulase R–10 preparation, respectively (Thayer 1985). The main structural role of cellulose and the presence of fuco-glucurono-xylans in the cell wall of brown algae explain the effectiveness of cellulase RS in *Hecatonema terminale* protoplast production. The addition of macerozyme R-10 to the enzyme mixture containing cellulase RS and alginate lyase did not improve protoplast yield. The effect of macerozyme R-10 inclusion in enzyme formulations has not been previously studied in brown algae. Reddy et al. (2006) found that this enzyme was inappropriate for protoplast isolation from *Ulva* and *Monostroma*. They demonstrated that macerozyme R-10 is unnecessary in enzyme mixtures if the algal cell walls do not contain pectin or its derivatives, which is the case for brown algae. Our results also suggest that macerozyme R-10 can be excluded when isolating protoplasts from Phaeophyceae.

The incomplete cell wall digestion reported in this study has been described in *Sphacelaria* sp. (Ducreux & Kloareg 1988), *Pylaiella littoralis* (Mejjad et al. 1992), and female gametophyte of *Macrocystis pyrifera* (Varvarigos et al. 2004). However, this was not an impediment to obtain true and viable protoplasts from *Hecatonema terminale*. Considering the fast degradation of cellulose during the isolation process, incomplete digestion might be due to the specificity of the commercial alginate lyase used in this study. According to the manufacturer, this lyase is a mannuronate lyase, which exhibits inefficient alginate gel disruption in comparison to the high activity of guluronate lyases (Formo et al. 2014). Despite this limitation, our results indicate that alginate lyase from Sigma, in combination with cellulase RS, is effective in protoplast isolation from *Hecatonema terminale*.

The addition of cation chelators has been reported to have a positive effect on protoplast production in Ectocarpales (Mejjad et al. 1992; Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989). However, this positive effect might be also affected by the concentration of the chelator, pH of the solution, incubation time, and alginate content in the sample (Butler et al. 1989; Chen & Shyu 1994a). In our study, the effect of chelation pre-treatment was dependent on the specific type of enzyme mixture. Only combinations containing alginate lyase showed significant increases following incubation in the chelating solution.

Our study characterized the growth of *Hecatonema terminale* suspension cultures by measuring the dry weight during 14 days in order to determinate growth phases. This approach is commonly used in plant cell suspension cultures (Mustafa et al. 2011) and it offers a better growth characterization in contrast with those reported using only growth rate (Björk et al. 1990, 1992; Gómez-Pinchetti et al. 1993). Ducreux & Kloareg (1988), Mejjad et al. (1992) and Benet et al. (1997) described a simplistic relationship between growth and protoplast yield. Our results showed that protoplast yield increased 1.94 times during the exponential phase, and remained constant from the day 8 to the end of the experiment. High percentages of viable and true protoplast were isolated throughout the culture period (98-100%). During cell division and growth, cell wall elasticity is necessary and, consequently, its composition changes (Burns et al. 1982a,b, 1984). Elasticity and rigidity of alginate in the cell wall is due to mannuronate (M) and guluronate (G) blocks respectively. Increasing the M-block content is expected during the exponential phase due to a high cell division. Then, culture enters into stationary phase and cell

division rate reduces leading to an increase in G block content (Kloareg & Quatrano 1988; Lee et al. 2012). The alginate lyase used in this study mainly cleave M blocks and, therefore, could digest better the cell wall during exponential phase. In yeasts and land plants, actively dividing cells have also produced higher amounts of protoplasts (Shahin 1972; Strauss & Potrykus 1980; Nakagawa et al. 1985). To the best of our knowledge, this is the first study on seaweeds to stablish a relationship between growth phases and protoplast yield.

Generally, the reported incubation periods for producing protoplast from Phaeophyceae range from 2-3 hours in *Undaria pinnatifida* and *Saccharina japonica* (Xiaoke et al. 2003; Inoue et al. 2011) to 24 hours in *Sargassum muticum* (Fisher & Gibor 1987) depending upon the types of enzyme used and cell wall complexity. Coelho et al. (2012) recommend 6 hours incubation time for protoplast isolation from *Ectocarpus*. In this study, incubation time did not affect protoplast yield and, for next experiments, it could be reduced to 6 hours without affecting viability and cell wall removal, as Coelho et al. (2012) indicated for *Ectocarpus* strains. This short incubation time can also reduce the negative effect of possible protease activity presents in cellulase RS (Inoue et al. 2011).

pH did not affect the protoplast production in *Hecatonema terminale*. A similar response was observed in *Sphacelaria*, where a pH of 7 did not increase the protoplast yield (Ducreux & Kloareg 1988). Previous studies on protoplasts from filamentous brown algae have used a pH range 5.8-6.5 (Ducreux & Kloareg 1988; Mejjad et al. 1992; Varvarigos et al. 2004; Coelho et al. 2012). It is known that the activity of enzymes used in protoplast isolation is pH dependent (Bhojwani & Razdan 1996). According to the manufacturer, the optimum pH for cellulase RS and alginate lyase is 5–6 and 6.3, respectively. Therefore, a pH range 5.8-6.3 is recommended for protoplast isolation in *H. terminale*.

Temperature affected the protoplast yield in our experiments. Although lower temperatures have been used in other brown filamentous algae (Ducreux & Kloareg 1988; Coelho et al. 2012), protoplasts from *Hecatonema terminale* could be obtained in higher amounts at 25 °C. High temperatures can impact negatively protoplast production and survival (Kovac & Subik 1970; Rao & Prakash 1995); however, in the case of *H. terminale*, it seems that protoplasts are not affected by temperature increase, probably due the fact it is a widespread algae (Guiry & Guiry 2020) and it can tolerate a wide range of temperatures in the nature.

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Driselase is a natural combination mixture of enzymes (e.g. cellulase, hemicellulase and pectinase) that can degraded mixed-linked glucan (MLG; Thibault & Rouau et al. 1990). Recently, MLG has been reported to be present in brown algal cell walls (Salmeán et al. 2017). However, its inclusion on the best enzymatic mixture did not improve protoplast yields. A simple combination of cellulase and alginate lyase was sufficient for producing protoplasts from *Hecatonema terminale*.

Protoplast production can be increased, directly or indirectly, by increasing osmolarity (Xiaoke et al. 2003; Huang et al. 2013). However; high osmolarities can also impair protoplasts metabolism and cell wall synthesis (Shepard & Totten 1977). In our experiments, osmolarity increase reduced significantly the protoplast amounts.

Regeneration ability is one of important parts in protoplast systems (Bhojwani & Razdan 1996). Protoplast was capable of cell wall regeneration and division, although cell division was affected by the initial protoplast densities and regeneration media. In filamentous brown algae, although single initial protoplast density in range from 1×10^2 to 5×10^5 protoplasts mL^{-1} has been used, its effect never has been tested (Ducreux & Kloareg 1988; Mejjad et al. 1992; Kuhlenkamp & Müller 1994; Benet et al. 1997). Our results showed an optimum density of 2.4×10^3 protoplasts mL^{-1} for protoplast regeneration. Higher initial protoplast densities decrease the regeneration ability probably because fast depletion of nutrients (Davey et al. 2005a) or toxins secreted by cells undergoing necrosis (Yeong et al. 2008). Our initial protoplast densities were also tested with different regeneration media (Table 2). RM1 medium containing $CaCl_2$ was the most effective for protoplast regeneration. Calcium may be an important factor for protoplast regeneration of *Hecatonema terminale*. Calcium is known as a crucial regulator in plant growth and development (Hepler 2005). RM4 medium without enrichment produced the lowest FPE. This suggests that enrichment might be necessary for increasing protoplast regeneration.

Cell wall regeneration started after 3 h of culture, which is similar to what was reported for female gametophyte of *Macrocystis pyrifera* (Varvarigos et al. 2004). Complete cell wall regeneration was delayed 1 or 2 days compared with *Sphacelaria* sp., *Pylaiella littoralis*, and *M. pyrifera* (Ducreux & Kloareg 1988; Mejjad et al. 1992; Varvarigos et al. 2004). However, regeneration time for whole plant was similar to Sphacelaria sp. (Ducreux & Kloareg 1988) and faster than ones in *Laminaria digitata* and *M. pyrifera* (Benet et al. 1997). The regeneration

pathway of *Hecatonema terminale* was mainly unipolar and characterized by an asymmetric first cell division after budding and outside of the protoplast. This is distinguished from the protoplast development reported for *Sphacelaria* sp., *Ectocarpus siliculosus*, *L. digitata*, and *M. pyrifera* (Ducreux & Kloareg 1988; Mejjad et al. 1992; Kuhlenkamp & Müller 1994; Benet et al. 1997; Varvarigos et al. 2004). The occurrence of *Mikrosyphar*-like plants has been only reported for *H. streblonematoides* (Loiseaux 1970). Further studies may be necessary to link this stage to the life cycle of *H. terminale*.

In conclusion, although a previous study using commercial enzymes reported low viability and survival of protoplasts from brown algae (Chen & Shyu 1994a), our results show that true protoplasts with high viability and regeneration capacity can be obtained by a simple mixture of commercial enzymes (cellulase RS and alginate lyase) with chelation pre-treatment.

CHAPTER 2. Optimization of protoplast isolation from the gametophytes of brown alga *Undaria pinnatifida* using response surface methodology[*]

1. Introduction

Protoplasts are living plant cells from which cell walls have been removed, usually by digesting with enzymes. These cells are potentially totipotent and represent an important biological tool for genetic improvement, tissue culture and physiological studies (Reddy et al. 2008; Baweja et al. 2009). Although protoplast isolation and culture techniques have been investigated for more than five decades (Cocking 1960), their utility in genome-editing and gene silencing technologies has led to a reemergence of protoplast systems over the past few years (Burris et al. 2016). The development of this type of systems is based on the establishment of reproducible protocols for protoplast isolation and culture (Bhojwani & Razdan 1996).

Undaria pinnatifida (Harvey) Suringar is one of the most important economic seaweeds worldwide. Its macroscopic sporophytic stage is commonly used in food and cosmetics, and extensive research has been done regarding its pharmacological properties (Yamanaka & Akiyama 1993; Wang et al. 2018). The microscopic gametophytic stage has been mainly investigated for aquaculture (Wu et al. 2004; Choi et al. 2005) and cryopreservation studies (Kuwano et al. 2004; Wang et al. 2011). However, recent works have shown that this stage can also produce useful bioactive compounds (Mori et al. 2004; Dwiranti et al. 2012; SEPPIC 2020). Likewise, it has the potential of being cultured in cell bioreactors for the production of biomolecules due to its simple anatomy, undifferentiated state, and easy isolation, culture and manipulation methods (Rorrer et al. 1995; Rorrer & Cheney 2004; Gao et al. 2005). These features make gametophytes suitable for cellular biotechnology techniques, and many of them rely on protoplasts (Reddy et al. 2008).

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Currently, several studies on protoplast isolation and culture from sporophytes and gametophytes of *Undaria pinnatifida* have been limited in using non-commercial enzymes (Wu 1988, Zha & Kloareg 1996, Benet et al. 1997, Matsumura et al., 2001, Xiaoke et al. 2003). Zha & Kloareg (1996) obtained high protoplast yields from *U. pinnatifida* gametophyte using non-commercial alginate lyases from marine herbivores and cellulase from filamentous fungi. Also, Benet et al. (1997) reported inconsistent yields using a mixture of commercial cellulase and non-commercial alginate lyases from *Haliotis tuberculata* and *Pseudomonas alginovora*. However, the use of non-commercial enzymes makes the isolation process expensive and time-consuming because they have to be produced. In addition, contaminating enzymes (e.g. protease, lipase, nuclease and carbohydrolase) in crude preparations and variation of the enzymatic activity from batch to batch compromise the reproducibility of the results (Gupta et al. 2011; Inoue et al. 2011). Thus, an optimal protocol for protoplast isolation using commercial enzymes is fundamental for the development of a protoplast system for this species.

In recent years, response surface methodology (RSM) has been successfully used for optimizing the protoplast yield from plants (Rezazadeh & Niedz 2015), fungi (Wei et al. 2016) and red seaweeds (Gupta et al. 2011). RSM is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. It is particularly applied when dealing with products or processes that are potentially influenced by several input variables. It can identify significant factors and their interactions in a faster and more economical way than the classical one-variable at a time or full factorial experimentation (Reddy et al. 2008; Myers et al. 2009; Krishnaiah et al. 2015). To date, RSM has not been used for protoplast production in brown seaweeds.

In this study, we assessed key parameters influencing protoplast isolation such as enzyme composition, chelation pretreatment, growth, temperature, incubation time, pH and osmolarity for male and female gametophytes of *Undaria pinnatifida* using commercial lytic enzymes. RSM via Box-Behnken design (BBD) was used to design the experiment, generate a model and optimize the protoplast isolation conditions. In addition, we described the regeneration pattern of male and female protoplasts.

2. Materials and methods

Establishment of gametophyte clones

Matured sporophytes were sampled at Geoje and Jindo islands, Korea, in March 1, 2013, and May 5, 2016, respectively. Specimens were transported to the laboratory in cool boxes (5–8 °C) within 48 h after collection. Cleaning of well-matured sori and sporulation were performed according to Redmond et al. (2014). After spore attachment, spores were cultured in PES at low density (10^2–10^1 spores mL^{-1}) in 12-well plates under 14:10-h light/dark photoperiod with light intensity 50–80 μmol photons m^{-2} s^{-1} at 13 °C. Male and female gametophyte clones were isolated after 2 weeks culture and thereafter cultured separately in 100×40 mm Petri dishes filled with 50 mL modified PES medium (m-PES), which did not contain Fe^{3+} supplementation because removing Fe^{3+} can effectively inhibit gametogenesis (Suzuki et al. 1995). Cultures were maintained in vegetative stage at 20 °C under 40 μmol photons m^{-2} s^{-1} of the blue LED (DyneBio Co., Korea) and the same photoperiod (Morita et al. 2003; Xu et al. 2005). Medium was renewed every two weeks. After 7 months in culture, gametophyte clones accumulated much biomass and were transferred into 1-L flat-bottomed round flasks filled with 1 L m-PES medium under aeration. Air was sterilized with 0.22-μm SFCA syringe filters (Corning, Germany). Medium was renewed every two weeks. Light intensity was 40–60 μmol photons m^{-2} s^{-1}. Clone spheres were fragmented monthly using an Ultra-Turrax homogenizer (T25, Ika Works Inc., USA) in order to maintain homogenous cell-filament suspension cultures.

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study were cellulase Onozuka RS and R-10, macerozyme R-10 (Yakult Co. Ltd., Japan), alginate lyase, pectinase and driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA). Different enzyme combinations were evaluated for male (from Geoje Island) and female gametophyte (from Jindo Island) clones and the optimal enzyme mixture was selected for the highest protoplast yield (Table 12). Various concentrations for each enzyme within the optimal mixture were also tested.

Protoplast isolation was performed as described in "General Materials and Methods" with approximately 10–20 mg of *Undaria pinnatifida* at 20 °C for 6 h in the dark. Protoplast isolation

was repeated four times in each treatment. Also, we tested the effect of chelation pre-treatment on protoplast yield.

Growth experiments

Gametophyte clones were inoculated in 1-L flat-bottomed round flasks containing 1 L m-PES at 1 g FW L^{-1}, cultured at 20 °C, under 40–60 µmol photons m^{-2} s^{-1} and 14:10-h light/dark photoperiod following being cut into short fragments (200–300 µm in length) with an Ultra-Turrax homogenizer. FW of gametophyte clones was measured at 2-day intervals after m-PES was squeezed out by hand using a 25-µm aperture nylon mesh. After FW measurement, protoplast isolation was performed using the optimal mixture. Culture duration was maintained over 18 days. The duration of the exponential phase was determined using the semi-logarithmic plot of FW (x) as a function of time (t). Four replicate flasks were used per treatment.

Multivariate optimization

All statistical calculations made for the optimization process were performed using Minitab version 17.1 (USA). The multivariate optimization of experiments was carried out in two steps for male and female gametophytes separately. The initial screening design used was a split-plot design, requiring 32 run experiments, with temperature as the whole plot factor and incubation time (h), pH and osmolarity as the sub-plot factors. Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. Each individual factor was set at two levels, high (+ 1) and low (− 1), based on the findings of preliminary experiments (Table 13). The factors which were significant at $p < 0.01$ were considered to have a significant effect on protoplast yields. A 3k factor BBD was thereafter applied to determine the optimum level of the significant factors identified by split-plot design. Fifteen run experiments were generated by Minitab 17.1 which included 12 base run experiments and triplicates at the center point to estimate experimental errors. The whole experiment was carried out in duplicate giving a total of 30 run experiments. For predicting the optimal condition, the quadratic polynomial equation was fitted to correlate the relationship between variables and response (i.e. protoplast yield), and estimated with the following equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} X_{ii} + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} X_i X_j$$

where Y is the predicted response, b_0 is a constant coefficient, b_i is a linear coefficient, b_{ii} is the quadratic equation, b_{ij} is the interaction coefficient, and X_i and X_j are the input variables. Statistical analysis of the resulted models for the optimum conditions of variables was evaluated by ANOVA at $p < 0.01$. The adequacy of the developed models was tested by performing coefficient of determination (R^2), adequate precision, the Mallow's C_p statistics and coefficient of variance (CV %) (Dawson & Martinez-Dawson 1998; Myers et al. 2009; Yetilmezsoy et al. 2009). The response surface plot was drawn to visualize the input-output relationships.

Validation of the model

The optimized conditions were validated for the maximum protoplast yield based on the values obtained using RSM. The experimental and predicted values were compared in order to determine the validity of the models. Protoplast isolation was repeated three times. Further validation was performed with additional gametophyte clones isolated from different sites in Korea.

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Table 12. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Undaria pinnatifida* gametophytes.

Commercial enzymes	Composition of enzyme mixtures								
	A	B	C	D	E	F	G	H	I
Cellulase RS (%)	2	-	1	1	2	2	2	2	2
Cellulase R-10 (%)	-	2	1	1	-	-	-	-	-
Macerozyme R-10 (%)	-	-	-	-	2	2	-	-	1.3
Alginate lyase (U mL^{-1})	3	3	-	3	3	-	3	3	3
Pectinase (%)	-	-	-	-	-	-	-	1.1	1.1
Driselase (%)	-	-	-	-	-	-	1	1	1

Table 13. Level of factors used in split-plot design for protoplast production from *Undaria pinnatifida* gametophytes. HEPES was used for adjusting pH at 7.5. Osmolarity was calculated as 1570 (initial osmolarity) and 3140 (2 times the initial osmolarity) mOsm L^{-1} H_2O for enzymatic solution at 1× and 2×, respectively.

Factor	Levels	
	Low (-1)	High (+1)
Temperature (C°)	15	25
Incubation time (h)	3	15
pH	5.8	7.5
Osmolarity	1×	2×

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as described in "General Materials and Methods".

Protoplast regeneration

Protoplast culture and regeneration was performed as described in "General Materials and Methods" using 2 mL of RM1 with some modifications. Protoplasts were cultured at 20°C in the dark and at initial protoplast density of 9×10^3 protoplasts mL^{-1} plus antibiotic mix (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin and 5 mg L^{-1} chloramphenicol). Although protoplasts could regenerate in antibiotic-free medium, antibiotic addition greatly reduced bacterial overgrowth allowing a better characterization of the regeneration process. After 4 days in the dark, osmotic pressure was reduced slowly using PES medium and cultures were gradually exposed to a final intensity of 40 µmol photons m^{-2} s^{-1} white LED. The culture medium was renewed weekly.

Statistical analysis

Normality and homoscedasticity were checked with Shapiro-Wilk and Levene tests, respectively, prior to conducting two-way ANOVA for the comparison of protoplast yield under different enzyme mixtures and chelation pre-treatment. One-way ANOVA was performed to examine the effects of different enzyme concentrations in the optimal mixture. Only treatments with at least three non-zero values were considered in these analyses. The effect of days in culture on protoplast yield was assessed using repeated-measures ANOVA. The Huynh-Feldt correction was used to adjust for sphericity violations when necessary. Tukey's post hoc test was used when the results of ANOVA were significant. In the case of repeated-measures ANOVA, the Bonferroni method was chosen for multiple comparisons (Park et al. 2009). Effect sizes (Sullivan & Feinn 2012) were presented for ANOVA analysis as *ω2* or η2p (Field 2009, Lakens 2013). All the statistical tests were performed using Minitab 17.1, with the exception of Mauchly's sphericity test and Huynh–Feldt correction which were conducted with R programming package within the graphical interface R-Studio (R Core Team 2016, https://www.R-project.org/).

3. Results

Protoplast isolation using enzymes

Our various mixtures of six enzymes (cellulase RS, cellulase R-10, macerozyme R-10, alginate lyase, pectinase and driselase) showed that, for male gametophyte, mixture G (cellulase RS, alginate lyase and driselase) with chelation pretreatment produced the highest number of protoplasts ($1.17 \pm 0.13 \times 10^5$ protoplasts g^{-1} FW), followed by mixture G without chelation pre-treatment ($0.44 \pm 0.25 \times 10^5$ protoplasts g^{-1} FW), mixture A with chelation pre-treatment ($0.30 \pm 0.24 \times 10^5$ protoplasts g^{-1} FW) and mixture D (cellulase RS, cellulase R-10 and alginate lyase) with chelation pre-treatment ($0.05 \pm 0.03 \times 10^5$ protoplasts g^{-1} FW). For female gametophyte, mixture G with chelation pre-treatment produced the highest number of protoplasts ($2.66 \pm 0.35 \times 10^5$ protoplasts g^{-1} FW), followed by mixture A with chelation pretreatment ($2.41 \pm 0.70 \times 10^5$ protoplasts g^{-1} FW), mixture A without chelation pre-treatment ($1.50 \pm 0.73 \times 10^5$ protoplasts g^{-1} FW) and mixture H (cellulase RS, alginate lyase, driselase and pectinase) with chelating pre-treatment ($1.45 \pm 0.47 \times 10^5$ protoplasts g^{-1} FW). Significant variation in protoplast yields was recorded for both gametophytes under different mixtures and chelation pretreatment. A significant interaction between the effects of both factors was observed only for male gametophyte (Table 14). Chelation pre-treatment showed positive effects with mixture G (1.7-fold increase) for male gametophyte, while mixture G (1.5-fold increase) and H (5.6-fold increase) for female gametophyte (Table 15). Removing cellulase RS or alginate lyase from the optimal mixture (mixture G) reduced significantly the protoplast production or gave inconsistent yields. Increasing or decreasing the enzyme concentrations did not affect the protoplast yield (Fig. 13 and 14). For the next experiments, enzyme concentrations in the optimal mixture were set at 1% cellulase RS, 2 U mL^{-1} alginate lyase and 1% driselase with chelation pre-treatment.

Effect of growth on protoplast yield

Male gametophyte presented a fast biomass increase during the first 10 days of culture (exponential phase), followed by a gradual decrease in the next 8 days (deceleration phase). Cultures did not reach the stationary phase during the experiments. Female gametophyte presented a lag phase during the first 4 days of culture, followed by a fast biomass increase for 8 days (exponential phase) and then by a stationary phase. Protoplast yield was affected by days

in culture (male gametophyte: $p < 0.001$, $\eta^2_p = 0.88$; female gametophyte: $p = 0.006$, $\eta^2_p = 0.85$), reaching its maximum values in the mid-exponential phase (day 6), for male gametophyte, and at the end of the lag phase (day 4), for female gametophyte (Fig. 15).

Optimization of protoplast yield by response surface methodology

For both gametophytes, the split-plot design revealed a significant negative effect of pH while osmolarity was found to have a positive effect. For female gametophyte, time presented a significant positive effect. The range of temperatures tested did not affect protoplast production (Fig. 16). For the design of optimization, parameters with significant effects were used for generating the BBD experimental design algorithm. The results derived from the BBD are displayed in Table 16, with the statistical analyses (ANOVA) of the results given in Table 17. The polynomial models were mathematically expressed as follows:

$$Y_{male} = -461 - 1.12X_1 + 93.20X_2 + 271.70X_3 - 0.1317X_1^2 - 6.88X_2^2 - 70.4X_3^2$$
$$+ 0.459X_1X_2 + 0.064X_1X_3 - 8.16X_2X_3$$

$$Y_{female} = -224.3 - 4.23X_1 + 44.0X_2 + 160.7X_3 - 0.0842X_1^2 - 3.42X_2^2 - 38.26X_3^2$$
$$+ 0.804X_1X_2 + 0.130X_1X_3 - 5.89X_2X_3$$

Y represents the response factor (protoplast yield) for male or female gametophytes, and X_1, X_2 and X_3 are time, pH and osmolarity, respectively. Using these formulas, the quadratic regression model was estimated by ANOVA. The model was statistically robust. In this case, X_2 and X_3^2 (male gametophyte) and X_2, X_1^2, X_3^2 and X_1X_2 (female gametophyte) were significant model terms. Lack of fit was not significant in both models. The R^2 coefficient, a measure of goodness of fit, showed that the models were able to explain 76.22% and 87.16% of the total variation for male and female gametophytes, respectively. Adequate precision measures the signal to noise ratio. In this study, the ratio was 6.88 and 11.63 for male and female gametophytes, respectively. The Mallow's C_p statistic was 10.00 for both models while values of CV% were 46.49% and 30.99% for male and female gametophytes, respectively.

There was a gradual and uniform decrease in protoplast yield for male gametophyte with the increase in pH from 5.8 to 7.5 (Fig. 17A). In female gametophyte, this decrease was more pronounced due to the significant interaction between pH and incubation time. The protoplast yield increased with the time over the period of 3 to 12 h followed by a slight decrease after 12 h (Fig. 17B). Osmolarity showed a quadratic effect in both models. Protoplast yield increased with osmolarity from $1\times$ (1570 mOsm L^{-1} H_2O) to $1.6\times$ (2512 mOsm L^{-1} H_2O) followed by a decrease over $1.6\times$ (Fig. 17A, C).

Determination and validation of optimum conditions

Applying the methodology of desired function, the optimum levels of various parameters that maximized the protoplast yield were obtained and it indicates that an incubation time of 6.63 h, pH of 6.06 and $1.58\times$ osmolarity (2481 mOsm L^{-1} H_2O) give 3.26×10^6 protoplasts g^{-1} FW for male gametophyte; and an incubation time of 3.8 h, pH of 5.8 and $1.65\times$ osmolarity (2591 mOsm L^{-1} H_2O) give 2.15×10^6 protoplasts g^{-1} FW for female gametophytes. Under these conditions, experimental values were found to be 3.12 ± 0.51 and $2.11 \pm 0.08 \times 10^6$ protoplasts g^{-1} FW for male and female gametophytes, respectively. These mean values were compared with the predicted values and indicated the suitability of the developed quadratic models. The percentage deviation of the experimental and theoretical results was found as -4.29% (male) and -1.86% (female). Protoplasts were released through apical or one-sided holes in the cell wall (incomplete cell wall digestion). They were spherical in shape with an average diameter of 9.50 ± 1.64 μm in male gametophyte and 12.38 ± 2.68 μm in female gametophyte, and with peripherally arranged chloroplasts. Calcofluor white staining confirmed the absence of cell walls and red chlorophyll autofluorescence showed they were viable (Fig. 18). Other gametophyte strains from Korea showed protoplast yields ranging from 2.57 to 8.17×10^6 protoplasts g^{-1} FW in male gametophyte and 2.74 to 7.91×10^6 protoplasts g^{-1} FW in female gametophyte (Table 18).

Table 14. Results of two-way ANOVA evaluating the effect of different enzyme mixtures and chelation pre-treatment (20 mM EGTA) on protoplast yield from *Undaria pinnatifida* gametophytes.

	Effects	SS	*df*	MS	*F*	*p*	ω^2
	Enzyme mixture	2.76	2	1.38	57.35	< 0.001	0.62
Male	Chelation pre-treatment	0.68	1	0.68	28.11	< 0.001	0.15
	Interaction	0.52	2	0.02	10.72	0.001	0.11
	Enzyme mixture	13.91	5	2.78	14.53	< 0.001	0.40
Female	Chelation pre-treatment	8.65	1	8.65	45.18	< 0.001	0.26
	Interaction	3.13	5	0.63	3.27	0.015	NS

SS, sum of squares; *df*, degrees of freedom; MS, mean square; *F*, F statistic; *p*, significance level; ω^2, omega squared (effect size); NS, no significant

Table 15. Protoplast yield from *Undaria pinnatifida* gametophytes obtained using different enzyme combinations with or without chelation pre-treatment (20 mM EGTA). Uppercase superscript letters indicate significant differences among pre-treatments for each enzyme mixture and lowercase superscript letters among enzyme mixtures ($p < 0.01$). Values are presented as mean ± SD (n = 4).

Enzyme mixture	Protoplast yield ($\times 10^5$ protoplasts g^{-1} FW)			
	Male		Female	
	With chelation pre-treatment	Without chelation pre-treatment	With chelation pre-treatment	Without chelation pre-treatment
A	0.30±0.24[a;A]	0.02±0.02[a;A]	2.41±0.70[a,c;A]	1.50±0.73[a;A]
B	0.03±0.02[a;A]	0.02±0.03[a;A]	0.51±0.39[b;A]	0.56±0.10[a,b;A]
C	IY	IY	IY	0.09±0.06[b]
D	0.05±0.03[a]	IY	1.41±0.41[a,b,c;A]	0.67±0.04[a,b;A]
E	0.05±0.04[a]	IY	1.17±0.38[b,c;A]	0.52±0.21[a,b;A]
F	NP	NP	IY	IY
G	1.17±0.13[b;A]	0.44±0.26[b;B]	2.66±0.35[a;A]	1.06±0.65[a,b;B]
H	IY	NP	1.45±0.47[a,b,c;A]	0.22±0.15[b;B]
I	IY	NP	0.46±0.37[b]	IY

IY, Inconsistent yield (at least two zero values per treatment); NP, No protoplasts

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Fig. 13. Effect of enzyme concentrations on protoplast yield from *Undaria pinnatifida* male gametophyte. (A) The effect of various concentrations of cellulase (%, w/v) on protoplast yield, in combination with 3 U mL^{-1} alginate lyase and 1% driselase. (B) The effect of various concentrations of alginate lyase (U mL^{-1}) on protoplast yield, in combination with 2% cellulase and 1% driselase. (C) The effect of various concentrations of driselase (%, w/v) on protoplast yield, in combination with 2% cellulase and 3 U mL^{-1} alginate lyase. Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different postscripts indicate a significant difference ($p < 0.01$). IY, inconsistent yield.

Fig. 14. Effect of enzyme concentrations on protoplast yield from *Undaria pinnatifida* female gametophyte. (A) The effect of various concentrations of cellulase (%, w/v) on protoplast yield, in combination with 3 U mL^{-1} alginate lyase and 1% driselase. (B) The effect of various concentrations of alginate lyase (U mL^{-1}) on protoplast yield, in combination with 2% cellulase and 1% driselase. (C) The effect of various concentrations of driselase (%, w/v) on protoplast yield, in combination with 2% cellulase and 3 U mL^{-1} alginate lyase. Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different postscripts indicate a significant difference ($p < 0.01$).

Fig. 15. Growth curve (▲) and protoplast yield (bars) from *Undaria pinnatifida* gametophytes. (A) Male gametophyte. (B) Female gametophyte. Different letters indicate significant differences for protoplast yield (n = 4, $p < 0.01$). Only mean values are presented for FW (n = 4). The duration of each growth phase is indicated.
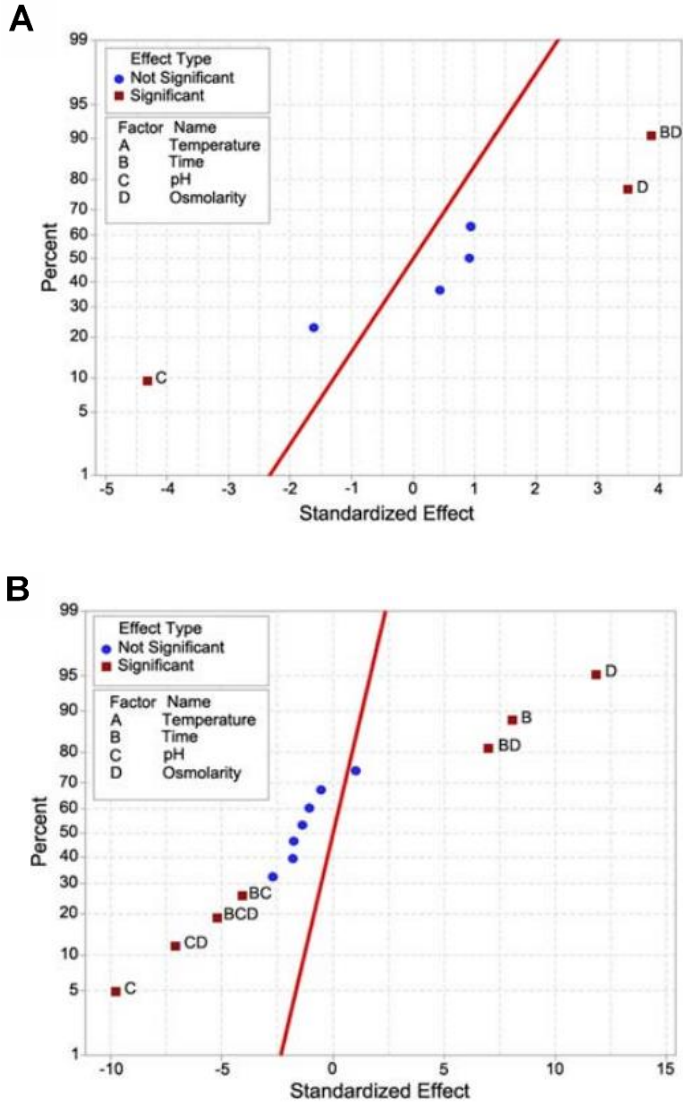
Fig. 16. Normal probability plot of the standardized effects for protoplast yield from *Undaria pinnatifida* gametophytes. (A) Male gametophyte. (B) Female gametophyte.

Table 16. Box-Behnken experimental design with three independent variables for protoplast production from *Undaria pinnatifida* gametophytes. HEPES was used for adjusting pH at 7.5. Osmolarity was calculated as 1570 (initial osmolarity), 2355 (1.5 times the initial osmolarity) and 3140 (2 times the initial osmolarity) mOsm L^{-1} H_2O for enzymatic solution at 1×, 1.5× and 2×, respectively. Each run was carried out in duplicate. Values are mean of duplicate runs.

Run	Incubation time (h)		pH		Osmolarity		Protoplast yield, Y (x 10^5 protoplasts g^{-1} FW)	
	X_1	Code	X_2	Code	X_3	Code	Male	Female
1	9	0	5.8	-1	1X	-1	4.63	1.73
2	9	0	5.8	-1	2X	+1	18.00	16.07
3	9	0	7.5	+1	1X	-1	3.91	0.96
4	9	0	7.5	+1	2X	+1	3.42	5.29
5	3	-1	6.65	0	1X	-1	4.83	3.18
6	3	-1	6.65	0	2X	+1	11.46	8.91
7	15	+1	6.65	0	1X	-1	3.60	1.21

Table 16. Continued.

Run	Incubation time (h)		pH		Osmolarity		Protoplast yield, Y (x 10^5 protoplasts g^{-1} FW)	
	X_1	Code	X_2	Code	X_3	Code	Male	Female
8	15	+1	6.65	0	2X	+1	11.00	8.50
9	3	-1	5.80	-1	1.5X	0	33.57	20.89
10	3	-1	7.50	+1	1.5X	0	14.67	7.81
11	15	+1	5.80	-1	1.5X	0	21.37	9.08
12	15	+1	7.5	+1	1.5X	0	11.83	12.40
13[a]	9	0	6.65	0	1.5X	0	32.30	18.05
14[a]	9	0	6.65	0	1.5X	0	25.82	17.04
15[a]	9	0	6.65	0	1.5X	0	32.10	19.45

[a] The left point was replicated three times.

Table 17. ANOVA table of the Box-Behnken design model for optimized parameters.

Factors	Statistics				
	SS	*df*	**MS**	*F*	*p*
Male gametophyte					
Model	3329.21	9	369.91	7.12	<0.001
X_1	69.90	1	69.90	1.35	0.260
X_2	477.94	1	477.94	9.20	0.007
X_3	181.08	1	181.08	3.49	0.077
X_1^2	166.11	1	166.11	3.20	0.089
X_2^2	182.63	1	182.63	3.52	0.075
X_3^2	2289.56	1	2289.56	44.09	<0.001
$X_1 X_2$	43.79	1	43.79	0.84	0.369
$X_1 X_3$	0.30	1	0.30	0.01	0.941
$X_2 X_3$	96.24	1	96.24	1.85	0.189
Residual	1038.68	20	51.93		
Lack of fit	88.07	3	29.36	0.53	0.671
Pure error	950.61	17	55.92		

Table 17. Continued.

Factors	Statistics				
	SS	*df*	MS	*F*	*p*
Female gametophyte					
Model	1306.38	9	145.153	15.09	<0.001
X_1	23.04	1	23.04	2.39	0.137
X_2	113.59	1	113.59	11.81	0.003
X_3	250.82	1	250.82	26.07	<0.001
X_1^2	67.83	1	67.83	7.05	0.015
X_2^2	45.15	1	45.15	4.69	0.043
X_3^2	675.61	1	675.61	70.22	<0.001
$X_1 X_2$	134.43	1	134.43	13.97	0.001
$X_1 X_3$	1.22	1	1.22	0.13	0.725
$X_2 X_3$	50.07	1	50.07	5.20	0.034
Residual	192.43	20	9.62		
Lack of fit	14.62	3	4.88	0.47	0.710
Pure error	177.81	17	10.46		

SS, sum of squares; *df*, degrees of freedom; MS, mean square; *F*, F statistic; *p*, significance level
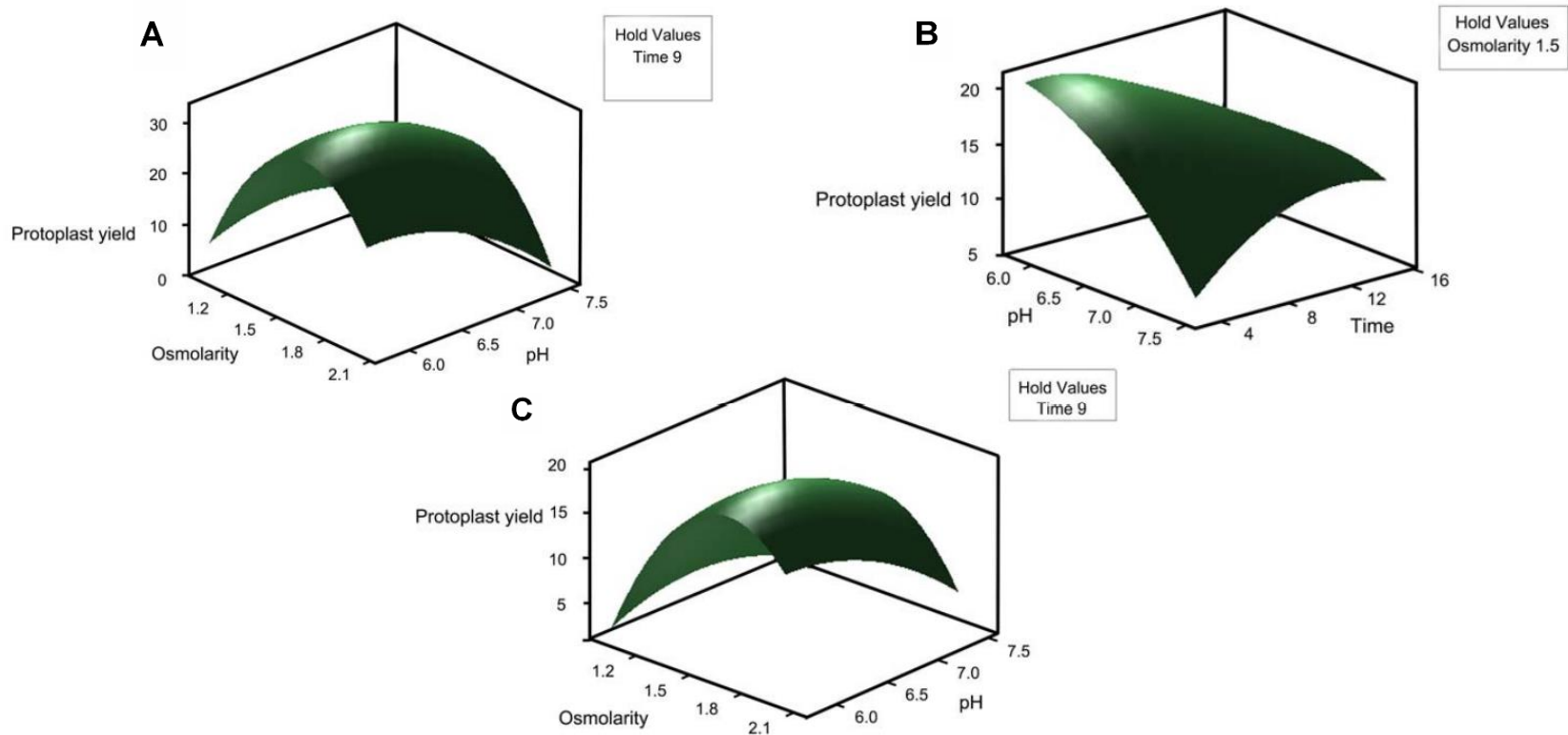
Fig. 17. Response surface plots for the maximum protoplast yield in *Undaria pinnatifida* gametophytes. Plots were generated using the data shown in Table x under the conditions established by the Box-Behnken design. (A) Protoplast yield ($\times 10^5$ protoplasts g^{-1} FW) as a function of osmolarity and pH in male gametophyte. (B) Protoplast yield ($\times 10^5$ protoplasts g^{-1} FW) as a function of pH and time (h) in female gametophyte. (C) Protoplast yield ($\times 10^5$ protoplasts g^{-1} FW) as a function of osmolarity and pH in female gametophyte.

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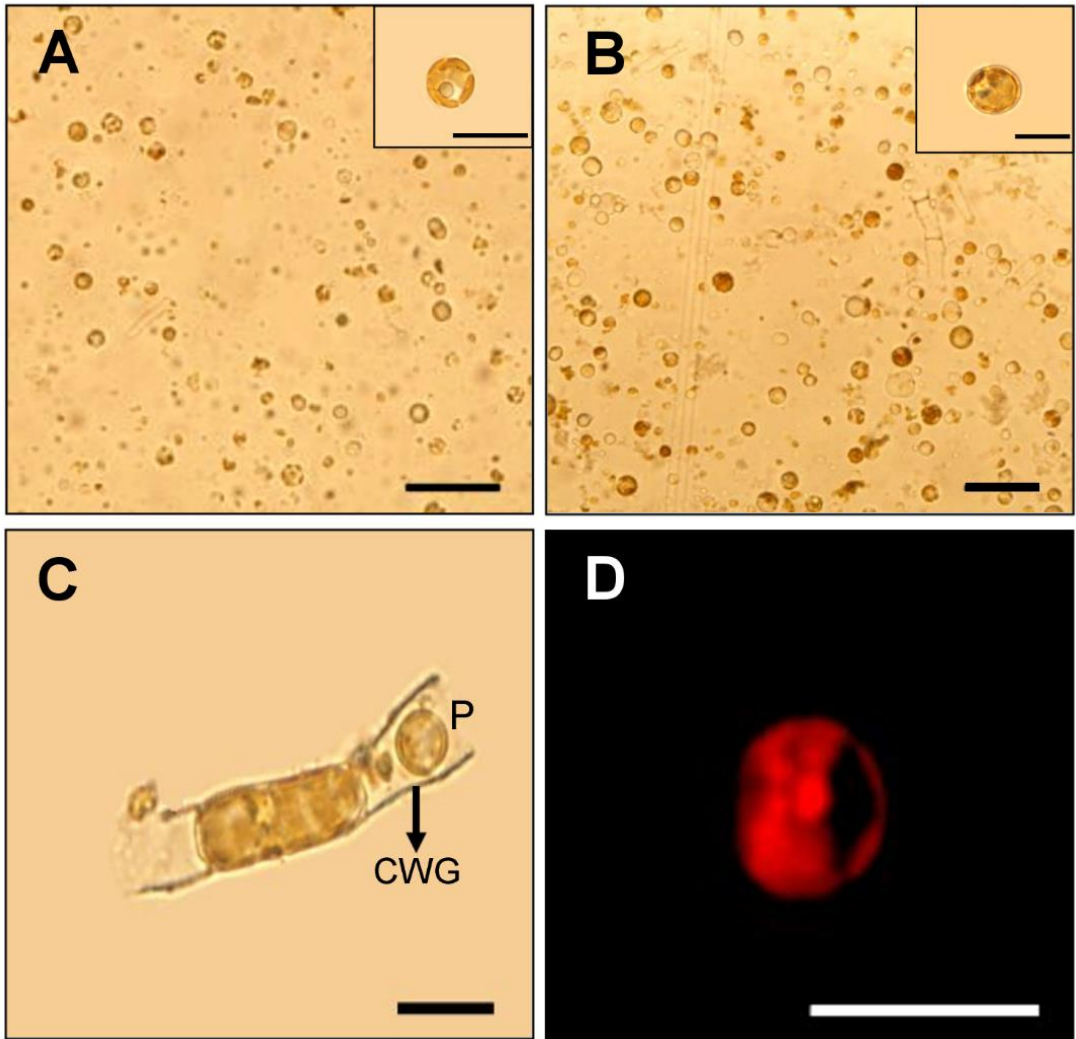
Fig. 18. Protoplast isolation from *Undaria pinnatifida* gametophytes. (A) Freshly isolated protoplasts from male gametophyte. Inset: closer view of a protoplast. (B) Freshly isolated protoplasts from female gametophyte. Inset: closer view of a protoplast. (C) Protoplast release. (D) Fluorescence image of a freshly isolated protoplasts showing red chlorophyll autofluorescence. P protoplast, CWG cell wall ghost. The scales in (A) and (B) are 50 μm; the scales in (C) and inset in (A) and (B) are 20 μm.

Table 18. Protoplast yields from different gametophyte strains of *Undaria pinnatifida* isolated in Korea. Values are presented as mean ± SD (n = 4)

Strain code	Collection site	Collection date	Sex	Protoplast yield (x 10^6 protoplasts g^{-1} FW)
1	Geomun Island, Yeosu, South Jeolla Province	25 March, 2016	Male	2.57±0.27
			Female	4.93±0.82
2	Gampo, Gyeongju, North Gyeongsang Province	1 April, 2016	Male	4.67±1.36
			Female	7.91±1.97
3	Jindo Island, Jindo, South Jeolla Province	5 May, 2016	Male	5.16±0.75
4	Heuksan Island, Sinan, South Jeolla Province	19 May, 2016	Male	8.17±1.74
			Female	2.74±0.95
5	Hondgo Island, Sinan, South Jeolla Province	20 May, 2016	Male	5.45±1.02
			Female	4.55±1.15

Protoplast culture and regeneration

After three hours in culture, cell wall formation started with blue fluorescent spots (positive staining) in the surface of the protoplasts (Fig. 19A). However, the entire surface of the protoplast was covered by a uniform cell wall after 2-6 days in culture (Fig. 19B).

Regeneration of protoplasts started by forming one bud in one pole of the cell prior first asymmetric cell division (Fig. 19C). Symmetric cell division was also observed but in lower percentages (14-20% of regenerated protoplasts) (Fig. 19D). In the case of female gametophyte, after 15 days in culture, most of protoplasts followed unipolar germination (81% of regenerated protoplasts) forming uniseriate filaments that early branched (Fig. 19E). Typical new thalli were clearly distinguished after 22 days in culture. In the case of male gametophyte, after 18 days in culture, 2 or more additional uniseriate filaments were produced from the initial protoplasts (Fig. 19F). Typical new thalli were clearly distinguished after 26 days in culture. Both regenerated gametophytes normally developed reproductive structures (Fig. 19G, H). Final platting efficiency values for female and male gametophytes were $76.01 \pm 3.16\%$ and $18.01 \pm 2.02\%$, respectively.
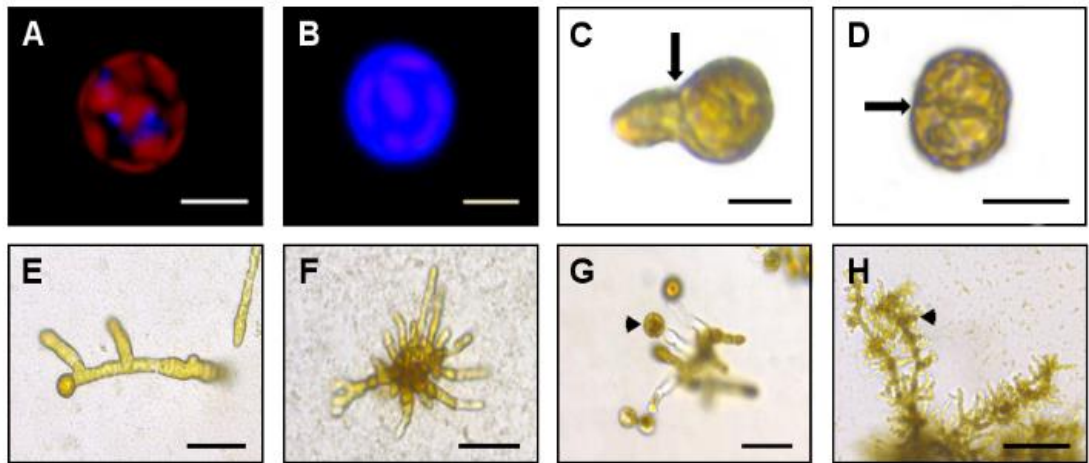
Fig. 19. Protoplast regeneration of *Undaria pinnatifida* gametophytes. (A) Fluorescence image of cell wall formation after 3 h of culture in a female protoplast. (B) Fluorescence image of cell wall formation after 48 h of culture in female protoplast. (C) First asymmetric cell division (arrow) in male gametophyte. (D) First symmetric cell division (arrow) in female gametophyte. (E) Branched filament (female gametophyte) after 15 days in culture. (F) Multiple rhizoid-like protrusions (male gametophyte) after 18 days in culture. (G) Regenerated female gametophyte producing oogonia (arrowhead) after 22 days in culture. (H) Regenerated male gametophyte producing spermatangia (arrowhead) after 26 days in culture. Areas showing bright blue fluorescence indicate cellulose deposition. The red autofluorescence of the chlorophyll reveals areas without cell wall. The scales in (A), (B) and (C) are 10 µm; the scale in (D) is 20 µm; the scales in (E) and (G) are 50 µm; the scale in (F) is 40 µm; the scale in (H) is 100 µm.

4. Discussion

The present study demonstrates a successful method for isolating a large number of viable protoplasts from male and female gametophytes of the economically important brown seaweed *Undaria pinnatifida* using commercial enzymes with chelation pre-treatment. Protoplast isolation conditions were optimized using RSM via BBD, representing the first report of this technique in the production of protoplasts from brown seaweeds.

The highest protoplast yields of 3.12 and 2.11 $\times 10^6$ protoplasts g^{-1} FW obtained in this study for male and female gametophytes, respectively, were superior to the number of protoplasts reported by Benet et al. (1997). However, Zha and Kloareg (1996) reported higher protoplast values than us (15 to 25 $\times 10^6$ protoplasts g^{-1} FW) with a mix of non-commercial enzymes which might have had additional active ingredients digesting the cell walls of gametophytes.

A simple mix of commercial cellulase RS (1%), alginate lyase (2 U mL^{-1}) and driselase (1%) was found to be the best enzymatic combination. Cellulase RS was more effective than cellulase R-10, especially in female gametophyte, mainly due to the higher xylanase and cellulase activities in the RS preparation (Thayer 1985). Alginate lyase (a mannuronate lyase according to the manufacturer) was also critical in the enzymatic mixture, although the incomplete cell wall digestion can be attributed to its inefficient degradation of alginate (Formo et al. 2014).

Driselase increased significantly the number of protoplasts in male gametophyte while marginally improved their release in the female gametophyte. Driselase is a natural mixture of enzymes (e.g. cellulase, hemicellulase and pectinase) that can cleave, among other polysaccharides, mixed-linked glucan (MLG) also known in fungi as lichenan (Thibault & Rouau 1990). Recently, Salmeán et al. (2017) demonstrated that MLG is common in brown algal cell walls, including *Undaria pinnatifida* sporophyte. This can explain the effectiveness of driselase on protoplast isolation, especially in male gametophyte.

The addition of macerozyme R-10 and pectinase to the best enzymatic mixture did not improve the protoplast release. Benet et al. (1997) reported that pectin-degrading enzymes were inefficient at increasing protoplast yields in gametophytes from Laminariales. Reddy et al. (2006) demonstrated that macerozyme R-10 is unnecessary in enzyme mixtures if the algal cell walls do not contain pectin or its derivatives, which is the case for brown algae (Cronshaw et al. 1958;

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Kloareg & Quatrano 1988). Our results suggest that macerozyme R-10 and pectinase can be excluded when isolating protoplasts from Phaeophyceae.

The addition of cation chelators has improved protoplast production in Ectocarpales (Mejjad et al. 1992; Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989). However, its effect has not been tested in gametophytes from Laminariales. In our study, the effect of chelation pretreatment was dependent on the specific type of enzymatic mixture. Only the best enzymatic combination (cellulase, alginate lyase and driselase) showed significant increases in both gametophytes following incubation in the chelating solution.

Our results showed that protoplast yield was affected by the growth phase of culture, reaching maximum values in the mid-exponential phase (male gametophyte) or just before the beginning of this one (female gametophyte). Differences in growth patterns can be attributed to distinct reproductive strategies in gametophytes (Destombe & Oppliger 2011). Actively dividing cells or fast-growing plants have produced higher amounts of protoplasts in yeasts (Shahin 1972), land plants (Strauss & Potrykus 1980; Nakagawa et al. 1985) and in green and red seaweeds (Björk et al. 1990, 1992; Gómez Pinchetti et al. 1993). During cell division and growth, cell wall elasticity is necessary and, consequently, its composition changes (Burns et al. 1982a, 1982b, 1984). Elasticity and rigidity of alginate in the cell wall are due to mannuronate (M) and guluronate (G) blocks, respectively. M-block content increase is expected during the exponential phase due to a high cell division (Kloareg & Quatrano 1988; Lee et al. 2012). The alginate lyase used in this study mainly cleaves M blocks, which can explain the better digestibility of the cell wall during the exponential phase.

The range of temperatures tested (15–25 °C) did not affect protoplast yield in *Undaria pinnatifida* gametophytes. The optimal temperature employed for isolating protoplasts is more closely related to that what species of macroalgae naturally grow (Huddy et al. 2013). Morita et al. (2003) showed that gametophytes from *U. pinnatifida* can grow in the range of 10 to 25 °C with an optimal of 20 °C. This wide range of temperatures might explain the lack of effect of incubation temperature on protoplast yield.

An enzymatic digestion period of 4–7 h was optimum according to the RSM. Benet et al. (1997) reported 15 h incubation time for gametophytes from Laminariales, while Varvarigos et al.

(2004) obtained high amounts of protoplasts from female gametophytes of *Macrocystis pyrifera* with a period of 4–5 h. The type of enzymes used, cell wall complexity and the physiological state of the explant can explain the variations of the time periods (Reddy et al. 2008). Interestingly, female gametophyte showed a shorter incubation time compared with male gametophyte, suggesting that cell wall composition may vary between them.

Optimal enzyme pH values were found to be 5.8–6.1. Previous studies on protoplast from gametophytes of Laminariales have used a pH 6.5 (Benet et al. 1997; Varvarigos et al. 2004). This difference could be due to the type of enzyme mixtures used because the activity of the enzymes is pH dependent (Bhojwani & Razdan 1996). According to the manufacturer, the optimum pH for cellulase RS and alginate lyase is 5–6 and 6.3, respectively. The optimal values obtained in this work were within this range. Optimal pH for the commercial enzymes used also explain the decrease of protoplast yield by increasing the pH.

The highest protoplast yields were obtained at 2481– 2591 mOsm L^{-1} H_2O (around 1.6× enzymatic solution). Although our optimal osmolarity values were higher than those ones used previously (Benet et al. 1997; Xiaoke et al. 2003; Varvarigos et al. 2004; Mussio & Rusig 2006), protoplasts did not show damages. Instead, high osmolarity favoured the isolation process probably due to the stimulation of alginate lyase activity by increasing salt concentrations (Huang et al. 2013), promotion of protoplast release through the holes in the cell wall, or protection of the protoplast membrane (Xiaoke et al. 2003).

The models developed using RSM were evaluated using several parameters. The R^2 coefficient for both models was superior to that one reported by Gupta et al. (2011) for protoplasts from *Gracilaria dura* and *G. verrucosa*. Male and female gametophytes reported adequate precision of 6.88 and 11.63, respectively. Values greater than 4 indicate an adequate signal and confirm that all predicted models can be used to navigate the design space (Anderson & Whitcomb 2017). The Mallow's C_p statistics were 10.00 for both gametophytes. C_p can be used to determine how many terms can be omitted from the response surface model. When $C_p \leq p$, where p is the number of parameters or variables in the regression model including the intercept term ($p = 10$ in our study), the prediction model is very good (Dawson & Martinez-Dawson 1998). Although the CV% values were somewhat elevated for both gametophytes, validation of the model showed less than 5% deviation between experimental and theoretical values indicating the suitability of

the developed quadratic models. Moreover, high numbers of protoplasts were isolated from other gametophyte strains using the optimum conditions.

Protoplast regeneration from gametophytes of Laminariales usually exhibits either symmetric or asymmetric cell division followed by uni or multipolar development (Benet et al. 1997). The present results shows that the great majority of protoplasts from *Undaria pinnatifida* gametophytes undergo a simple regeneration with asymmetric first cell division. Although symmetric cells occurred, these ones were present in lower percentages. Gametophytes differed on their regeneration pathways: female protoplasts regenerated through unipolar development, while male ones, through multipolar development. These differences have not been pointed out in previous reports (Zha and Kloareg 1996, Benet et al. 1997).

In conclusion, high amounts of viable and true protoplasts could be obtained from *Undaria pinnatifida* gametophytes cultures in exponential phase using RSM and a simple mixture of commercial enzymes (cellulase RS, alginate lyase and driselase) with chelation pre-treatment. RSM via BBD can be applied as a useful method for increasing the protoplast production in brown algae.

CHAPTER 3. Protoplast production from *Sphacelaria fusca* (Sphacelariales, Phaeophyceae) using commercial enzymes[*]

1. Introduction

The filamentous brown alga genus, *Sphacelaria*, is a common epiphyte on macroalgae and marine plants (Piazzi et al. 2015; Varisco et al. 2015), as well as epizoic on sea turtles (Velasco-Charpentier et al 2016). It provides food and habitat for animals, playing an important role in coastal benthic communities (Pavia et al. 1999; Karez et al. 2000; Viejo & Åberg 2003). *Sphacelaria* represents an important component of the diet of grazers such as the isopod *Dynamene magnitorata* (Arrontes 1990), spider crab *Leucippa pentagona* (Varisco et al. 2015), and the "key herbivore" *Diadema antillarum* (Hernández et al. 2007). It is known that grazers can control the proliferation of epiphytic algae and thus they help to avoid a decrease in the performance of the host (Whalen et al. 2013). *Sphacelaria* can be also be infected in the wild by fungal parasites such as the chytrid *Chytridium polysiphonae* (Raghukumar 1987). Abiotic factors (e.g. intertidal elevation) can affect the distribution of *Sphacelaria* in its host, as it has been reported for other epiphytic filamentous brown algae (Longtin et al. 2009) Besides *Sphacelaria* has been used as a model organism for plant morphogenesis due to its apical growth and ease of cultivation (Dworetzky et al. 1980; Charrier et al. 2012; Bogaert et al. 2013). Thus, *Sphacelaria* is a suitable organism for exploring different stressors and environmental inputs, both in controlled and non-controlled conditions.

Transcriptome analysis can help to understand the molecular basis of physiological responses to environmental stressors (Imadi et al. 2015). Recently, single-cell RNA sequencing has emerged as a novel approach to measure transcriptome with high resolution using different cell types of animal cells (Hwang et al. 2018). However, the presence of cell walls has hindered the application of this approach to plant cells (Shulse et al. 2019). One way to overcome this problem

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[*] Modified from the published article: Avila-Peltroche J. & Won B.Y. 2020. Protoplast production from *Sphacelaria fusca* (Sphacelariales, Phaeophyceae) using commercial enzymes. *Journal of Marine Bioscience and Biotechnology* 12(1): 50-58.

is using protoplasts, which are plant cells whose cell wall has been removed by enzymatic methods (Reddy et al. 2008). The high amounts of protoplasts are more important than their regeneration ability in single-cell RNA sequencing (Efroni & Birnbaum 2016; Shulse et al. 2019). Moreover, they represent an important biotechnological tool for genome-editing and gene-silencing technologies (Burris et al. 2016), as well as, for the production of useful secondary metabolites, such as dictyopterenes from the seaweed *Dictyopteris prolifera* (Fujimura et al. 1994).

In brown algae, protoplast isolation has been reported in 31 species (Fig. 2, Table 2). To date, protoplasts from *Sphacelaria* have only been isolated in low amounts using commercial and non-commercial enzymes for microtubule analysis during regeneration (Ducreux & Kloareg 1988; Rusig et al. 1994). The protoplast isolation becomes more expensive and time-consuming because these non-commercial enzymes need to be extracted from marine herbivores or microorganisms (Cocking 1972). Thus, protoplast isolation protocols using commercial enzymes are important for the application of single-cell RNA sequencing in *Sphacelaria.*

In this study, we report for the first time the protoplast isolation from *Sphacelaria fusca* using a simple mix of commercial enzymes. We tested the effect of osmolarity and driselase inclusion on protoplast production to determine the best conditions for this process. Also, we assessed the effect of light exposure during the first days of culture on protoplast survival and regeneration.

2.   Materials and methods

Isolation and culture of the strain

Sphacelaria fusca (MBRB0073TC18209C1) was collected by hand from *Sargassum muticum* in Jindo Island, Jeollanam-do, Korea, on July 27, 2017. Apical filaments of *S. fusca* were cultured in 12 well plates containing PES medium under 14:10-h light/dark photoperiod at 20°C with light intensity 40 μmol photons $m^{-2} s^{-1}$ of blue LED (DyneBioCo, Korea). The medium was renewed weekly. After 3 months, plants were transferred to 100×40 mm Petri dishes and were cultured under the same culture conditions. Two months later, plants accumulated much biomass and were transferred into 1-L flat-bottomed round flasks filled with 1-L PES medium under aeration. Light intensity was 40-60 μmol photons $m^{-2} s^{-1}$ of white fluorescent light. The air was sterilized

using 0.22-µm SFCA syringe filters (Corning, Germany). The medium was renewed every 2 weeks.

Identification of the culture strain

Taxonomic identification was performed using morphological characters (Keum et al. 2010) from cultures maintained in 60×15 mm Petri dishes without agitation. Photomicrographs were taken using a Leica inverted microscope (DMi8; Leica, Germany) equipped with a Leica DFC450C camera. Genomic DNA extraction, PCR amplification, DNA purification, and sequencing were performed as previously described (Keum et al. 2005; Bustamante et al. 2016) using cultured samples. The plastid-encoded RuBisCo spacer region was amplified using the primer combinations G1 and G2 (Destombe & Douglas 1991). The amplified region sequences were compared to the GenBank nucleotide database using the BLAST program (Altschul et al. 1997).

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study included cellulase Onozuka RS (Yakult Co. Ltd., Japan), alginate lyase, and driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA). Different enzyme combinations and conditions are shown in Table 19.

Protoplast isolation was performed as described in "General Materials and Methods" with approximately 100–300 mg plants (from 3-day-old cultures) at 20 °C, pH 6 for 6 h in the dark. Enzyme mixtures contained cellulase Onozuka RS and alginate lyase, either with or without driselase. The osmolarity of the enzymatic solution was tested in two levels: normal osmolarity ($1\times = 1570$ mOsm L^{-1} H_2O) and increased osmolarity ($1.6\times = 2512$ mOsm L^{-1} H_2O). Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. Also, we tested the effect of chelation pre-treatment on protoplast yield. Protoplast isolation was repeated four times in each treatment.

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Table 19. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Sphacelaria fusca*.

Commercial enzymes	Composition of enzyme mixtures			
	A	B	C	D
Cellulase Onozuka RS (%)	1	1	1	1
Alginate lyase (U/mL)	4	4	4	4
Driselase (%)	1	-	1	-
Osmolarity	1×	1×	1.6×	1.6×

1×, 1570 mOsm L^{-1} H$_2$O; 1.6×, 2512 mOsm L^{-1} H$_2$O

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as described in "General Materials and Methods".

Protoplast regeneration

Protoplast culture and regeneration was performed as described in "General Materials and Methods" using 2 mL of RM1 with some modifications. Protoplasts were cultured at 20 °C in the dark and at initial protoplast density of 9×10^3 protoplasts mL^{-1} plus antibiotic mix (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin and 5 mg L^{-1} chloramphenicol). As the protocol used for *Hecatonema terminale* was not effective for regenerating protoplast from *Sphacelaria fusca*, the effect of osmolarity reduction and light exposure on cell wall regeneration, survival and division was assessed in six conditions (Table 20). Under the best condition, the regeneration media used by Ducreux and Kloareg (1988) in *Sphacelaria* sp., which includes glucose and sucrose as carbon source, was compared with RM1 in order to choose the best medium.

Statistical analysis

To evaluate the effect of driselase addition and osmolarity on protoplast yield a generalized linear model with a binomial negative error distribution (GLM.nb) was used as a traditional method for handling overdispersed data. The analyses were performed using "MASS" package in R (Venables & Ripley 2002). The proportion of cell types on protoplast preparations were compared to controls (undigested *Sphacelaria fusca* filaments) using beta regressions since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari & Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto & Zeileis 2010). The *p*-value was corrected by the Bonferroni method to compensate for the effect of multiple hypotheses testing. Protoplast isolation was repeated four times in each treatment.

Table 20. Osmolarity reduction and light exposure conditions in protoplast cultures of *Sphacelaria fusca*. In all cases, white fluorescent light was used.

Condition	Start of osmolarity reduction	Light exposure
C1	After 2 days in culture	Protoplast were exposed first to 1-2 µmol photons $m^{-2} s^{-1}$, and then to 40 µmol photons $m^{-2} s^{-1}$
C2	After 1 day in culture	Protoplast were exposed first to 1-2 µmol photons $m^{-2} s^{-1}$, and then to 40 µmol photons $m^{-2} s^{-1}$
C3	After 1 day in culture	Protoplast were exposed directly to 40 µmol photons $m^{-2} s^{-1}$
C4	After 3 days in culture	Protoplast were exposed directly to 40 µmol photons $m^{-2} s^{-1}$
C5	After 3 days in culture	Protoplast were maintained at 1-2 µmol photons $m^{-2} s^{-1}$
C6	After 1 day in culture	Protoplast were exposed first to 1-2 µmol photons $m^{-2} s^{-1}$, and then to 10 µmol photons $m^{-2} s^{-1}$

3. Results

Identification

The vegetative characteristics of *Sphacelaria fusca* are shown in Fig. 20A-C. Cultures (with or without aeration) did not present reproductive structures during the study. They showed three-armed propagules, typical morphological feature of this species (Figure 20B; Keum et al. 2005). Our morphological identification of *S. fusca* was confirmed by molecular analysis. A 585-bp portion was sequenced for the strain (CUK18209 (= MBRB0073TC18209C1) in Chosun University Herbarium in Korea; MT009225 in GenBank) of *S. fusca*. The RuBisCo spacer region of our strain was 100% identical to *S. fusca* clone A2 (FJ710148; Lee et al. 2011).

Protoplast isolation using enzymes

Protoplast yields ranged from 0-15.08 $\times 10^4$ protoplasts g^{-1} FW. Mixture C (cellulase RS, alginate lyase and driselase with 1.6$\times$ osmolarity) with chelation pre-treatment produced the highest number of protoplasts (15.08 $\pm$ 5.31 $\times 10^4$ protoplasts g^{-1} FW), followed by mixture D (cellulase RS and alginate lyase with 1.6$\times$ osmolarity) with chelation pre-treatment (7.55 $\pm$ 4.16 $\times 10^4$ protoplasts g^{-1} FW). The effect of osmolarity and driselase inclusion is shown in Figure 21. Osmolarity increase had a highly significant and positive effect on protoplast production ($p <$ 0.001). Under normal osmolarity (1$\times$), protoplast yields were low (<100 protoplasts g^{-1} FW) and inconsistent. By elevating the osmolarity, protoplast could be isolated in larger and consistent amounts. The inclusion of driselase to the enzymatic mix did not affect protoplast yield ($p =$ 0.030); however, it slightly increased the protoplast number. There was no interaction between both factors ($p = 0.454$). As an effort to simplify our protoplast isolation protocol, chelation pre-treatment was skipped. However, this resulted in more than 10 times reduction of protoplast yield (<10^4 protoplasts g^{-1} FW).

Cell wall digestion was not complete and "cell-wall ghosts" were still visible at the end of the enzymatic treatment (Figure 20F). However, protoplasts were released through holes in the cell walls due to filament fragmentation (Figure 20E, F). Protoplasts were spherical shape and very heterogeneous in size and pigmentation, corresponding to the various cell types in *Sphacelaria*. Protoplasts from apical cells were 30-50 µm in diameter and contained numerous physodes

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(Figure 20G). Protoplasts from subapical cells were similar in size but did not contain large numbers of physodes (Figure 20H). Smaller protoplasts (10-30 µm) were from nodal and internodal cells (Figure 20I). Compared with the proportions of the different cell types in the non-digested filaments (Figure 20D), protoplast preparations were enriched in subapical, nodal, and intermodal-cell protoplasts. These values are shown in Table 21. True protoplast percentages were 98-100% with calcofluor white staining, while the viability of freshly isolated protoplasts was approximately 98% with red chlorophyll autofluorescence (Figure 20J).

Protoplast culture and regeneration

Protoplasts started to regenerate their cell wall as soon as 12 h in culture. After 72 h, 66% of protoplasts, especially apical and subapical-cell protoplasts, showed complete regeneration of their cell walls (Fig. 22A). The starting time of osmolarity reduction and light exposure were critical factors for protoplast survival and regeneration, but not for cell wall regeneration (Table 22). Protoplasts were not able to survive if the osmolarity reduction started after 2 or 3 days in culture and/or they were exposed, directly or not, to 40 µmol photons $m^{-2} s^{-1}$. The best condition for protoplast survival and regeneration was C6: osmolarity reduction starting after 1 day in culture and gradual exposure to 10 µmol photons $m^{-2} s^{-1}$. Under this condition, apical-cell protoplasts divided once and developed a filament that reached five to ten cells after 2 weeks in culture (Fig. 22B-D). Five fully grown plants were observed after 2 months in culture (FPE = 0.06%; Fig. 22E). Protoplasts cultured in the regeneration media used by Ducreux and Kloareg (1988) did not divided and, ultimately, died because of extensive bacterial contamination.

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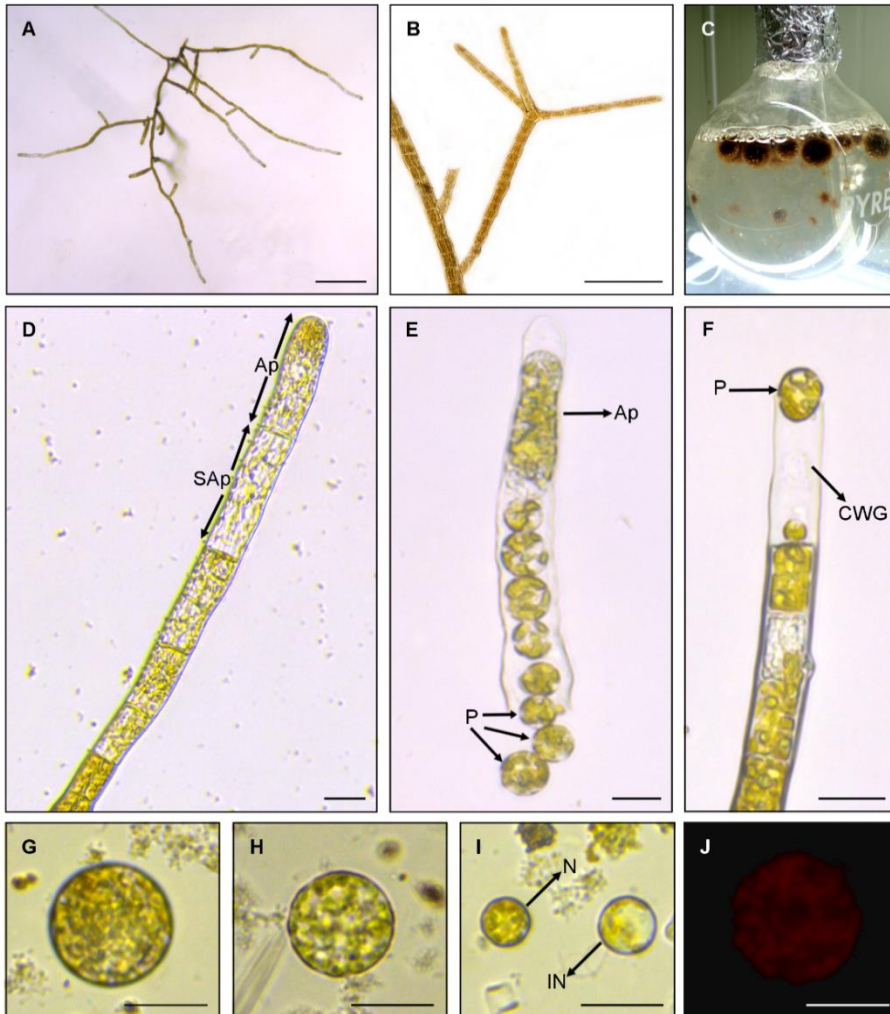
Fig. 20. Protoplast production from *Sphacelaria fusca*. (A) Piece of *S. fusca* from unialgal cultures without aeration. (B) Three-armed propagule. (C) A 1-month old culture in 1-L flat-bottomed round flask with aeration. (D) Control (non-digested apex) showing apical (Ap) and subapical (Sap) cells. (E) Protoplast (p) release from an apical portion. Notice that the apical cell wall is intact (Ap). (F) Nodal-cell protoplast (p) being released from the middle part of a filament. Notice the "cell-wall ghost" (CWG). (G) Apical-cell protoplast. (H) Subapical-cell protoplast. (I) Nodal (N) and internodal-cell (IN) protoplasts. (J) True and viable protoplast showing red chlorophyll autofluorescence. The scale in (A) is 400 μm; the scale in (B) is 200 μm; the scales in (D), (E), (F), (G), (H), (I) and (J) are 25 μm.
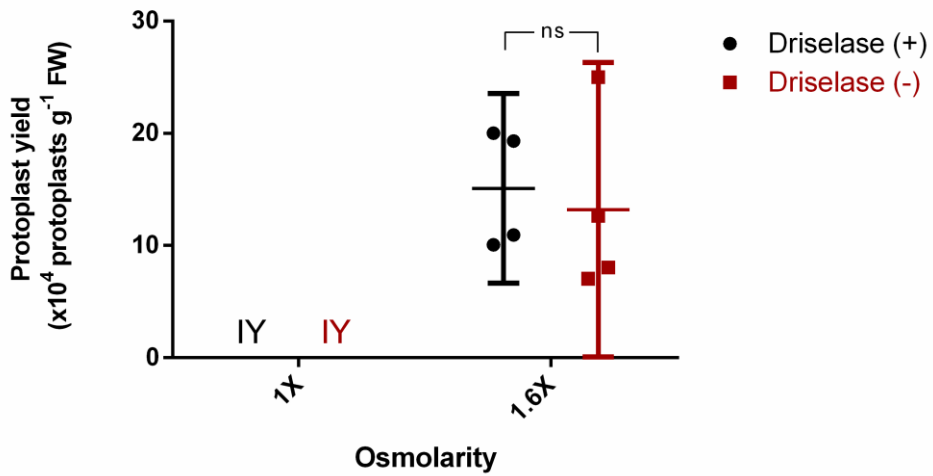
Fig. 21. Effect of osmolarity and driselase inclusion on protoplast yield from *Sphacelaria fusca*. Independent data points and averages (horizontal lines) are shown (n=4). Error bars represent 95% confidence intervals. IY, inconsistent yield; ns, no significant difference ($p > 0.01$).

Table 21. Relative proportions (%) of the various cell types in the controls (undigested *Sphacelaria fusca* filaments) and protoplast preparations. Superscript letters indicate highly significant differences among treatments ($p < 0.001$). Values are presented as mean $\pm$ SD (n = 4).

	Apical	Subapical	Nodal	Internodal
Controls	20.38 ± 17.55^a	1.62 ± 1.46^a	0.44 ± 0.43^a	77.56 ± 18.25^a
Protoplasts	27.16 ± 2.74^a	19.62 ± 2.79^b	36.08 ± 1.69^b	17.14 ± 5.33^b
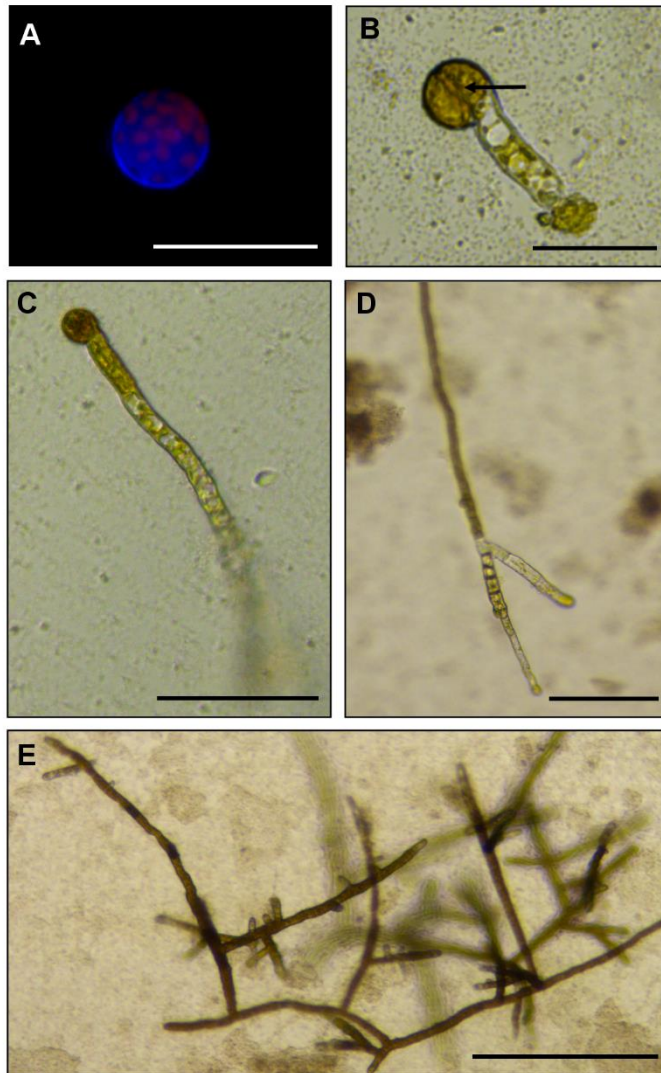
Fig. 22. Protoplast regeneration of *Sphacelaria fusca*. (A) Internodal-cell protoplast with complete cell wall regeneration (bright blue fluorescence) after 48 h in culture. Areas showing red autofluorescence of the chlorophyll indicate the chloroplasts. (B) First division of apical-cell protoplast (arrow) with an emerging filament. (C) Unbranched filament after 2 weeks in culture. (D) First branch near the apical portion of a filament after 3 weeks in culture. (E) Whole plant regeneration after 2 months in culture. The scale in (A) is 25 µm; the sale in (B) is 50 µm; the scale in (C) is 100 µm; the scale in (D) is 200 µm; the scale in (E) is 500 µm.

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Table 22. Protoplast cultures from *Sphacelaria fusca* under different conditions of osmolarity reduction and light exposure.

Conditions	Observations
C1	Protoplasts were found dead but with regenerated cell walls by the start of osmolarity reduction
C2	Protoplasts, especially from apical and subapical cells, showed fast cell wall regeneration by the start of osmolarity reduction. However, extensive lysis occurred by the last day of osmolarity reduction (day 3). Fragmented chloroplasts were also observed
C3	Protoplasts, especially from apical and subapical cells, showed fast cell wall regeneration by the start of osmolarity reduction. However, extensive lysis occurred by the last day of osmolarity reduction. Fragmented chloroplasts were also observed
C4	Protoplasts were found dead but with regenerated cell walls by the end of osmolarity reduction
C5	Protoplasts were found dead but with regenerated cell walls by the start of osmolarity reduction
C6	Protoplasts regenerated their cell walls and most of them survived by the last day of osmolarity reduction. Uniseriate filaments were observed after 2 weeks in culture. Whole plants (5) were observed after 2 months in culture

4. Discussion

The highest protoplast amount obtained in this study was 15.08×10^4 protoplasts g^{-1} FW, which represents more than 30 times the highest value reported previously for *Sphacelaria* by Ducreux and Kloareg (1988). These authors used two commercial enzymes and one non-commercial enzyme for the isolation process (Table 23). Similarly, Coelho et al. (2012) used a combination of commercial and non-commercial enzymes for protoplast isolation from *Ectocarpus*. Our protocol used commercial enzymes: cellulase Onozuka RS, alginate lyase, and driselase from Sigma. Using only commercial enzymes is desirable when developing protocols for establishing protoplast systems (Bhojwani & Razdan 1996). Therefore our results will helpful for future studies.

Cell walls in brown algae are composed of alginate, fucoidans, fuco-glucorono-xylans, and a small amount (1-8%) of cellulose (Cronshaw et al. 1958; Kloareg & Quatrano 1988). Recently, the presence of mixed-linked glucan (MLG) in brown algal cell walls, including the related species *Stypocaulon scoparium*, has been demonstrated (Salmeán et al. 2017). Although MLG can be degraded by driselase (Thibault & Rouau et al. 1990), its inclusion on the enzymatic mixture was not crucial for improving protoplast yields. The combination of cellulase and alginate lyase was sufficient for releasing true protoplasts from all cell types. Chelation pre-treatment was necessary for improving protoplast yield. The use of cation chelators has shown positive effect in protoplast isolation from Ectocarpales (Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989).

Table 23. Comparison of protoplast isolation between the previous study and this study.

	Ducreux and Kloareg (1988)	**This study**
Species	*Sphacelaria* sp.	*Sphacelaria fusca*
Chelation pre-treatment	No	Yes
Commercial enzymes	2% Cellulysin, 0.5% pectolyase Y23	1% cellulase Onozuka RS, 1% driselase, 4 U mL^{-1} alginate lyase
Non-commercial enzymes	2% alginate lyase from *Haliotis tuberculata*, *Patella vulgata* or *Aplysia punctata*	Not used
Incubation time	12 h	6 h
Osmolarity (mOsm L^{-1} H$_2$O)	1800	2512
pH	5.8	6
Protoplast yield ($\times 10^4$ protoplasts g^{-1} fresh weight)	0.46	15.8
Cell types* in protoplast preparation	All, but apical-cell protoplast enriched	All, but subapical and nodal-cell protoplast enriched

*Apical, subapical, nodal and internodal cells.

Our results also showed that high osmolarity (2512 mOsm L^{-1} H_2O) favored the isolation process, probably due to the stimulation of alginate lyase activity by increasing salt concentrations (Huang et al. 2013); promotion of protoplast release through the holes in the cell wall; or protection of the protoplast membrane (Xiaoke et al. 2003). Even though protoplast isolation protocols commonly use lower osmolarities (Ducreux & Kloareg 1988; Benet et al. 1997; Coelho et al. 2012), the increased osmolarity did not damage the protoplasts. These changes give better results in protoplast isolation.

Unlike other filamentous brown algae, plants of *Sphacelaria* present four different cell types: apical, subapical, nodal and internodal. Differences among types are not only reflected by their morphology but also their morphogenetic competences. *Sphacelaria* growth is mainly directed by the large apical cell, which has the ability to regenerate a whole plant (Ducreux & Kloareg 1988). Protoplast preparations obtained in this study showed all the cell types reported for *Sphacelaria*, with higher proportions of subapical and nodal-cell protoplasts. Interestingly, our enzymatic combination could not degraded apical cell walls, which have been reported to be more digestible by other authors (Ducreux & Kloareg 1988). Protoplasts from different cell types could allow to explore the transcriptome profile in different cell populations (Shulse et al. 2019).

Protoplast isolation process inevitably results in multiple type of stress for vegetative cells due to cell wall removal, considerable cell death, and loss of cell to cell communication. In protoplasts from the red seaweed *Chondrus crispus* and the brown seaweed *Laminaria digitata*, expression of stress genes, such as heat shock proteins and enzymes involved with detoxification, were enhanced (Roeder et al. 2005; Collén et al. 2006). Although we did not assess gene expression levels in the protoplast of *Sphacelaria fusca*, we expect a similar trend. These changes in gene expression do not limit the use protoplasts in single-cell RNA sequencing as protoplast-inducible genes are filtered out during the analysis (Shulse et al. 2019).

Protoplasts from *Sphacelaria fusca* were able to divide when the osmotic pressure was quickly reduced and they were exposed to low light intensity (10 μmol photons m^{-2} s^{-1}) during the first days of culture. It is known that maintaining protoplasts in high osmotic medium and high light intensities applied from the beginning of the culture can inhibit their growth (Ochatt & Power 1992; Chawla 2019). In a previous report (Ducreux & Kloareg 1988), protoplasts from *Sphacelaria* sp. were cultured at low light (0.8 W m^{-2} ≈ 3.68 μmol photons m^{-2} s^{-1}); however,

osmotic pressure was not reduced, at least, during the first days of culture. Also, the use of glucose and sucrose as carbon source in the regeneration medium was detrimental for protoplast survival due to bacterial overgrowth. This is similar to what was reported for protoplasts from Laminariales (Benet et al. 1997).

In conclusion, this is the first report of protoplast isolation and regeneration from *Sphacelaria fusca*. Our result showed that a simple mix of commercial enzymes (cellulase Onozuka RS, alginate lyase, and driselase) with increased osmolarity (2512 mOsm L^{-1} H_2O) and chelation pre-treatment was capable of producing high amounts of true and viable protoplasts from all cell types of *S. fusca*. These features make the present protocol useful for single-cell RNA sequencing, transcriptomics, gene editing, gene silencing technology, microtubule analysis and natural production from algae.

CHAPTER 4. Improved method for protoplast isolation and culture from *Undaria pinnatifida* sporophyte (Laminariales, Phaeophyceae)

1. Introduction

Undaria pinnatifida (Harvey) Suringar is one of the most important farmed seaweeds worldwide (FAO 2020). Its sporophytic phase is used in food, medicine, cosmetics and pharmaceutical applications in countries like Japan, China and Korea. Recently, the interest of culturing and using this edible seaweed has expanded into places where it has been introduced, such as Spain and Argentina (Peteiro et al. 2016; Salomone & Riera 2019).

Protoplasts (i.e. naked plant cells) are especially useful in economic cultivars because it offers the possibility of *in vitro* manipulation and crop improvement bypassing sexual reproduction (Davey et al. 2005a). Protoplast isolation has been extensively investigated in *Undaria pinnatifida* (Fujita & Migita 1985; Wu 1988; Yamaguchi 1989; Matsumura et al. 2001; Xiaoke et al. 2003); however, most of the studies have used non-commercial enzymes or crude extracts, which make the isolation process expensive, time consuming and/or low reproducible (Cocking 1972; Gupta et al. 2011; Inoue et al. 2011).

Successful protoplast regeneration has been previously reported in this species (Matsumura et al. 2001). Nevertheless, this protocol presents two main disadvantages: 1) the use of abalone acetone powder, a crude extract that is no longer available commercially, for protoplast isolation; and 2) the fact that protoplast culture is time consuming during the first month, as it involves changing the medium several times a day, increasing the chances of contamination due to manipulation (Leifert et al. 1991).

In this study, we report an improved protocol for protoplast isolation and successful regeneration from *Undaria pinnatifida* sporophytes using a simple mixture of commercial enzymes. We tested the effect of osmolarity, driselase inclusion, chelation pre-treatment, incubation time, origin of explants and previous isolation protocols on protoplast production. Also, we assessed the effect of regeneration medium, temperature and antibiotic mixture in protoplasts cultures to determine the best conditions for regeneration.

2. Materials and methods

Male and female gametophyte cultures were established from matured sporophytes sampled at Heuksan (May 19, 2016) and Jindo islands (May 5, 2016), Korea, respectively. Sporulation, isolation and maintenance of the cultures were performed as described in chapter 2. Young sporophytes (0.5-1 cm in length) were obtained after 2-3 months in culture at 16 °C by crossing both strains using the method described by Shan et al. (2016).

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study included cellulase Onozuka RS (Yakult Co. Ltd., Japan), alginate lyase, and driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA). Different enzyme combinations and conditions are shown in Table 24.

Protoplast isolation was performed as described in "General Materials and Methods" with explants of 4 mm^2 from cultured sporophytes of *Undaria pinnatifida* (0.5-1 cm in length) at 20 °C, pH 6 for 6 h in the dark. Enzyme mixtures contained cellulase Onozuka RS and alginate lyase, either with or without driselase. The osmolarity of the enzymatic solution was tested in two levels: normal osmolarity ($1\times = 1570$ mOsm L^{-1} H$_2$O) and increased osmolarity ($1.6\times = 2512$ mOsm L^{-1} H$_2$O). Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. Also, we tested the effect of chelation pre-treatment, incubation time, and the origin of the explant on protoplast yields. For assessing the last factor, explants of 4-6 mm^2 from the rhizoid, stipe, basal meristem and distal blade were excised from a field sample of *U. pinnatifida* (20 cm in length) collected at Jindo Island in April 14, 2018. Protoplast isolation was repeated four times in each treatment.

Protoplast isolation was also performed following the protocols of Kevekordes et al. (1993), also called non-enzymatic method; and Chen & Shyu (1994a), using 4% cellulase RS and 2% macerozyme R-10. Their protoplast yields were compared to the values obtained using our protocol under optimal conditions.

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively,

as described in "General Materials and Methods". The protoplast viability was further confirmed by using 2.4 µM FDA (Sigma, USA) and observed under a Leica DMi8 inverted microscope equipped with a 540/46 nm emission filter and a 590 nm suppression filter.

Protoplast regeneration

Protoplast culture and regeneration was performed as described in "General Materials and Methods" using 1 mL of regeneration medium in 24-well tissue culture test plates with an initial protoplast density of 2.4 x 10^3 protoplasts mL^{-1} in the dark. Osmolarity reduction started after 3 days in culture. Protoplasts were exposed to 10 µmol photons m^{-2} s^{-1} by the end of osmolarity reduction. Ten days later, light intensity was increased to 20 µmol photons m^{-2} s^{-1}. Preliminar experiments showed that direct exposure to 20-40 µmol photons m^{-2} s^{-1} was detrimental to protoplast survival. PES medium was renewed every 2-3 days during the first month in culture, and then once a week.

Two experiments were conducted to investigate the effect of regeneration medium, antibiotic mixture and temperature on protoplast cultures. In experiment 1, four regeneration media were tested (Table 25) at 16 °C using ES antibiotic mixture (Table 26) at the beginning of the culture. After 2 weeks, cultures were checked for the presence of alive cells (cells showing brown pigmentation). In experiment 2, three antibiotic mixtures (Table 26) and two temperatures (16 °C and 20 °C) were evaluated in three repetitions using the best regeneration medium from the previous experiment. The percentage (%) of alive and dividing cells were calculated after 1 and 2 months in culture, respectively. Cultures were checked every week thereafter until sporophyte regeneration. For a better comparison with the protoplast regeneration process reported previously (Matsumura et al. 2001), FPE was defined as the percentage of the originally plated protoplast (P_0) that had proliferated into PDAFs (called gametophyte-like filaments by Matsumura et al. 2001) (P_{pdaf}) after 3 months of culture. FPE (%) was calculated using the following equation:

$$FPE(\%) = \frac{P_{pdaf}}{P_0} \times 100$$

The regenerated sporophyte yield (RSY), which is the amount of regenerated sporophytes that can be obtained from protoplasts produced by one gram of explant, was calculated using the following equation:

$$RSY = \frac{PY \times Sp}{IPP}$$

where PY is the protoplast yield expressed as protoplasts g^{-1} FW, Sp is the total amount of regenerated sporophytes after 3 months in culture, and IPP is the total initial amount of plated protoplasts. RSY was expressed as sporophytes g^{-1} FW. Only normal sporophytes (i.e. sporophytes without outgrowths in the margins consisting of undifferentiated cells) were considered for RSY calculation.

Statistical analysis

Normality and homoscedasticity were examined by using the Shapiro–Wilk and Levene tests, respectively, prior to conducting parametric tests. Two-way analysis of variance (ANOVA) was used for the comparison of protoplast yield under driselase inclusion and osmolarity. One-way ANOVA was performed to examine the effect of chelation pre-treatment, incubation time, origin of the explant and isolation protocols on protoplast yields. Welch's ANOVA tests were used when data did not meet homoscedasticity assumption. Effect sizes (Sullivan & Feinn 2012) were presented as ω^2 (Field 2009, Lakens 2013) in case of significant results. All these analyses were performed using "car" (Fox & Weisberg 2019) and "userfriendlyscience" (Peters 2018) packages in R.

Tukey's post hoc test was used when the results were significant. In case of heteroscedastic data, Games-Howell test was performed instead. Post-hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.

Table 24. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Undaria pinnatifida* sporophyte.

Commercial enzymes	Composition of enzyme mixtures			
	A	B	C	D
Cellulase Onozuka RS (%)	1	1	1	1
Alginate lyase (U mL^{-1})	4	4	4	4
Driselase (%)	1	-	1	-
Osmolarity	1×	1×	1.6×	1.6×

1×, 1570 mOsm L^{-1} H$_2$O; 1.6×, 2512 mOsm L^{-1} H$_2$O

Table 25. Regeneration media used in protoplast cultures of *Undaria pinnatifida* sporophyte. Components in bold are the osmotica of each medium. Osmolarities were calculated as 1570 mOsm L^{-1} H_2O for RM1, RM2, RM3, and RM6; 1300 mOsm L^{-1} H_2O for RM4; and 1662 mOsm L^{-1} H_2O for RM5. Calcofluor was included at a final concentration of 10 μg mL^{-1}.

Regeneration medium	Component	Reference
RM1	PES with 285 mM **NaCl** and 5 mM CaCl$_2$	Mejjad et al. 1992
RM2	PES with 285 mM **NaCl**, 0.7 mM sucrose, and 1 mM glucose	Ducreux and Kloareg 1988
RM3	PES with 570 mM **sorbitol**	Cheng and Shyu 1994a
RM4	Seawater with 50mM **MgCl$_2$** and 75 mM **KCl**	Benet et al. 1997
RM5	Seawater with 150 mM **MgCl$_2$**, 100 mM **KCl**, 4 mM NaHCO$_3$, 2 mM KNO$_3$ and 100 μM NaH$_2$PO$_4$	Coelho et al. 2012
RM6	Enzymatic solution (without enzymes) used for protoplast isolation	Matsumura et al. 2001

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Table 26. Antibiotic mixtures used in protoplast cultures of *Undaria pinnatifida* sporophyte. Mixtures were added once at the beginning of the cultures. Final concentrations of each component are showed.

Antibiotic mixture	Component	Reference
PSC	Penicillin G (50 mg L^{-1}), streptomycin (25 mg L^{-1}), and chloramphenicol (5 mg L^{-1})	Coelho et al. 2012
ES	Erythromicin (50 mg L^{-1}) and streptomycin (50 mg L^{-1})	Varvarigos et al. 2004
ES-half	Erythromicin (25 mg L^{-1}) and streptomycin (25 mg L^{-1})	Varvarigos et al. 2004

The percentage of alive and dividing cells were analyzed as proportions with beta regressions, since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari & Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto & Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson & Verkuilen 2006). Treatments with no cell survival or division were excluded from the analysis.

3. Results

Protoplast isolation using enzymes

Protoplast yields ranged from 10-26 $\times$ 10^6 protoplasts g^{-1} FW. Mixture A (cellulase RS, alginate lyase and driselase with 1$\times$ osmolarity) produced the highest number of protoplasts (26.33 $\pm$ 8.82 $\times$ 10^6 protoplasts g^{-1} FW), followed by mixture B (cellulase RS and alginate lyase with 1$\times$ osmolarity) with 20.89 $\pm$ 4.36 $\times$ 10^6 protoplasts g^{-1} FW. The effect of osmolarity and driselase inclusion is shown in Figure 23A. Osmolarity increase had a negative effect on protoplast production ($p = 0.002$; $\omega^2 = 0.49$). Under normal osmolarity (1$\times$), protoplast yields were 1.69-2.07 times higher than in increased osmolarity (1.6$\times$). The inclusion of driselase to the enzymatic mix did not affect protoplast yield ($p = 0.077$). The interaction between both factors was not significant ($p = 0.999$).

In an effort to simplify our protoplast isolation protocol, chelation pre-treatment and different incubation times were tested. Our experiments showed that pre-treatment did not have a significant effect on protoplast yield ($p = 0.733$; Fig. 23B). Also, incubation time could be reduced to 2-4 h without compromising the protoplast numbers (Fig. 23C). Isolation of protoplasts was also possible from a field sample; however, the origin of the explants had highly significant effect on protoplast yields ($p < 0.001$; $\omega^2 = 0.96$). Those ones coming from the basal meristem yielded the highest protoplast amount (47.02 $\pm$ 3.62 $\times$ 10^6 protoplasts g^{-1} FW; Fig. 23D). Our protocol produced more than 7 times the amount of protoplasts obtained with two previously reported protocols (Fig. 23E). It is worth to notice that, in subsequent experiments, the non-enzymatic method was not successful in isolation protoplasts.

Explants were totally digested after 2-4 h in the enzymatic mixture (Fig 24A, B), except for the rhizoidal part (Fig 24C). Numerous protoplasts were isolated from the epidermis, cortex and medulla of the blades and stipe (Fig. 24D). Protoplasts were pale yellow-brown, spherical shape with several discoid chloroplasts. They were 18.40 ± 4.55 μm (range, 8-32 μm) in diameter. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% with red chlorophyll autofluorescence and about 65% with FDA staining (Fig 24E, F).
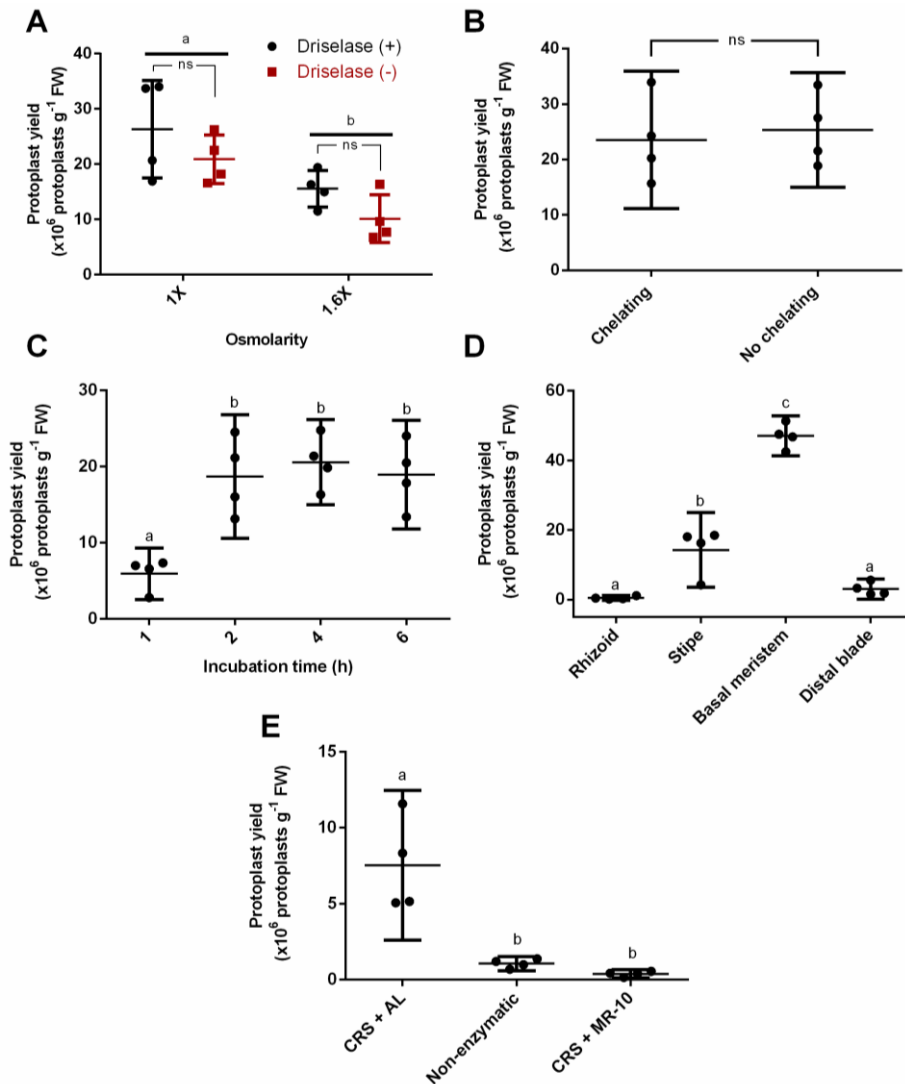
Fig. 23. Effect of different isolation conditions on protoplast yield from *Undaria pinnatifida* sporophyte. (A) Effect of osmolarity and driselase inclusion. (B) Effect of chelation pre-treatment. (C) Effect of incubation time. (D) Effect of origin of explants using a field sample. (E) Effect of three different isolation protocols: CRS+AL (this study); non-enzymatic (Kevekordes et al. 1993); and CRS+MR-10 (Chen & Shyu 1994a). Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different letters indicate significant differences between means ($p < 0.01$). CR, cellulase RS; AL, alginate lyase; MR-10, macerozyme R-10; ns, no significant difference ($p > 0.01$).
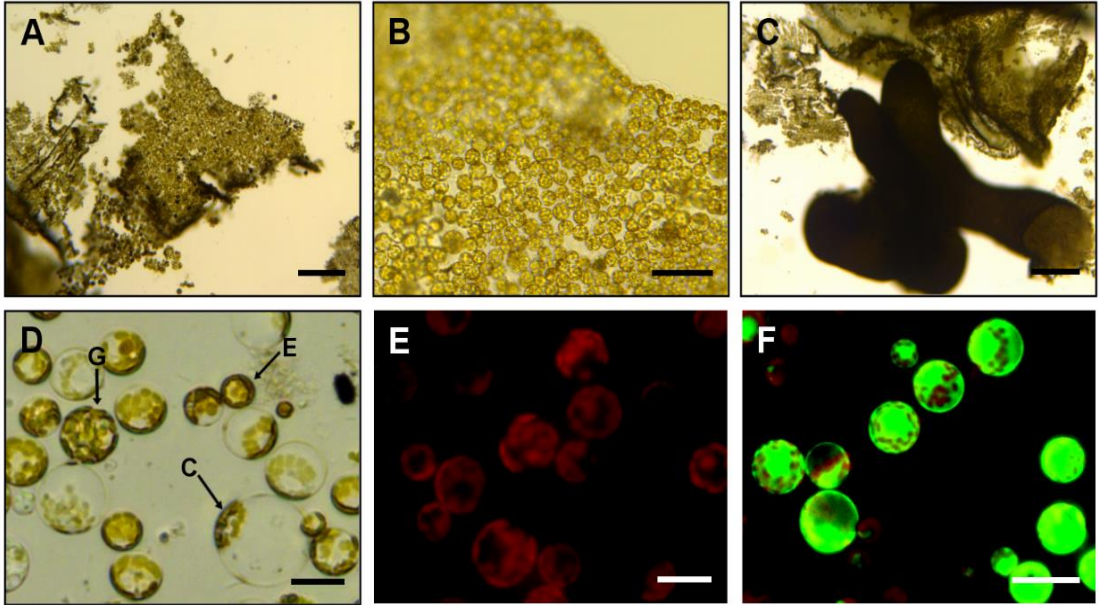
Fig. 24. Protoplast production from *Undaria pinnatifida* sporophyte. (A) Blade after 2 h of enzymatic digestion. Note the disintegration of the explant. (B) Close up of the same blade. Note the spherical protoplasts. (C) Rhizoid after 2 h of enzymatic digestion without any sign of softening. (D) Protoplasts from epidermis (E), gland cells (G), and cortex (C). (E) True protoplasts showing red chlorophyll autofluorescence. (F) Viable protoplasts stained with FDA showing green fluorescence. The scale in (A) and (C) is 200 µm; the scale in (B) is 50 µm; the scales in (D), (E) and (F) are 10 µm.

Protoplast culture and regeneration

Staining with calcofluor white revealed that protoplasts formed cell walls within 3-4 days in culture regardless their initial size. About 50% of protoplasts showed cell walls with homogenous distribution of cellulose (i.e. normal cell wall regeneration) during the next 4 days (Fig 25). Upon culture in different regeneration media, protoplasts were able to survive only in RM1 and RM2 after two weeks in culture (Table 27). For the next experiment, RM1 was chosen because it had simpler composition and lacked sugars, which could help to control bacterial growth. As protoplasts cultured without antibiotics could not survive more than 1 month in culture, two antibiotic mixtures were tested. Cell survival was better with PSC at 16 °C ($73.82 \pm 18.28\%$) and 20 °C ($87.99 \pm 10.40\%$). Low survival ($< 1\%$) was mostly found in cultures with ES (Fig. 26A). After 2 months in culture, only PSC treatment presented cells in division at both temperatures tested (10-16%). ES and ES-half treatments showed significantly lower values of dividing cells (~5%) at 16 °C, while division was not observed at 20 °C (Fig. 26B). Protoplast cultures with PSC were selected for monitoring the regeneration process during the rest of the experiment.

The protoplasts regenerated into normal sporophytes mostly at 20 °C. Two different development processes were observed depending on the temperature.

Indirect development through formation callus-like masses at 16 °C: Protoplasts underwent symmetric cell division after a month in culture (Fig. 27A), and then formed uniseriate filaments through successive cell divisions during the next 15 days (Fig. 27B). Callus-like masses developed after 2 months in culture (Fig. 27C). The masses were composed of unpigmented outer cells and pigmented inner cells with various sizes (15-58 μm in diameter), and shapes (rectangular to spherical, Fig. 27D). These masses became visible by naked eye during the next 15-30 days (~1 mm in diameter, Fig. 27E). Small blades emerged from the masses during the same period; however, these readily formed dedifferentiated cells in the margins that did not allow further growth of the blade (Fig. 27F).

Indirect development through formation of PDAFs at 20 °C: Protoplasts mostly underwent asymmetric cell division after 1-2 months in culture (Fig. 28A), and then formed rhizoid-like protrusions in one pole of the cell (Fig. 28B). Filaments resembling gametophytes (from now on referred to as PDFAs) developed during the next 15 days (Fig 28C). PDFAs were monoecious

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and formed antheridia and oogonia (Fig. 28D). Cells were 27.20 ± 12.98 um in length x 6.05 ± 1.48 um in width. Callus-like masses were also observed at 20 °C but in very low percentage (5.2%). After 3 months in culture, normal sporophytes developed from vegetative cells or released egg cells from PDFAs (Fig. 28E). FPE and RSY values were 0.51-0.77% and 1.2-3.0 x 10^4 sporophytes g^{-1} FW, respectively. Sporophytes (about 0.5 cm in length) were transferred to 1-L flat-bottomed round flasks filled with 1-L PES medium under aeration at 12 °C and 40-60 µmol photons m^{-2} s^{-1} of white fluorescent light. Under this conditions, they reached about 9 cm in size, with 0.5 cm stipes after 1-1.5 months (Fig. 28F).

Callus-like masses could be fragmented (friable) and further propagated in 100×40 mm Petri dishes containing 50 mL PES at 16 °C and 20 °C (Fig 29A). Abnormal sporophytes were sometimes observed emerging from the masses. Four out of the five cultures maintained at 20 °C developed filaments that resembled PDFAs after 11 months in culture (Fig 29B.). PDFAs were also propagated under the previously described conditions (Fig 29C). Sporophyte production was not sustained during the whole period of culture. PDFAs kept producing normal sporophytes during the first two subcultures; however, sporophytes were rarely or not observed in the subsequent subcultures (Table 28). Callus-likes masses and PDFAs cultures have been maintained in our laboratory for more than a year.

Table 27. Protoplast cultures of *Undaria pinnatifida* sporophyte in different regeneration media. Culture conditions were 16 °C, with an initial protoplast density of 2.4 x 10^3 protoplasts mL^{-1}, at 10 μmol photons m^{-2} s^{-1} of white fluorescent light, and ES antibiotic mixture. Protoplast survival was checked after 2 weeks in culture.

Regeneration medium	Observations
RM1	Protoplasts survived
RM2	Protoplasts survived
RM3	Protoplasts did not survive (lysis)
RM4	Few alive protoplasts with fragmented chloroplasts
RM5	Protoplasts did not survive (lysis)
RM6	Protoplasts did not survive (lysis)
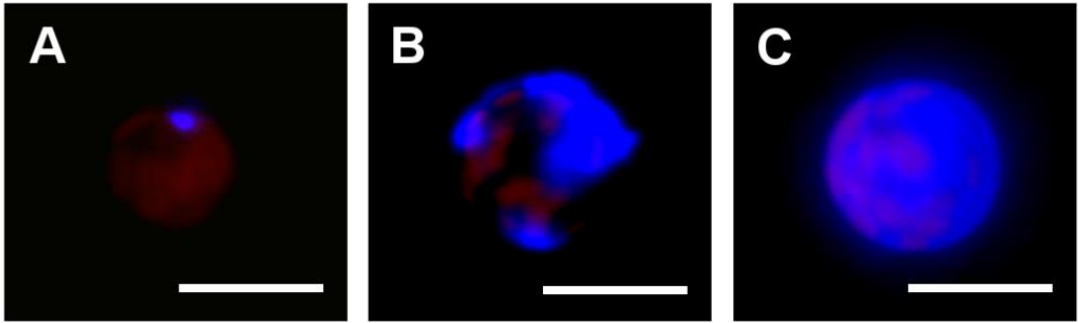
Fig. 25. Cell wall formation of protoplasts from *Undaria pinnatifida* sporophyte. (A) Fluorescence image of cell wall formation after 3 h of culture. (B) Fluorescence image of cell wall formation after 26 h of culture. (C) Fluorescence image of cell wall formation after 1 week of culture. Areas showing bright blue fluorescence indicate cellulose deposition. The red autofluorescence of the chlorophyll reveals areas without cell wall. The scales in (A), (B) and (C) are 10 μm.
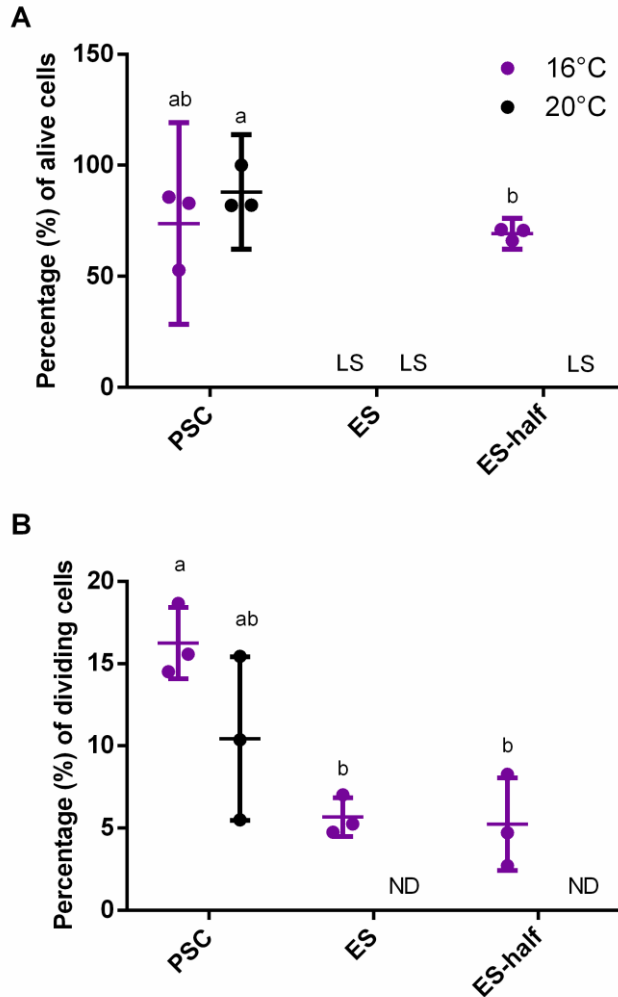
Fig. 26. Effect of temperature and antibiotic mixtures on protoplast survival and cell division from *Undaria pinnatifida* sporophyte. (A) Effect of temperature and antibiotic mixtures on the percentage (%) of alive cells after one month in culture. (B) Effect of temperature and antibiotic mixtures on the percentage (%) of dividing cells after two months in culture. Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different letters indicate significant differences between means ($p < 0.01$). PSC, Penicillin G + Streptomycin + Chloramphenicol; ES, Erythromycin + Streptomycin; ES-half, half concentration of ES mixture; LS, low survival ($\leq$ 1%); ND, no cell division; ns, no significant difference ($p > 0.01$).
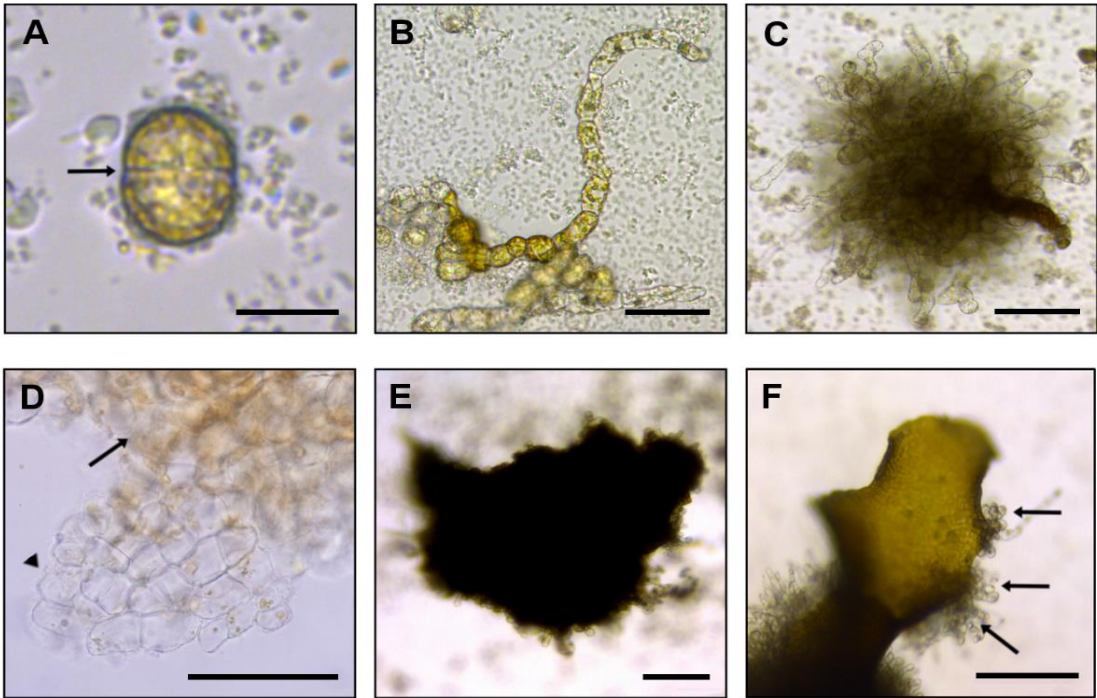
Fig. 27. Indirect development of protoplasts from *Undaria pinnatifida* sporophyte through formation of callus-like masses at 16 °C. (A) First symmetric cell division after a month in culture. The arrow indicates the division plane. (B) Uniseriate filament formed through successive cell divisions after 1 and half month in culture. (C) Callus-like mass after 2 months in culture. (D) Unpigmented outer cells (arrowhead) and pigmented inner cells (arrow) from a callus-like mass. (E) Friable callus-like mass after 2 and half months in culture. (F) Abnormal sporophyte from a callus-like mass showing dedifferentiated cells growing at the margins of the blade (arrows). The scale in (A) is 10 µm; the scale in (B) is 50 µm; the scales in (C) and (D) are 100 µm; the scale in (E) is 400 µm; the scale in (F) is 500 µm.
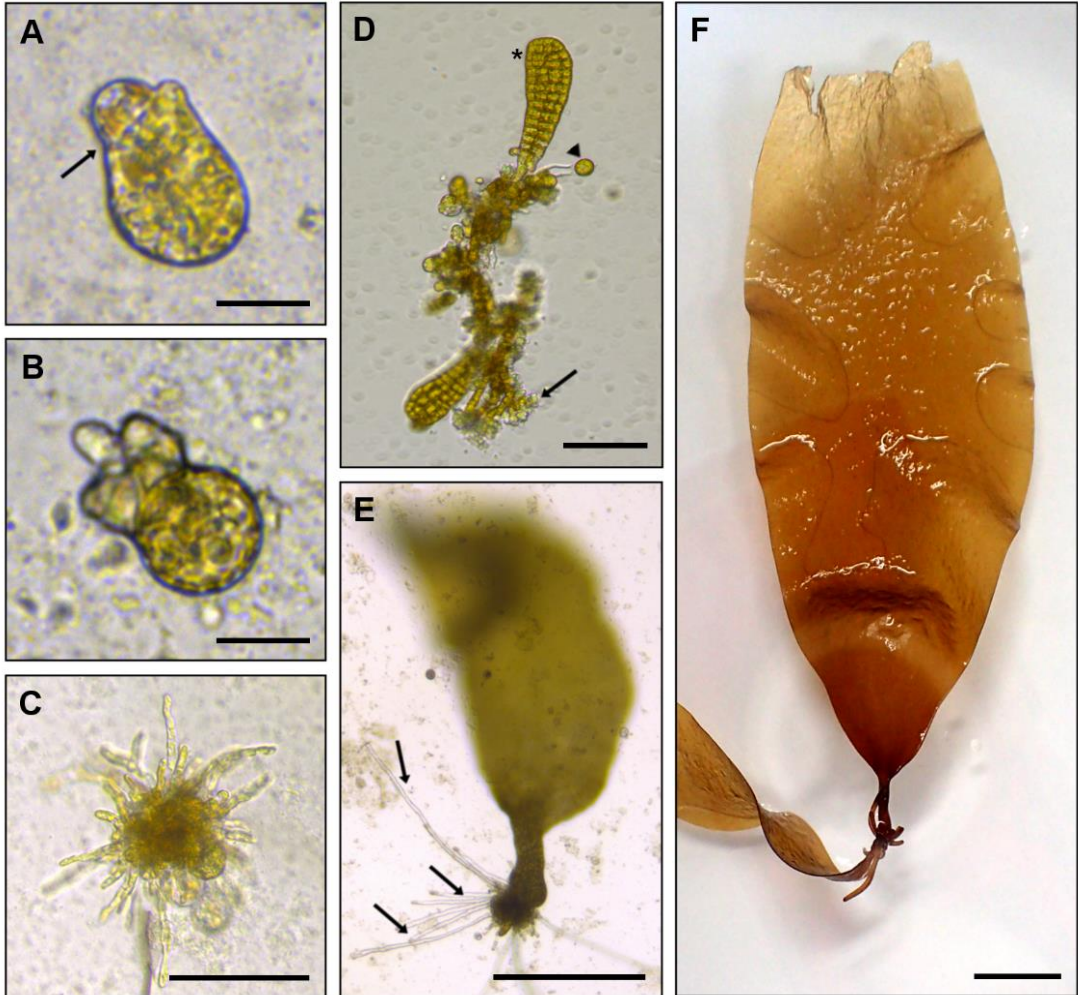
Fig. 28. Indirect development of protoplasts from *Undaria pinnatifida* sporophyte through formation of aposporous filaments at 20 °C. (A) First asymmetric cell division after 2 months in culture. The arrow indicates the division plane. (B) Rhizoid-like protrusions from the initial protoplasts after 2 months in culture. (C) Protoplast-derived aposporous filaments (PDAFs) after 2 and half months in culture. (D) Antheridia (arrow) and an oogonium (arrowhead) from PDFAs. Asterisk indicates a young sporophyte. (E) A sporophyte with well-developed rhizoids (arrows) after 3 months in culture. (F) Sporophyte after 1 month in culture at 12°C and constant aeration. The scales in (A) and (B) are 10 µm; the scales in (C) and (D) are 100 µm; the scale in (E) is 500 µm; the scale in (F) is 1 cm.

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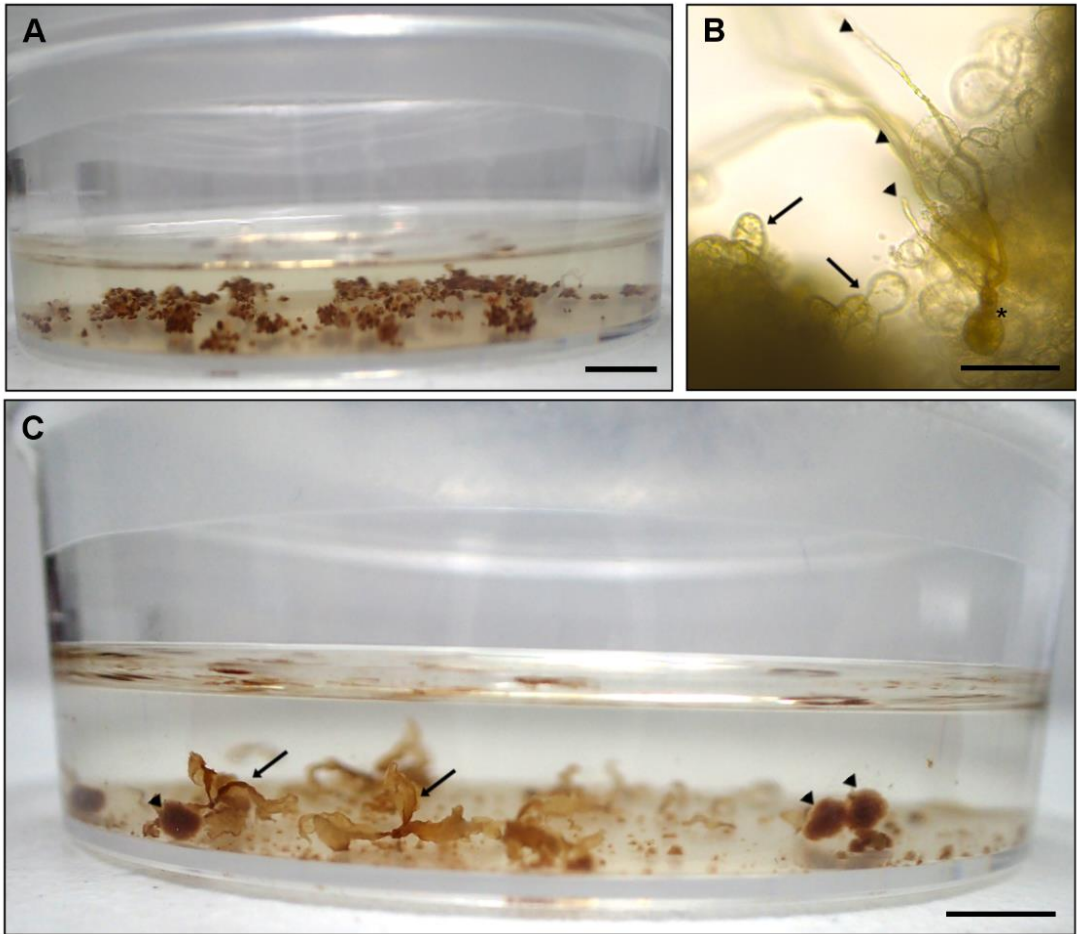
Fig. 29. Propagation of callus-like masses and protoplast-derived aposporous filaments (PDAFs) from *Undaria pinnatifida* sporophyte after 11 months in 50 mL cultures. (A) Callus-like masses at 16 °C. (B) Callus-like mass (arrows) at 20 °C forming filaments resembling PDAFs (arrowheads). Asterisk indicates the cell in the callus-like mass that produces the filaments. (C) Sporophytes (arrows) arising from PDAFs (arrowheads) at 20 °C. The scales in (A) and (C) are 1 cm; the scale in (B) is 100 µm.

Table 28. Sporophyte production from protoplast-derived aposporous filament subcultures of *Undaria pinnatifida* at 20 °C, under 40-60 μmol photons m^{-2} s^{-1} of white fluorescent light. The total amount of regenerated sporophytes were counted after 1 month of establishing the subculture. PDFAs were subcultured 5 times over the course of one year.

	Culture volume (mL)	Total amount of regenerated sporophytes
Initial culture	1	26
1st subculture	50	55
2nd subculture	5	56
3rd subculture	5	2
4th subculture	5	0
5th subculture	5	0

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4. Discussion

The present study presents an improved method for protoplast isolation and regeneration of sporophytes from *Undaria pinnatifida* using commercial enzymes.

Protoplast isolation from *Undaria pinnatifida* has been reported by several authors (Fujita & Migita 1985; Wu 1988; Yamaguchi 1989; Matsumura et al. 2001; Xiaoke et al. 2003). The enzyme mixtures used in these works are composed by alginate lyases or crude extracts from bacteria or the digestive system of marine herbivores. Our results show that is possible to isolate protoplast from *U. pinnatifida* using only two commercial enzymes, cellulase RS and alginate lyase. Furthermore, our protoplast yields ($2\text{-}4 \times 10^7$ protoplasts g^{-1} FW) are the highest reported so far.

Chen & Shyu (1994a) showed that a commercial enzyme mixture (4% cellulase RS and 2% macerozyme R-10) was effective for obtaining protoplasts from *Undaria pinnatifida* sporophyte. We could also isolate protoplasts using the same enzyme combination; however, the yields were almost 20 times less than the values obtained using our protocol. A non-enzymatic method has been reported for producing protoplasts from some kelp species (Kevekordes et al. 1993). We also tried this method on *U. pinnatifida*. Our protocol gave 7 times more protoplasts than the non-enzymatic one. Although successful at first, the non-enzymatic method gave no protoplasts in subsequent experiments, showing that it is not reliable for protoplast production in *U. pinnatifida.*

Chelation pre-treatment has shown positive effects on protoplast production in other species of Laminariales (Butler et al. 1989; Kloareg et al. 1989); however, Chen & Shyu (1994a) reported that this pre-treatment reduced the amount of protoplasts from *Undaria pinnatifida*. In our experiments, the addition of cation chelators prior enzymatic treatment did not have a significant effect on protoplast yield. Thus, chelation pre-treatment can be skipped when isolating protoplasts from *U. pinnatifida*.

Incubation times from 2-6 h are reported for enzymatic digestion in *Undaria pinnatifida* (Wu 1988; Chen & Shyu 1994a; Matsumura et al. 2001). Xiaoke et al. (2003) found that 2 h was optimal for this species, and that longer times reduced the protoplast yields. This is similar to

what was found in our experiments, where incubation time could be reduced to 2-4 h without compromising protoplast production.

In complex brown algae, the origin of explants can influence the isolation process as the cell wall composition varies among different regions (Kloareg & Quatrano 1988). In *Saccharina latissima*, explants from the meristematic and distal area gave about 10 times higher amounts of protoplasts than those ones from the holdfast and stipe (Benet et al. 1997). Our results showed that the basal meristem of *Undaria pinnatifida* is more suitable for isolating high yields of protoplasts. This might be explained by the increment of M blocks in the alginate of the cell walls from meristematic regions (Kloareg & Quatrano 1988; Lee et al. 2012), where cell wall elasticity is necessary for cell division and growth (Burns et al. 1982a,b, 1984). According to the manufacturer, M blocks are the main target of the alginate lyase used in our protocol.

Matsumura et al. (2001) reported, for first time, the successful regeneration of sporophytes from protoplasts of *Undaria pinnatifida* after 3-4 months in culture. In this protocol, protoplast were isolated using a mix of commercial cellulase and abalone acetone powder, a crude extract that is no longer available, with chelation pre-treatment. Our protocol was also able to regenerate normal sporophytes from protoplasts obtained by commercial enzymes without the necessity of chelation pre-treatment. Regeneration time (3-4 months) was similar to what was reported by Matsumura et al. (2001). Furthermore, our method is less time consuming during the first month of protoplast culture and uses only two media during this period. The improved protocol gave higher values of protoplast yield and similar FPE (Table 29).

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Table 29. Comparison of protoplast isolation and regeneration from *Undaria pinnatifida* sporophyte between the previous protocol and our improved protocol.

	Matsumura et al. (2001)	This study
Commercial enzymes	Cellulase Onozuka RS	Cellulase Onozuka RS Alginate lyase (Sigma)
Crude extracts	Abalone acetone powder	No
Chelation pre-treatment	Yes	No
Incubation time	2 h	2-4 h
Protoplast yield (protoplasts g^{-1} fresh weight)	1.9×10^7	$2\text{-}4 \times 10^7$
Number of media used during protoplast culture	3 (regeneration medium, half-PES, PES)	2 (regeneration medium, PES)
Frequency of culture medium change during the first month	Several times a day during first 10 days, and then each 2 days	Once day during day 3 to 5, and then each 2 or 3 days
Final plating efficiency (%)	0.68	0.51-0.77

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An antibiotic mixture of penicillin G, streptomycin, and chloramphenicol was key for protoplast survival and cell division. The same mixture was applied in protoplast culture of the model organism *Ectocarpus* (Coelho et al. 2012). Despite the fact that Matsumura et al. (2001) did not include antibiotics in their protocol, their use is recommended for avoiding bacterial overgrowth and assuring protoplast survival. Protoplasts from kelps are very sensitive to microbial contamination (Benet et al. 1997).

The regeneration processes were similar to those ones reported by Matsumura et al. (2001); however, in our experiments, PDAFs were inhibited at 16 °C and only sporophytes produced from PDAFs were able to grow normally. These authors also suggested that PDAFs could be indefinitely propagated by subculturing for mass production of *Undaria pinnatifida*. In fact, clonal propagation through a diploid cell-filament suspension culture has been reported in other kelp, *Laminaria digitata* (Asensi 2001). Nevertheless, our results suggest that PDFAs have a limited potential for sporophyte production. After the second subculture, PDFAs apparently lost their capacity for sporophyte regeneration. Further studies are needed to determine optimal conditions for sustained production of *U. pinnatifida* sporophytes from PDFAs.

In conclusion, high yields of protoplasts could be obtained from *Undaria pinnatifida* using commercial cellulase RS and alginate lyase, without chelation pre-treatment, and after 2-4 h of enzymatic digestion. Protoplasts regenerated into normal sporophytes with the same efficiency previously reported using a culture protocol that is less time consuming and involves less manipulation.

CHAPTER 5. Protoplast isolation and regeneration from the potential economic brown alga *Petalonia fascia* (Ectocarpales, Phaeophyceae)

1. Introduction

Petalonia fascia (O.F.Müller) Kuntze is a brown algae distributed along temperate coasts worldwide. Its thallus is mostly epiphytic and shows a polystichous structure, with blades up to 30 cm high attached to the substratum by small discoid holdfasts (Boo 2010). Cultivation of *P. fascia* was proposed by Lee et al. (2003) in Korea as a new economic species for the country. They stated that *P. fascia* could be easily cultivated on ropes, and that its market value was promising, either as raw or dried good. In addition, chemical compounds or extracts from *P. fascia* has been used in nine patents for biomedical applications. For example, fucoidan from this species was used for enhancing stem cell mobilization and proliferation (Kim & Jung 2019). Thus, *P. fascia* represents a potential economic brown algae with multiple uses.

Protoplasts are naked plant cells that can be obtained by enzymatic digestion of the cell walls. These are widely used for studying plant genetics, breeding, and, more recently, for genome-editing and gene silencing technologies (Davey et al. 2005a; Burris et al. 2016). Considering the economic potential of *Petalonia fascia*, this species is a good candidate for protoplast technology. In brown algae, studies on protoplast isolation and regeneration has been accomplished in 31 species (Fig. 2, Table 2). High yields of protoplasts have been obtained from *P. fascia* using a combination of cellulase RS and macerozyme R-10 (Chen & Shyu 1994a). However, their regeneration ability, an important prerequisite in protoplast technology, has not been assessed.

In this study, we report the protoplast isolation and successful regeneration from *Petalonia fascia* using a simple mixture of commercial enzymes. We tested the effect of osmolarity, driselase inclusion, chelation pre-treatment, incubation time, and previous isolation protocols on protoplast production. Also, we assessed the effect of antibiotics and temperature in protoplasts cultures to determine the best conditions for regeneration.

2. Materials and methods

Isolation and culture of the strain

Petalonia fascia was isolated from crude cultures of filamentous brown algae collected in Geoje Island, Gyeongsangnam-do, Korea, on March 16, 2018. Young germlines were cultured in 60×15 mm Petri dishes containing PES medium under 14:10-h light/dark photoperiod at 20 °C with light intensity 40 µmol photons m^{-2} s^{-1} of blue LED (DyneBioCo.Korea). The medium was renewed weekly. In this conditions, prostrate thalli were obtained. Erect thalli (blades) developed upon cultivation in 1-L flat-bottomed round flasks filled with 1-L PES medium under aeration with a light intensity of 40–72 µmol photons m^{-2} s^{-1}. Temperature and photoperiod were the same as indicated above. The air was sterilized using 0.22-µm SFCA syringe filters (Corning, Germany). The medium was renewed every 2 weeks. After a month in culture, blades reached about 8 cm in length before start forming plurilocular sporangia.

Identification of the culture strain

Taxonomic identification was performed using morphological characters (Boo 2010) from blades maintained in 1-L flat-bottomed round flasks with aeration. Photomicrographs were taken using a Leica inverted microscope (DMi8; Leica, Germany) equipped with a Leica DFC450C camera. Genomic DNA extraction, PCR amplification, DNA purification, and sequencing were performed according to Bustamante et al. (2016) using cultured samples. The plastid *rbc*L was amplified using the primer combinations described by Kogame et al. (1999). The amplified gene sequences were compared to the GenBank nucleotide database using the BLAST program (Altschul et al. 1997).

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study included cellulase Onozuka RS (Yakult Co. Ltd., Japan), alginate lyase, and driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA). Different enzyme combinations and conditions are shown in Table 30.

Protoplast isolation was performed as described in "General Materials and Methods" with explants of 4-6 mm² from cultured blades of *Petalonia fascia* (about 8 cm in length) at 20 °C, pH

6 for 6 h in the dark. Immature blades were used in all experiments. Enzyme mixtures contained cellulase Onozuka RS and alginate lyase, either with or without driselase. The osmolarity of the enzymatic solution was tested in two levels: normal osmolarity ($1\times = 1570$ mOsm L^{-1} H_2O) and increased osmolarity ($1.6\times = 2512$ mOsm L^{-1} H_2O). Osmolarity was increased by increasing the component concentrations in the enzymatic solution keeping their same proportions. Also, we tested the effect of chelation pre-treatment and incubation time on protoplast yields. Protoplast isolation was repeated four times in each treatment.

Protoplast isolation was also performed following the protocols of Kevekordes et al. (1993), also called non-enzymatic method; and Chen & Shyu (1994a), using 4% cellulase RS and 2% macerozyme R-10. Their protoplast yields were compared to the values obtained using our protocol under optimal conditions.

Table 30. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Petalonia fascia*.

Commercial enzymes	Composition of enzyme mixtures			
	A	B	C	D
Cellulase Onozuka RS (%)	1	1	1	1
Alginate lyase (U mL^{-1})	4	4	4	4
Driselase (%)	1	-	1	-
Osmolarity	1×	1×	1.6×	1.6×

1×, 1570 mOsm L^{-1} H$_2$O; 1.6×, 2512 mOsm L^{-1} H$_2$O

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as described in "General Materials and Methods".

Protoplast regeneration

Protoplast culture and regeneration was performed as described in "General Materials and Methods" using 1 mL of RM1 (PES + NaCl + CaCl$_2$) in 24-well tissue culture test plates with an initial protoplast density of 9 x 10^3 protoplasts mL^{-1} in the dark. Osmolarity reduction started after 2 days in culture. Protoplasts were exposed to 2-4 μmol photons m^{-2} s^{-1} by the second day of osmolarity reduction. Light intensity was increased to 20-25 μmol photons m^{-2} s^{-1} by the end of the osmolarity reduction. PES medium was renewed once a week. White fluorescent light was used in all the cultures.

The addition of PSC antibiotic mixture (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin, and 5 mg L^{-1} chloramphenicol) and three temperatures (10 °C, 16 °C and 20 °C) were evaluated in three repetitions. The percentage (%) of dividing cells were calculated after 3 weeks in culture. Morphological forms (i.e. prostrate thallus, discoid thallus, mixed thallus and cell clumps) obtained in each treatment combination were recorded as the number of forms per well after 4 weeks in culture. FPE (%) was defined as the percentage of the originally plated protoplast (P_0) that had proliferated into blades (P_{bl}) after 4 weeks of culture. FPE (%) was calculated using the following equation:

$$FPE(\%) = \frac{P_{bl}}{P_0} \times 100$$

Statistical analysis

Normality and homoscedasticity were examined by using the Shapiro–Wilk and Levene tests, respectively, prior to conducting parametric tests. Two-way analysis of variance (ANOVA) was used for the comparison of protoplast yield under driselase inclusion and osmolarity. One-way ANOVA was performed to examine the effect of chelation pre-treatment, incubation time, and

isolation protocols on protoplast yields. Effect sizes (Sullivan & Feinn 2012) were presented as ω^2 (Field 2009, Lakens 2013) in case of significant results. All these analyses were performed using "car" (Fox & Weisberg 2019) and "userfriendlyscience" (Peters 2018) packages in R.

Tukey's post hoc test was used when the results were significant. Post-hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.

The percentage (%) of dividing cells and FPE (%) were analyzed as proportions with beta regressions, since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari & Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto & Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson & Verkuilen 2006).

The number of each morphological form per well was analyzed using either negative binomial or Poisson regression model. Likelihood ratio test was used for deciding which count regression model to use. If zeros were present, Voung test for non-nested data was carried out to check if a zero-inflated regression was needed (Elhai et al. 2008). The analyses were performed using "pscl" (Jackman 2015) and "MASS" (Venables & Ripley 2002) packages in R.

3. Results

Identification

The vegetative characteristics of the cultured blades matched with the description of *Petalonia fascia* (Boo 2010), although they were somewhat twisted due to culture conditions (suspension cultures; Fig 31A). Our morphological identification of *P. fascia* was confirmed by molecular analysis. A 1333-bp portion of the 1476-bp *rbc*L gene was sequenced for our strain of *P. fascia*. The *rbc*L sequence of our strain was 99% identical to field samples of *P. fascia* from Japan reported by Matsumoto et al. (2014).

Protoplast isolation using enzymes

Protoplast yields ranged from 28-85 $\times 10^6$ protoplasts g^{-1} FW. Mixture C (cellulase RS, alginate lyase and driselase with 1.6$\times$ osmolarity) produced the highest number of protoplasts (85.16 $\pm$

29.62 × 10⁶ protoplasts g⁻¹ FW), followed by mixture D (cellulase RS and alginate lyase with 1.6× osmolarity) with $48.80 \pm 12.45 \times 10^6$ protoplasts g⁻¹ FW. The effect of osmolarity and driselase inclusion is shown in Figure 30A. Osmolarity increase had a positive effect on protoplast production ($p = 0.008$; $\omega^2 = 0.36$). Under increased osmolarity (1.6×), protoplast yields were around 1.8 times higher than in normal osmolarity (1×). Although there was a tendency toward higher protoplast yields when driselase was included in the enzymatic mix, this was not statistically significant ($p = 0.011$). The interaction between both factors did not have a significant effect ($p = 0.999$).

In an effort to simplify our protoplast isolation protocol, chelation pre-treatment and different incubation times were tested. Our experiments showed that pre-treatment had a significant effect on protoplast yield ($p = 0.002$; $\omega^2 = 0.77$; Fig. 30B). Also, incubation time could be reduced to 4 h without compromising the protoplast numbers (Fig. 30C). Our protocol produced more than 14 times the amount of protoplasts obtained with two previously reported protocols (Fig. 30D).

Explants were totally digested after 2-4 h in the enzymatic mixture. Numerous protoplasts were isolated from the cortex and medulla of the blades (Fig. 31B). Protoplasts were pale yellow-brown, spherical shape with a single discoid chloroplast. They were 14.43 ± 5.91 μm (range, 7-36 μm) in diameter. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% (Fig 31C).

Protoplast culture and regeneration

Staining with calcofluor white revealed that protoplasts started regenerating their cell walls as soon as 12-24 h in culture (Fig. 31D). Some protoplasts showed complete re-synthesis of their cell walls after 4 days in culture (Fig. 31E). However, complete cell wall regeneration was extensively observed after 1 week and 5 days in culture. A week later, protoplasts underwent first asymmetric cell division and progressed into a 3-celled stage (Fig. 31F). Addition of PSC antibiotic mixture at the beginning of protoplast culture showed significant effect on cell division after 3 weeks in culture ($p = 0.002$, PRE = 0.01), as well as its interaction with temperature ($p = 0.002$, PRE = 0.05). The highest value of dividing cells were found at 10°C with antibiotics ($11.66 \pm 1.91\%$). After 4 weeks in culture, four main morphologies were observed: 1) prostrate thallus; 2) discoid thallus; 3) mixed thallus; and 4) cell clumps (Fig. 31G-J). Addition of

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antibiotics and temperature did not have significant effect on the formation of these morphological forms ($p > 0.01$). Prostrate thallus was the predominant form in all the cultures and blades arose almost exclusively from this one (Fig. 31M). The highest FPE was found at 20°C without antibiotics ($8.45 \pm 7.35\%$); however, FPE values were not significantly affected by the addition of antibiotics and temperature ($p > 0.01$; Table 31). In one culture, we could observed successive cell divisions within some spherical protoplast-cells, and the subsequent formation of "spores" that were later released (Fig. 31K). This "spores" were 4.85 ± 0.97 µm (range, 3-6 µm), 3 times smaller than protoplasts (Fig. 31L). They were able to germinate but their further development was not followed.
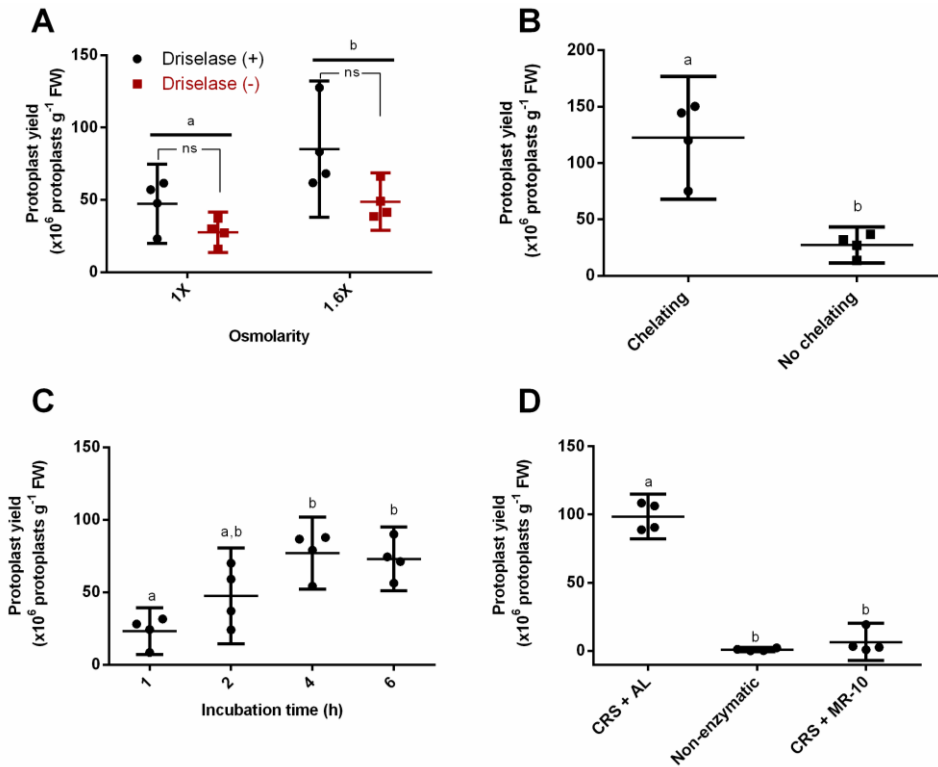
Fig. 30. Effect of different isolation conditions on protoplast yield from *Petalonia fascia*. (A) Effect of osmolarity and driselase inclusion. (B) Effect of chelation pre-treatment. (C) Effect of incubation time. (D) Effect of three different isolation protocols: CRS+AL (this study); non-enzymatic (Kevekordes et al. 1993); and CRS+MR-10 (Chen & Shyu 1994a). Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different letters indicate significant differences between means ($p < 0.01$). CR, cellulase RS; AL, alginate lyase; MR-10, macerozyme R-10; ns, no significant difference ($p > 0.01$).

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Table 31. Effect of the addition of antibiotics and temperature on protoplast regeneration of *Petalonia fascia* after 3 and 4 weeks in culture. Superscript letters indicate significant differences among treatments ($p < 0.01$) at each response variable (i.e. dividing cells, morphological forms and final plating efficiency). Values are presented as mean $\pm$ SD (n = 3).

Antibiotics (PSC mixture)*	Temperature	3 weeks in culture	4 weeks in culture					Final plating efficiency (%)
			Morphological forms					
		Dividing cells (%)	Prostrate thalli (protonemata well^{-1})	Discoid thalli (thalli well^{-1})	Mixed thalli[†] (thalli well^{-1})	Cell clumps (clumps well^{-1})		
With	10 °C	11.67 ± 1.91^a	7 ± 5^a	2 ± 2^a	4 ± 4^a	0.3 ± 1^a	0.04 ± 0.00^a
	16 °C	4.55 ± 1.15^b	17 ± 13^a	3 ± 4^a	1 ± 1^a	1 ± 1^a	0.10 ± 0.09^a
	20 °C	$6.17 \pm 3.57^{a,b}$	3 ± 2^a	1 ± 2^a	2 ± 2^a	1 ± 1^a	0.06 ± 0.03^a
Without	10 °C	4.33 ± 2.08^b	13 ± 12^a	1 ± 2^a	1 ± 1^a	0^a	0.07 ± 0.04^a
	16 °C	5.27 ± 2.64^b	6 ± 2^a	6 ± 10^a	2 ± 3^a	0.3 ± 1^a	0.10 ± 0.02^a
	20 °C	4.38 ± 0.98^b	6 ± 4^a	0^a	0^a	1 ± 1^a	0.11 ± 0.07^a

*PSC mixture was added once at the beginning of the culture. It contained 50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin, and 5 mg L^{-1} chloramphenicol; [†]Mixed thalli consisted of prostrate and discoid thalli arising from one single spot.

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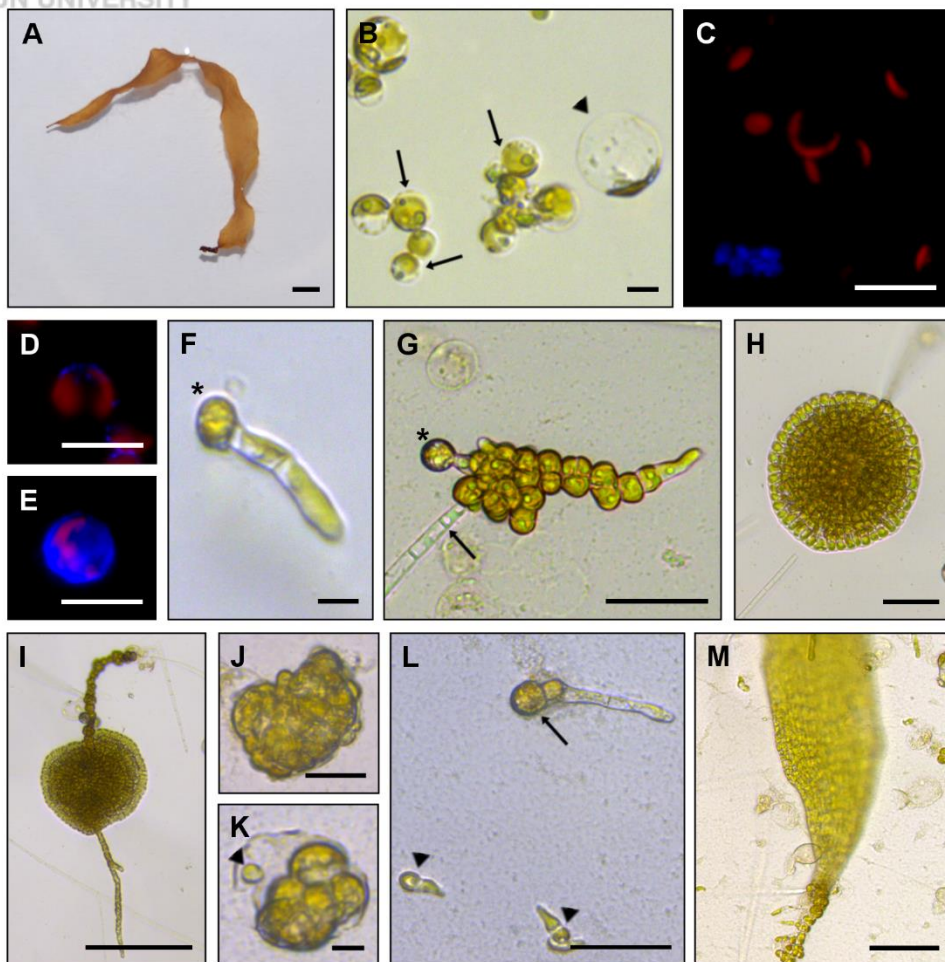
Fig. 31. Protoplast isolation and regeneration from *Petalonia fascia.* (A) A blade from suspension culture in 1-L flat-bottomed round flasks with aeration. (B) Protoplasts from the cortex (arrows) and medulla (arrowhead). (C) True protoplasts showing red chlorophyll autofluorescence. (D) Cell wall formation after 24 h of culture. (E) Complete cell wall regeneration after 4 days of culture. (F) 3-celled stage after 3 weeks of culture. (G) Prostrate thallus with a phaeophycean hair (arrow) after 4 weeks of culture. (H) Discoid thallus. (I) Mixed thallus showing prostrate filaments and disc thallus. (J) A cell clump. (K) A "spore" (arrowhead) being released from a group of cells derived of successive cell divisions within a spherical protoplast-cell. (L) Germination of spores (arrowheads) and a protoplast (arrow). (M) A blade formed from prostrate thallus after 4 weeks of culture. The blue fluorescence in (C) indicates cell wall material. Areas showing bright blue fluorescence in (D) and (E) indicate cellulose deposition. The asterisks in (F) and (G) indicate the initial protoplast. The scales in (A) is 1 cm; the scales in (B), (C), (D), (E), (F) and (K) are 10 µm; the scales in (G), (H) and (L) are 50 µm; the scale in (I) is 200 µm; the scale in (J) is 25 µm; the scale in (M) is 100 µm.

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4. Discussion

The present study reports the isolation of high amounts of protoplasts (10^7 protoplasts g^{-1} FW) from *Petalonia fascia* using commercial enzymes. Also, this study is the first presenting the successful regeneration of protoplasts from *P. fascia*.

A simple mix of commercial cellulase RS and alginate lyase could completely digest *Petalonia fascia* explants releasing high yields of protoplasts. The inclusion of driselase showed a positive effect on protoplast production; however, this was not statistically significant. Driselase is a natural mixture of enzymes (e.g. cellulase, hemicellulase and pectinase) that can cleave mixed-linked glucan (MLG), also known in fungi as lichenan (Thibault & Rouau 1990). Although the presence of MLG in brown algal cell walls has been reported (Salmeán et al. 2017), our results suggest that driselase might not be crucial for improving protoplast yields.

In our experiments, chelation pre-treatment improved the protoplast yield of *Petalonia fascia.* This positive effect has also been reported in protoplast isolation from Ectocarpales (Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989). However, in a previous report, this pre-treatment did not enhance protoplast release from *P. fascia* (Chen & Shyu 1994a). It is known that the pH of the EGTA solution used for pre-treatment is a critical factor. Butler et al. (1989) indicated that values over pH 5.5 caused extensive tissue damage in the brown algae *Laminaria*. The pH for pre-treatment used in our work and the previous report was 5.5 and 6.5, respectively. This could explain the difference in the effectiveness of the pre-treatment in *P. fascia*. Our results also showed an optimal incubation time of 4 h, which is the range of values reported for *Petalonia* and other blade forms (2-5 h; Chen & Shyu 1994a; Matsumura 1998)

Chen & Shyu (1994a) showed that a commercial enzyme mixture (4% cellulase RS and 2% macerozyme R-10) could produce high amounts of protoplasts (10^8 protoplasts g^{-1} FW) from *Petalonia fascia*. We also isolated protoplasts using the same protocol; however, the yields were almost 15 times less than the values obtained using our enzymatic combination. Kevekordes et al. (1993) reported a non-enzymatic method for producing protoplasts from some kelp species. Although we could isolate protoplasts using this method from *P. fascia*, our protocol gave about 100 times more protoplasts.

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Protoplast regeneration of *Petalonia fascia* involved the formation of prostrate, discoid and mixed thalli, as well as cell clumps. Among the four forms, the first one was the predominant in cultures. Erect thalli (blades) arose usually from the prostrate thalli after 4 weeks of culture. Discoid thalli and cell clumps have been also found in protoplast cultures from *P. binghamiae*. In this species, blades only emerged from discoid thalli formed by protoplasts from immature blades and young plantlets (Chen & Shyu 1994b). Prostrate and discoid thalli have been reported in the life cycle of *P. fascia* (Hsiao 1969; Kogame 1997; Lee et al. 2003). The occurrence of "spores" from group of cells formed within the spherical protoplast-cell has not been reported in protoplast regeneration of brown algae. Chen & Shyu (1994b) indicated that outer cells in clumps detached in later stages of protoplast culture; however, this does not seem to be the case in our cultures, as these "spores" were smaller than the other cells in the group. A detail examination of this process is needed for understanding the nature of these "spores".

Temperature did not have a significant effect on the development of the morphological forms and blades. A similar trend was found by Hsiao (1970), who reported that prostrate and discoid thalli, as well as blades, were present in cultures from 6 °C to 20 °C. The addition of antibiotics at the beginning of the culture did not enhance regeneration in *Petalonia fascia*. This suggests that protoplasts from this species are not very sensitive to microbial contamination, which differs from those ones of kelps (Laminariales; Benet et al. 1997).

In conclusion, high amounts of protoplasts could be obtained from *Petalonia fascia* using a simple mix of commercial enzymes (cellulase RS + alginate lyase), short incubation time (4 h), chelation pre-treatment, and increased osmolarity. Protoplasts regenerated into blades through the formation of prostrate thalli after 4 weeks in culture. Other forms were also observed but in less frequency. Antibiotics were not needed for improving regeneration and temperatures from 10 °C - 20 °C were suitable for protoplast culture. This is the first report of successful protoplast regeneration from the potential economic brown alga *P. fascia.*

CHAPTER 6. Protoplast isolation from three brown algal species (*Scytosiphon lomentaria*, *Dictyopteris pacifica* and *Ecklonia cava*) of economic importance

1. Introduction

Protoplasts technology offers a wide range of applications for basic and applied research, including genetic manipulation and crop improvement from species of economic importance (Davey et al. 2005a; Reddy et al. 2008; Burris et al. 2016). The success of this technology rely on reproducible protocols for protoplast isolation, which are mainly achieved by using commercial enzymes (Cocking 1972; Gupta et al. 2011; Inoue et al. 2011).

Brown algae are a diverse group of photosynthetic organisms (Bringloe et al. 2020). They are mainly distributed in marine environments and encompass species that are used in human food, animal feed, and traditional medicine (Liu et al. 2012; Sanjeewa & Jeon 2018). Protoplast isolation has been reported in 31 species of marine brown algae (Fig. 2, Table 2), including some commercial species like *Undaria pinnatifida* and *Saccharina japonica* (Matsumura et al. 2000, 2001). However, protoplast production in other economic brown algae has remained largely unexplored as works on this topic have become scarce during the last 8 years.

In an effort to expand protoplast technology in commercial brown algae, we selected three economic species (*Scytosiphon lomentaria*, *Dictyopteris pacifica* and *Ecklonia cava*) for protoplast production using commercial enzymes. These species are used as food and/or for traditional medicine in Asia, and have shown important biological properties (Matsukawa et al. 1997; Boo 2010; Zhuang et al. 2014; Sanjeewa & Jeon 2018). Also, we tested the effect of effect of osmolarity, driselase inclusion and chelation pre-treatment to determine the best isolation conditions for these species.

2. Materials and methods

Material

Scytosiphon lomentaria was collected at Gijang, Busan, Korea on February 20, 2019. The sample was cleaned with sterile seawater in the laboratory and it started to release spores after 10 min. These were isolated using the hanging-drop technique and then transferred to a 60×15 mm Petri dishes containing PES medium under 14:10-h light/dark photoperiod at 20°C with light intensity 40 μmol photons m^{-2} s^{-1} of white LED (DyneBioCo.Korea). To avoid further diatoms contamination, GeO_2 at a final concentration of 6 mg L^{-1} was added to the culture. These cultures produced prostrate filamentous thalli. After 2-3 months in culture, they were transferred to a 1-L flat-bottomed round flasks filled with 1-L PES medium under aeration with a light intensity 40–72 μmol photons m^{-2} s^{-1} of white fluorescent light. Temperature and photoperiod were the same as indicated above. Under these conditions, macrothalli of about 2-3 cm in length were formed after 2 months in culture.

Dictyopteris pacifica was collected at Uljin, Gyeongsangbuk-do, Korea on May 1, 2018. Monosporangial germlines were easily detached from old sporophytes in the lab and cultured in 60×15 mm Petri dishes containing PES medium under the same conditions for prostrate thalli of *Scytosiphon lomentaria*. Filamentous thalli were the predominant morphology in these cultures. Some small foliose thalli were also observed arising from the filamentous one but they quickly started to produce filaments (Fig. 35A). Upon culture in 1-L flasks, under the same conditions for suspension cultures of *S. lomentaria*, they produce foliose thalli of about 4-5 cm in length after 1 month and 1 week (Fig. 35B).

A juvenile thallus of *Ecklonia cava* (15 cm in length; Fig. 36A) was collected at Namhae Island, Gyeongsangnam-do, Korea on May 18, 2018. The thallus was transported to the laboratory in cool boxes (5–8 °C) within 48 h after collection. Once in the lab, it was cleaned from epiphytes and rinsed several times with sterile seawater prior protoplast isolation experiments.

Identification of the samples

Taxonomic identification was performed using morphological characters according Boo (2010), Cho (2010) and Lee & Hwang (2010), and confirmed molecularly. Genomic DNA extraction,

PCR amplification, DNA purification, and sequencing were performed according to Bustamante et al. (2016) using cultured (*Scytosiphon. lomentaria* and *Dictyopteris pacifica*) and field samples (*Ecklonia cava*). The plastid *rbc*L was amplified using the primer combinations described by Kogame et al. (1999). The amplified gene sequences were compared to the GenBank nucleotide database using the BLAST program (Altschul et al. 1997).

Protoplast isolation

The commercially available cell wall lytic enzymes used for this study included cellulase Onozuka RS (Yakult Co. Ltd., Japan), alginate lyase, and driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA). Different enzyme combinations and conditions are shown in Table 32 and 33.

Protoplast isolation was performed as described in "General Materials and Methods" with explants of 4-6 mm^2 from cultured macrothalli of *Scytosiphon lomentaria* (2-3 cm in length) and *Dictyopteris pacifica* (4-5 cm in length), and from the meristem region of *Ecklonia cava*. Enzymatic digestion was carried out at 20 ℃, pH 6 for 6 h in the dark. After preliminary experiments in *S. lomentaria* and *D. pacifica*, incubation time could be reduced to 4 h using normal osmolarity ($1\times = 1570$ mOsm L^{-1} H$_2$O). Enzyme mixtures contained cellulase Onozuka RS and alginate lyase, either with or without driselase. In *S. lomentaria* and *D. pacifica*, the inclusion of driselase was assessed together with chelation pre-treatment, whereas in *E. cava*, it was tested together with the osmolarity of the enzymatic solution in two levels: normal osmolarity ($1\times = 1570$ mOsm L^{-1} H$_2$O) and increased osmolarity ($1.6\times = 2512$ mOsm L^{-1} H$_2$O). Protoplast isolation was repeated four times in each treatment.

Viability and cell wall removal

The viability of protoplasts and cell wall removal were assessed by the red chlorophyll autofluorescence and staining with calcofluor white M2R (Sigma-Aldrich, USA), respectively, as described in "General Materials and Methods". The protoplast viability of *Scytosiphon lomentaria* and *Dictyopteris pacifica* was further confirmed by using 2.4 µM FDA (Sigma, USA) and observed under a Leica DMi8 inverted microscope equipped with a 540/46 nm emission filter and a 590 nm suppression filter.

Statistical analysis

Normality and homoscedasticity were examined by using the Shapiro–Wilk and Levene tests, respectively, prior to conducting parametric tests. Two-way analysis of variance (ANOVA) was used for the comparison of protoplast yield under driselase inclusion and chelation pre-treatment (in *Scytosiphon lomentaria* and *Dictyopteris pacifica*) or osmolarity (in *Ecklonia cava*). Effect sizes (Sullivan & Feinn 2012) were presented as ω^2 (Field 2009, Lakens 2013) in case of significant results. All these analyses were performed using "car" (Fox & Weisberg 2019) and "userfriendlyscience" (Peters 2018) packages in R.

Tukey's post hoc test was used when the results were significant. Post-hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.

3. Results

Identification

In our cultures, matured macrothalli of *Scytosiphon lomentaria* were smaller (2-3 cm in length) and with narrower hollow tubes (less than 1 mm in diameter; Fig. 34A, B) than the description provided by Womersley (1987) for field samples. Although the thalli did not show marked constrictions as reported by Boo (2010), molecular analysis using a 1342-bp portion of the 1476-bp *rbc*L showed that our sequence was 99.85% identical to a field sample of *S. lomentaria* from Japan (Cho et al. 2007). The cultured foliose thalli of *Dictyopteris pacifica* was about 4-5 cm in length and subdichotomous (Fig. 35B). The blades lacked of midrib, a key character for this species (Lee & Hwang 2010). Our morphological identification of *D. pacifica* was confirmed by molecular analysis. The *rbc*L region (1300-bp) of our strain was 100% identical to field samples of *D. pacifica* from Korea and Japan (Hwang et al. 2004). The young specimen of *Ecklonia cava* was identified based on molecular analysis. The *rbc*L sequence of this specimen (1349-bp) showed 99.92% of similarity with *E. cava* reported from Japan (Kawai et al. 2020).

Table 32. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Scytosiphon lomentaria* and *Dictyopteris pacifica*.

Commercial enzymes	Composition of enzyme mixtures			
	A	B	C	D
Cellulase Onozuka RS (%)	1	1	1	1
Alginate lyase (U mL^{-1})	4	4	4	4
Driselase (%)	1	-	1	-
Chelation pre-treatment	No	No	Yes	Yes

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Table 33. Combinations and concentrations of enzyme mixtures for protoplast isolation from *Ecklonia cava*.

Commercial enzymes	Composition of enzyme mixtures			
	E	F	G	H
Cellulase Onozuka RS (%)	1	1	1	1
Alginate lyase (U/mL)	4	4	4	4
Driselase (%)	1	-	1	-
Osmolarity	1×	1×	1.6×	1.6×

1×, 1570 mOsm L^{-1} H_2O; 1.6×, 2512 mOsm L^{-1} H_2O

Protoplast isolation using enzymes

Protoplast yields from *Scytosiphon lomentaria* ranged from $58\text{-}77 \times 10^6$ protoplasts g^{-1} FW. Mixture C (cellulase RS, alginate lyase and driselase with chelation pre-treatment) produced the highest number of protoplasts ($76.54 \pm 20.27 \times 10^6$ protoplasts g^{-1} FW), followed by mixture B (cellulase RS and alginate lyase without chelation pre-treatment) with $74.64 \pm 32.49 \times 10^6$ protoplasts g^{-1} FW. The effect of chelation pre-treatment and driselase inclusion is shown in Figure 32A. Pre-treatment did not improve protoplast production ($p = 0.664$), while the inclusion of driselase was not critical for enhancing protoplast yields ($p = 0.548$). After enzymatic digestion, two distinct populations were found with different sizes: protoplasts originating from vegetative cells of the cortex, and more or less mature zooids from digested plurilocular sporangia (Fig. 33, 34C, E). Medulla remained mostly undigested (Fig 34D). Numerous protoplasts were isolated from the cortex and medulla of the blades (Fig.34E). Protoplasts were pale yellow-brown, spherical shape with a single discoid chloroplast. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% with red chlorophyll autofluorescence and 83% with FDA staining (Fig 34F, G).

Protoplast yields from *Dictyopteris pacifica* ranged from $1\text{-}5 \times 10^6$ protoplasts g^{-1} FW. Mixture C (cellulase RS, alginate lyase and driselase with chelation pre-treatment) produced the highest number of protoplasts ($4.85 \pm 1.21 \times 10^6$ protoplasts g^{-1} FW), followed by D (cellulase RS and alginate lyase with chelation pre-treatment) with $4.83 \pm 2.08 \times 10^6$ protoplasts g^{-1} FW. The effect of chelation pre-treatment and driselase inclusion is shown in Figure 32B. Pre-treatment had a significant effect on protoplast production ($p < 0.001$; $\omega^2 = 0.63$). Explants pre-treated with chelation solution reported about 3 times more protoplasts than those ones without pre-treatment. The inclusion of driselase did not improve protoplast yields ($p = 0.573$). The interaction between both factors did not have a significant effect ($p = 0.587$). Numerous protoplasts were isolated from the cortex and medulla of the blades. Protoplasts were pale yellow-brown to dark brown, spherical shape with several discoid chloroplast (Fig. 35C, D). They were 26.59 ± 5.90 μm (range, 13-52 μm) in diameter. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% with red chlorophyll autofluorescence and 70% with FDA staining (Fig 35E, F).

Protoplast yields from *Ecklonia cava* ranged from 2-10 × 10^6 protoplasts g^{-1} FW. Mixture F (cellulase RS and alginate lyase with 1× osmolarity) produced the highest number of protoplasts (10.05 ± 3.22 × 10^6 protoplasts g^{-1} FW), followed by E (cellulase RS, alginate lyase and driselase with 1× osmolarity) with 6.61 ± 1.34 × 10^6 protoplasts g^{-1} FW. The effect of osmolarity and driselase inclusion is shown in Figure 32C Osmolarity increase had a negative effect on protoplast production ($p < 0.001$; $\omega^2 = 0.75$). Under increased osmolarity (1.6×), protoplast yields were around 2-4 times less than in normal osmolarity (1×). There was a tendency toward higher protoplast yields when driselase was included in the enzymatic mix at normal osmolarity; however, this was not statistically significant ($p = 0.083$). The interaction between both factors did not have a significant effect ($p = 0.081$). Although we did not test the effect of chelation pre-treatment, further experiments showed that protoplast yields were still high (13.45-18.50 × 10^6 protoplasts g^{-1} FW) even without pre-treatment. Protoplasts could be isolated from the cortex and medulla of the meristematic region (Fig. 36B). Protoplasts were pale yellow-brown, spherical shape with numerous discoid chloroplast. They were 7.35 ± 2.01 μm (range, 5-17 μm) in diameter. True protoplast percentages were 99–100% with calcofluor white staining. The viability of freshly isolated protoplasts was 98–100% with red chlorophyll autofluorescence (Fig. 36C).

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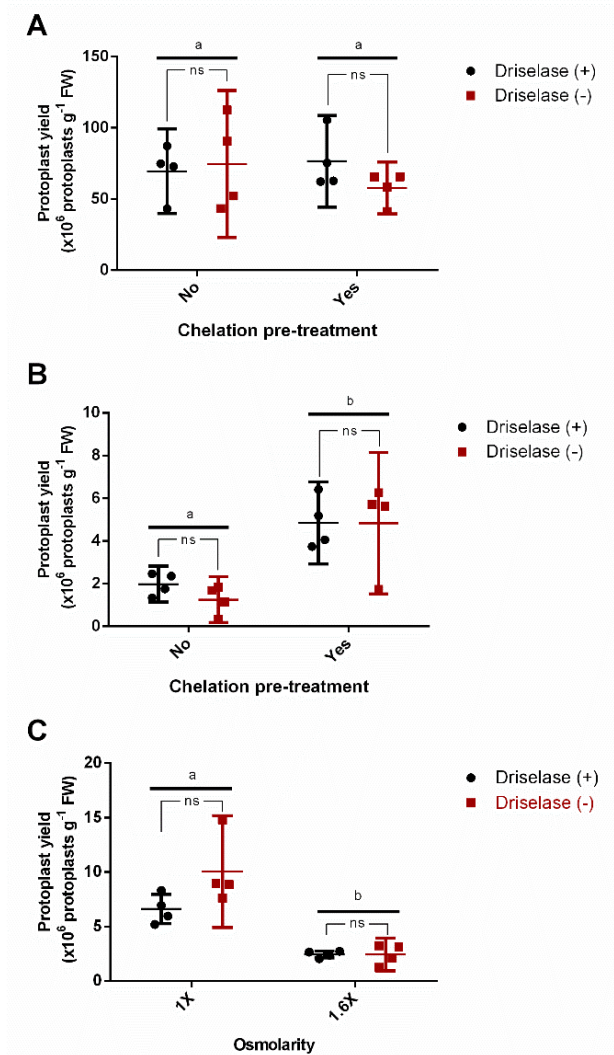
Fig. 32. Effect of different isolation conditions on protoplast yields from *Scytosiphon lomentaria, Dictyopteris pacifica* and *Ecklonia cava*. (A) Effect of chelation pre-treatment and driselase inclusion on protoplast yield from *S. lomentaria.* (B) Effect of chelation pre-treatment and driselase inclusion on protoplast yield from *D. pacifica*. (C) Effect of osmolarity and driselase inclusion on protoplast yield from *E. cava*. Independent data points and averages (horizontal lines) are shown (n = 4). Error bars represent 95% confidence intervals. Different letters indicate significant differences between means ($p < 0.01$). ns, no significant difference ($p > 0.01$).
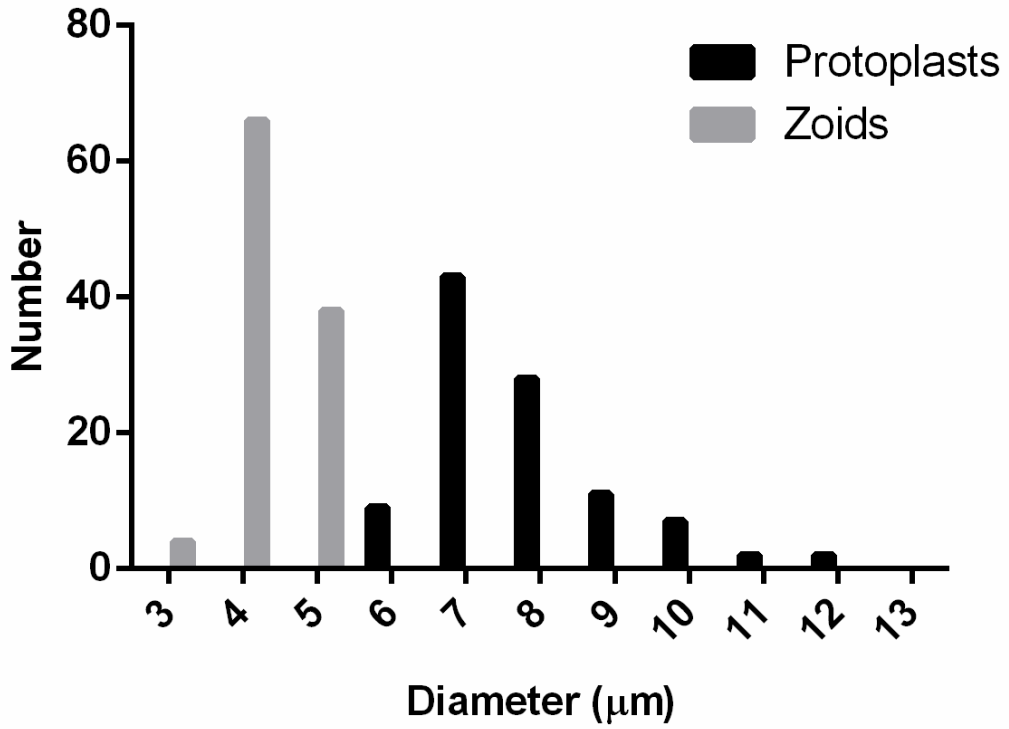
Fig. 33. Distribution of the size of somatic cell protoplasts and zooids
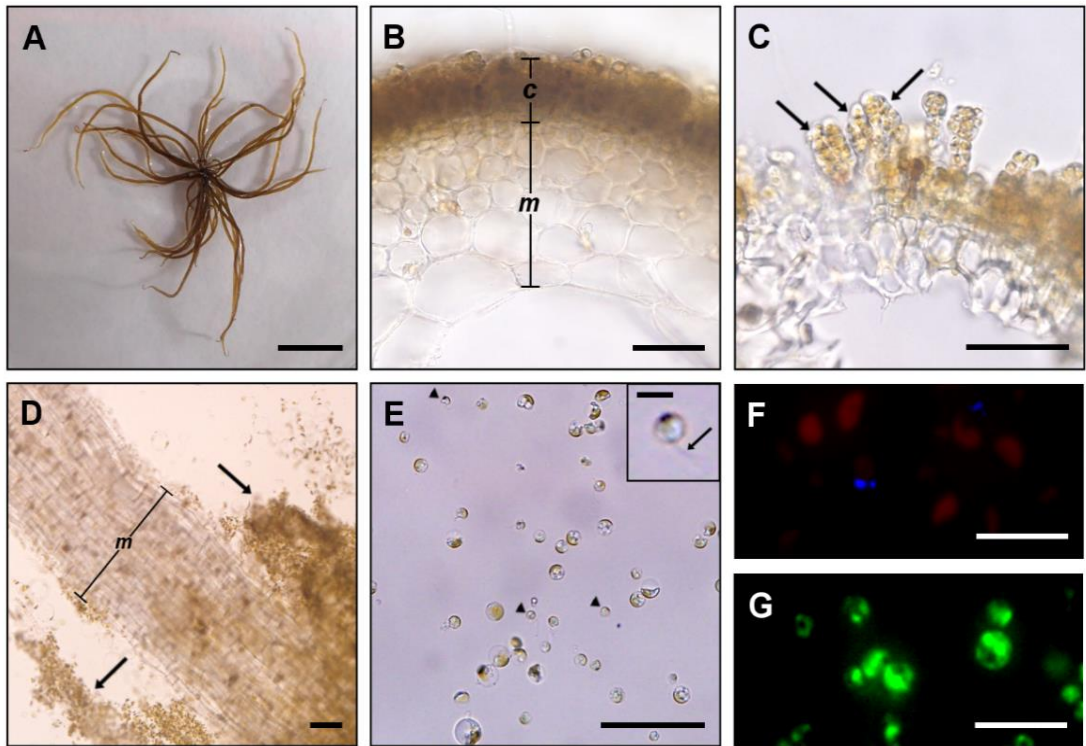
Fig. 34. Protoplast isolation from *Scytosiphon lomentaria.* (A) Mature macrothallus after 2 months in suspension culture. (B) Cross section of mature macrothallus showing cortex (c) and medulla (c) layers. (C) Plurilocular sporangia (arrows) from a mature macrothallus. (D) Protoplast release (arrows) after 2 h of enzymatic digestion. Note that the medulla (m) remains intact. (E) Freshly isolated protoplasts from somatic cells and zooids (arrowheads) from digested plurilocular sporangia. Inset: closer view of a zooid and its flagellum (arrow). (F) True protoplasts showing red chlorophyll autofluorescence. The blue fluorescence indicates cell wall remnants. (G) Viable protoplasts stained with FDA showing green fluorescence. The scales in (A) is 1 cm; the scales in (B), (C) and (E) are 50 µm; the scale in (D) is 100 µm; the scales in (F) and (G) are 10 µm; the scale in inset in (E) is 5 µm.
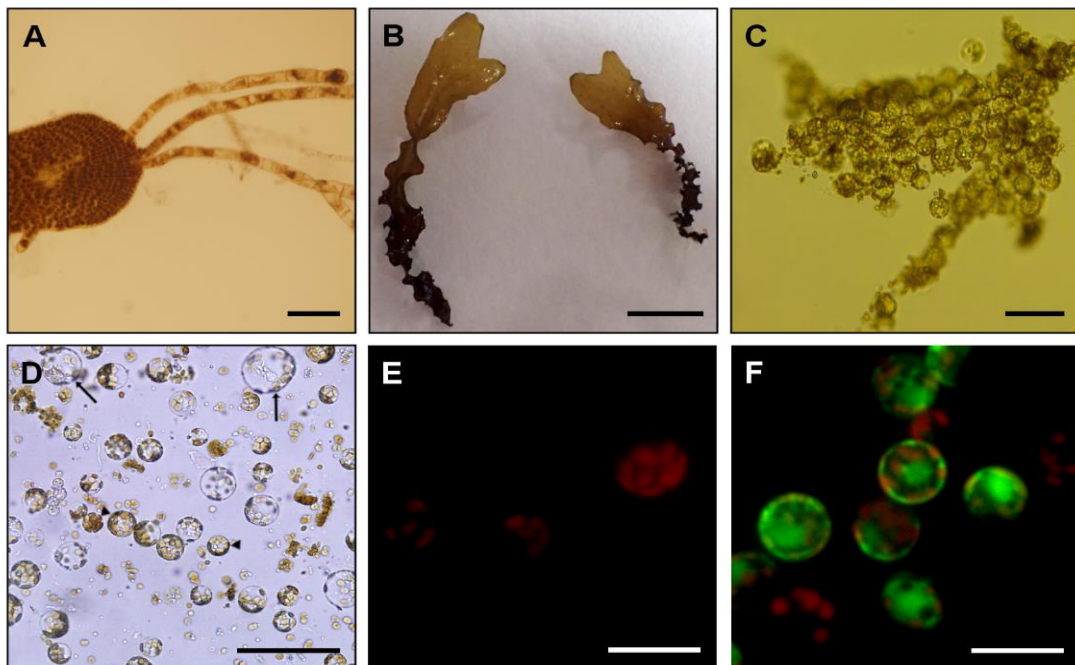
Fig. 35. Protoplast isolation from *Dictyopteris pacifica.* (A) A blade producing filaments in cultures without aeration. (B) Foliose thalli after 1 month in suspension culture. (C) Blade after 2 h of enzymatic digestion. Note the spherical protoplasts. (D) Freshly isolated protoplasts from the cortex (arrowheads) and medulla (arrows). (E) True protoplasts showing red chlorophyll autofluorescence. (F) Viable protoplasts stained with FDA showing green fluorescence. The scales in (A) is 200 µm; the scale in (B) is 1 cm; the scales in (C) and (D) are 100 µm; the scales in (E) and (F) are 10 µm.
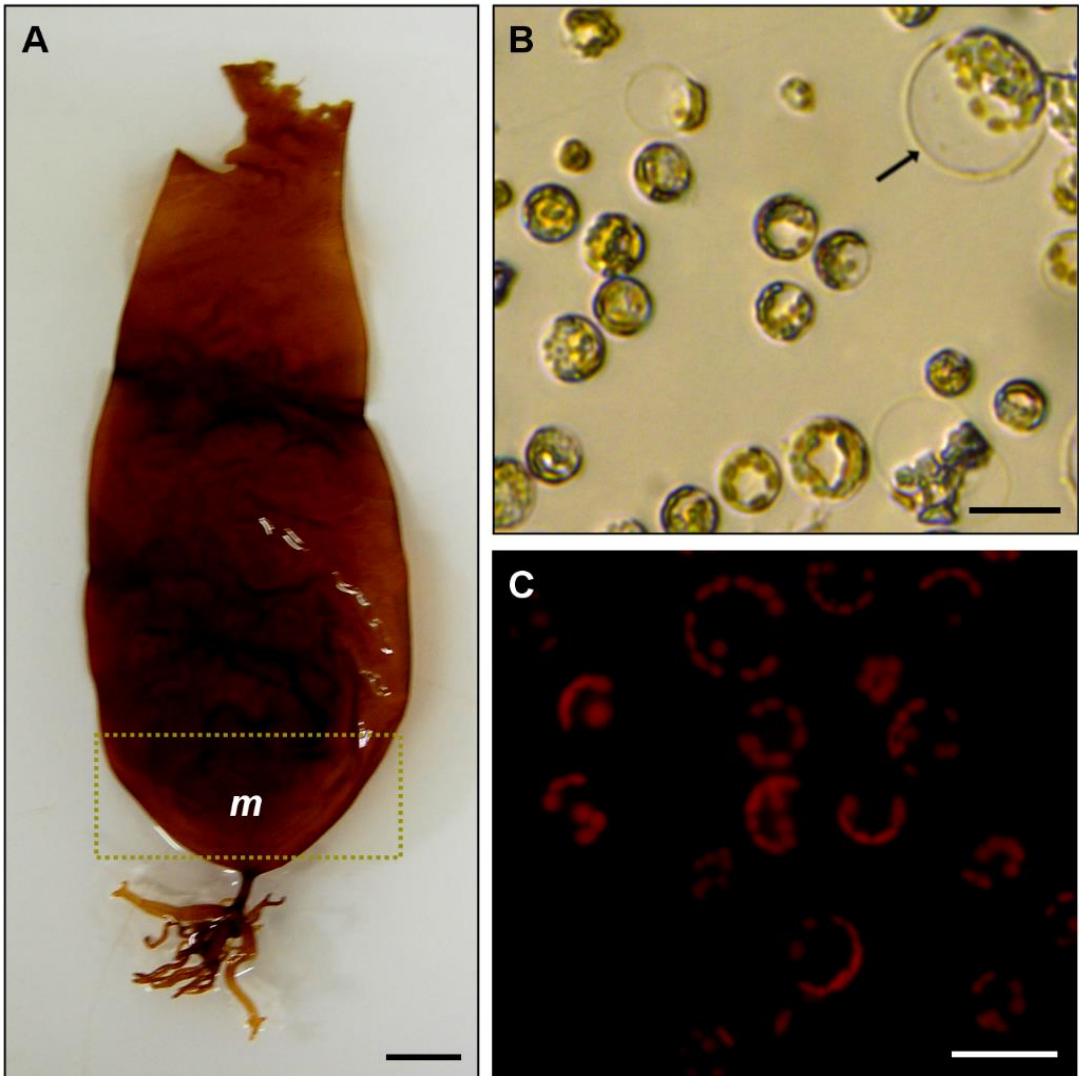
Fig. 36. Protoplast isolation from *Ecklonia cava*. (A) A young field-collected sporophyte. The dotted-line rectangle delimits the basal meristem (m), which is the area used for protoplast isolation experiments. (B) Freshly isolated protoplasts from the cortex. A large protoplast (arrow) from the medulla can be also observed. (C) True protoplasts showing red chlorophyll autofluorescence. The scales in (A) is 1 cm; the scales in (B) and (C) are 10 µm.

4. Discussion

Protoplasts from *Scytosiphon lomentaria* could be isolated in high yields (5.8-7.7×10^7 protoplasts g^{-1} FW) using a simple combination of cellulase RS and alginate lyase, without pre-treatment. Yamaguchi *et al*. (1989) isolated 10^4-10^5 protoplasts g^{-1} FW from *S. lomentaria* using a complex mixture of commercial enzymes and hepatopancreas extract from the marine herbivore *Trochus maculatus*. Our protocol produced 200-300 times more protoplasts than this previous report. Also, the enzyme mixture used could only isolated protoplast from the cortical layer, suggesting a different cell wall composition for cortical and medullary cells. For instance, in *Saccharina japonica*, epidermal and cortical cell walls differed by the crystallinity of cellulose and its content (Inoue et al. 2001). As we used mature thalli, zooids were also isolated from plurilocular sporangia; however, they were easily distinguished based on its size, as they were smaller compared to protoplasts. This difference was also pointed out by Mejjad et al. (1992) when isolating protoplasts and zooids from the filamentous brown alga *Pylaiella littoralis*.

We could isolated, for first time, protoplasts from *Dictyopteris pacifica* in high numbers (1-5×10^6 protoplasts g^{-1} FW) using commercial cellulase RS and alginate lyase. Our values were inferior to protoplast yields from *D. prolifera* (3.3×10^7 protoplasts g^{-1} FW; Fujimura et al. 1995) and *D. undulata* (8.2×10^6 protoplasts g^{-1} FW; Kajiwara et al. 1988). However, in these species, protoplast were isolated using a complex mixture of commercial and crude extracts from marine herbivores. Chelation pre-treatment was necessary for improving protoplasts yields in *D. pacifica*. This positive effect has been also reported in Ectocarpales (Mejjad et al. 1992; Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989).

Our attempts for isolating protoplasts using commercial enzymes (cellulase RS and alginate lyase) from *Ecklonia cava* were successful. This represents the first report of protoplast production from this species. Our protoplast yields (up to 1.05×10^7 protoplasts g^{-1} FW) were slightly inferior to the values reported for *E. radiata* (5×10^7 protoplasts g^{-1} FW; Kevekordes et al. 1993) and similar to the related species *Eisenia byciclis* (2.3×10^7 protoplasts g^{-1} FW; Wakabayashi et al. 1999). Osmolarity increase have shown a positive effect on protoplasts production (Xiaoke et al. 2003; Huang et al. 2013). However, our experiments showed that this is not the case for protoplasts from *E. cava*. Protoplast numbers were significantly reduced when

the osmolarity of the enzymatic solution was increased. A similar trend was found by Gupta et al. (2011) in protoplasts from the red seaweeds *Gracilaria dura* and *G. verrucosa*.

In conclusion, high amount of true and viable protoplasts could be isolated from the economic brown algae *Scytosiphon lomentaria*, *Dictyopteris pacifica* and *Ecklonia cava* using a simple mixture of commercial enzymes (cellulase RS and alginate lyase). In all cases, a short incubation time (4-6 h) and normal osmolarity were enough for obtaining high yields. In *D. pacifica*, chelation pre-treatment was critical for improving protoplast production. Our work proposes an improved method for isolating protoplasts from *S. lomentaria*. Also, this is the first report of protoplast production from *D. pacifica* and *E. cava*.

CHAPTER 7. Optimal conditions for protoplast technology in brown algae

Throughout chapters 1 to 6 of part 1, protoplasts have been successfully isolated from a total of seven species (*Dictyopteris pacifica*, *Ecklonia cava Hecatonema terminale*, *Petalonia fascia*, *Scytosiphon lomentaria*, *Sphacelaria fusca* and *Undaria pinnatifida*) encompassing different levels of anatomical complexity, from filamentous to parenchymatous forms. *H. terminale*, *D. pacifica*, *E. cava* and *S. fusca*, represented new reports for protoplast production. In addition, conditions for whole plant regeneration have been described for four of these seven species (*H. terminale*, *P. fascia*, *Sp. fusca* and *U. pinnatifida*). Successful regeneration was achieved, for first time, in *H. terminale*, *P. fascia* and *Sp. fusca*. In order to give an overview of the most important factors affecting protoplast technology in brown algae, this chapter summarizes the optimal conditions for protoplast isolation, culture and regeneration in the above-mentioned brown algal species. Additionally, we compare our findings with studies dealing with protoplasts from other organisms, especially from plants and algae.

Optimal conditions for protoplast isolation

Fig. 37. shows the highest protoplast yields and optimal conditions for protoplast production in seven brown algae species. The lowest yield was reported for the filamentous brown algae *Sphacelaria fusca*, while the highest one, for the immature blades of *Petalonia fascia*. Protoplast numbers were usually higher (10^7-10^8 protoplasts g^{-1} FW) in recently evolved groups (e.g. Laminariales and Ectocarpales), than in more basal ones (e.g. Sphacelariales and Dictyotales; 10^4-10^6 protoplasts g^{-1} FW). This might suggest that protoplast yields are affected by the phylogenetic origin of the explant. In fact, this factor, together with cell morphotype, exerted significant influence on the success of cryopreservation protocols in green microalgae (Fernandes et al. 2019). However, previous studies in algal protoplasts have not tested the effect of phylogeny on protoplast production. Also, the comparison of protoplast yields from studies with different protocols might lead to erroneous conclusions, as different isolation conditions could mask the effect of phylogeny. Thus, further studies including a wider range of orders and with similar protocols must be conducted to clarify the effect of phylogeny on protoplast production in brown algae

Besides phylogeny, the morphology also seemed to affect protoplast yields. With exception of *Dictyopteris pacifica*, filamentous forms showed lower protoplasts values (10^4-10^6 protoplasts g^{-1} FW) than those ones from species with more complex anatomy (10^7-10^8 protoplasts g^{-1} FW). Also, incomplete cell wall digestion was observed in all filamentous forms. Protoplasts in these species were isolated through holes in the cell wall. In contrast, most of the explants from more complex species were completely digested by the enzymatic mixture used. It has been suggested that filamentous brown algae have simple cell wall compositions (Kloareg & Quatrano 1988), and that protoplasts from them can be isolated using simple enzyme combinations (Chen & Shyu 1994a). Despite their simplicity, incomplete cell wall digestion has also been reported in other filamentous forms, such as in *Sphacelaria* sp. (Ducreux & Kloareg 1988), *Pylaiella littoralis* (Mejjad et al. 1992), and female gametophyte of *Macrocystis pyrifera* (Varvarigos et al. 2004). Altogether, it seems that cell walls from filamentous brown algae are usually difficult to digest; however, this is not an impediment to obtain true and viable protoplasts.

One of the most important factors during protoplast production are the type of cell-wall lytic enzymes used. In our experiments, the commercial available enzymes cellulase Onozuka RS (Yakult Co. Ltd) and alginate lyase (Sigma) proved to be ideal for obtaining high protoplast yields in all the species tested. This simple mixture of commercial enzymes represents an improvement over most of the previously published protocols in brown algal species. These are mainly composed by complex combinations of commercial and non-commercial enzymes, or crude extracts from marine organisms. Inoue et al. (2011) suggested that at least three components were essential for protoplast isolation from *Saccharina japonica*: cellulase, alginate lyase, and protease. Our results showed that only cellulase and alginate lyase were sufficient for successful protoplast isolation in brown algae. Despite the presence of mixed-linked glucan (MLG) in brown algal cell walls (Salmeán et al. 2017), the use of driselase, a natural enzyme mixture that degrades MLG (Thibault & Rouau 1990), did not improve protoplast yields, with exception of male gametophytes of *Undaria pinnatifida*.

A simple combination of commercial enzymes, like the one we propose in this work, is essential for establishing reproducible protocols for protoplast isolation. For example, in green marine macroalgae, a simple protocol using only cellulase RS has been recently established for consistent production of large amounts of protoplasts (Gupta & Reddy 2018). Similarly, simple

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combinations of commercial cellulase, macerozyme and/or pectinase are routinely used for protoplast isolation in higher plants, such as Brassicaceae species, cell suspension cultures of rice (*Oryza sativa*), and the model organism *Arabidopsis thaliana* (Yoo et al. 2007; Neumann et al. 2009; Davey et al. 2010)

Experiments conducted in *Hecatonema terminale* (chapter 1) and gametophytes of *U. pinnatifida* (chapter 2) allowed us to determine that growth was a crucial factor for protoplast isolation, as cultures in exponential phase showed the highest protoplast yields. In addition, higher amount of protoplasts could be isolated from the basal meristem of *U. pinnatifida*, supporting the idea that areas with actively growing cells are recommended for protoplast production. Similar findings have been reported in yeasts (Shahin 1972), land plants (Strauss & Potrykus 1980; Nakagawa et al. 1985) and in green and red seaweeds (Björk et al. 1990, 1992; Gómez Pinchetti et al. 1993). In actively growing cells or meristem areas, an increase of M blocks in the alginate of brown algal cell walls, and the subsequent augment in their elasticity, is expected (Burns et al. 1982a,b, 1984; Kloareg & Quatrano 1988; Lee et al. 2012). This might explain our results, as the M blocks are the main target of the alginate lyase used in our protocol. Thus, cultures in exponential phase, young samples and explant from meristem areas are recommended for protoplast isolation in brown algae.

Our experiments showed that chelation pre-treatment was not always necessary when isolating protoplasts from brown algae. All filamentous forms showed higher protoplast numbers when the explants were pre-treated. However, in the case of more complex forms, only *Dictyopteris pacifica* and *Petalonia fascia* were benefited with the pre-treatment. Although the addition of cation chelators has improved protoplasts yields in Ectocarpales (Mejjad et al. 1992; Coelho et al. 2012) and Laminariales (Butler et al. 1989; Kloareg et al. 1989), this positive effect can be influenced by other factors, such as the concentration of the chelator, pH of the solution, incubation time, and alginate content in the sample (Butler et al. 1989; Chen & Shyu 1994a). It is clear from our work that the necessity of a pre-treatment must be tested independently for each brown algal species.

The effect of osmolarity showed variable effects on the species tested. In *Sphacelaria fusca*, *Undaria pinnatifida* gametophytes and *Petalonia fascia*, increased osmolarity ($1.6\times = 2512$ mOsm L^{-1} H_2O) improved protoplast production. In *Scytosiphon lomentaria* and *Dictyopteris*

pacifica, normal osmolarity ($1\times = 1570$ mOsm L^{-1} H$_2$O) was enough for obtaining high protoplast yields. In *Hecatonema terminale*, *U. pinnatifida* sporophyte and *Ecklonia cava*, increased osmolarity reduced protoplast numbers. High osmolarities have shown opposite effects in plant and algal protoplasts. They can stimulate alginate lyase activity and/or protect protoplast membrane in brown algae (Xiaoke et al. 2003; Huang et al. 2013), but can also reduce protoplasts numbers in the red seaweeds *Gracilaria dura* and *G. verrucosa* (Gupta et al. 2011), and impair metabolism in plant protoplasts (Shepard & Totten 1977). It is worth to note that osmolarities higher than 2512 mOsm L^{-1} H$_2$O reduced significantly the protoplast yields in *U. pinnatifida* gametophytes. This indicates that further osmolarity increase is not recommended for improving protoplast isolation. As in the case of chelation pre-treatment, the effect of osmolarity is species-specific and it must be evaluated when optimizing protoplast isolation conditions in each brown algal species.

   The influence of pH were analyzed in detail for *Hecatonema terminale* and *Undaria pinnatifida* gametophytes. The results suggested that pH 6, incubation time of 4-7 h and a temperature of 20 °C were ideal for protoplast isolation. Enzymatic activity greatly depends on the pH of the enzymatic solution (Bhojwani & Razdan 1996). Optimal pH was 5-6, for cellulase RS, and 6.3, for alginate lyase. The multivariate analysis performed in *U. pinnatifida* gametophytes showed that optimal enzyme pH values were 5.8–6.1. Thus, pH 6 was set up for protoplast production in the other brown algal species.

   Our protocols reported incubation times of 6-7 h, which could be reduced to 4 h in species like *Dictyopteris pacifica*, *Petalonia fascia*, *Scytosiphon lomentaria* and *Undaria pinnatifida* sporophyte. In brown algae, incubation times range from 2-3 h to 24 h depending on the cell wall complexity and enzyme mixtures used (Fisher & Gibor 1987; Xiaoke et al. 2003; Inoue et al. 2011). Taking this into account, our protocols presented short enzymatic digestion period.

   Although a temperature of 25 °C increased protoplast yields in *Hecatonema terminale*, multivariate analysis in *Undaria pinnatifida* gametophytes showed that this factor was not significant. Huddy et al. (2013) mentioned that the optimal temperature for protoplast isolation is more closely related to that what species of macroalgae naturally grow. As most of the species used in our experiments came from laboratory cultures at 20 °C, this temperature was set up as the ideal one for protoplast isolation experiments.
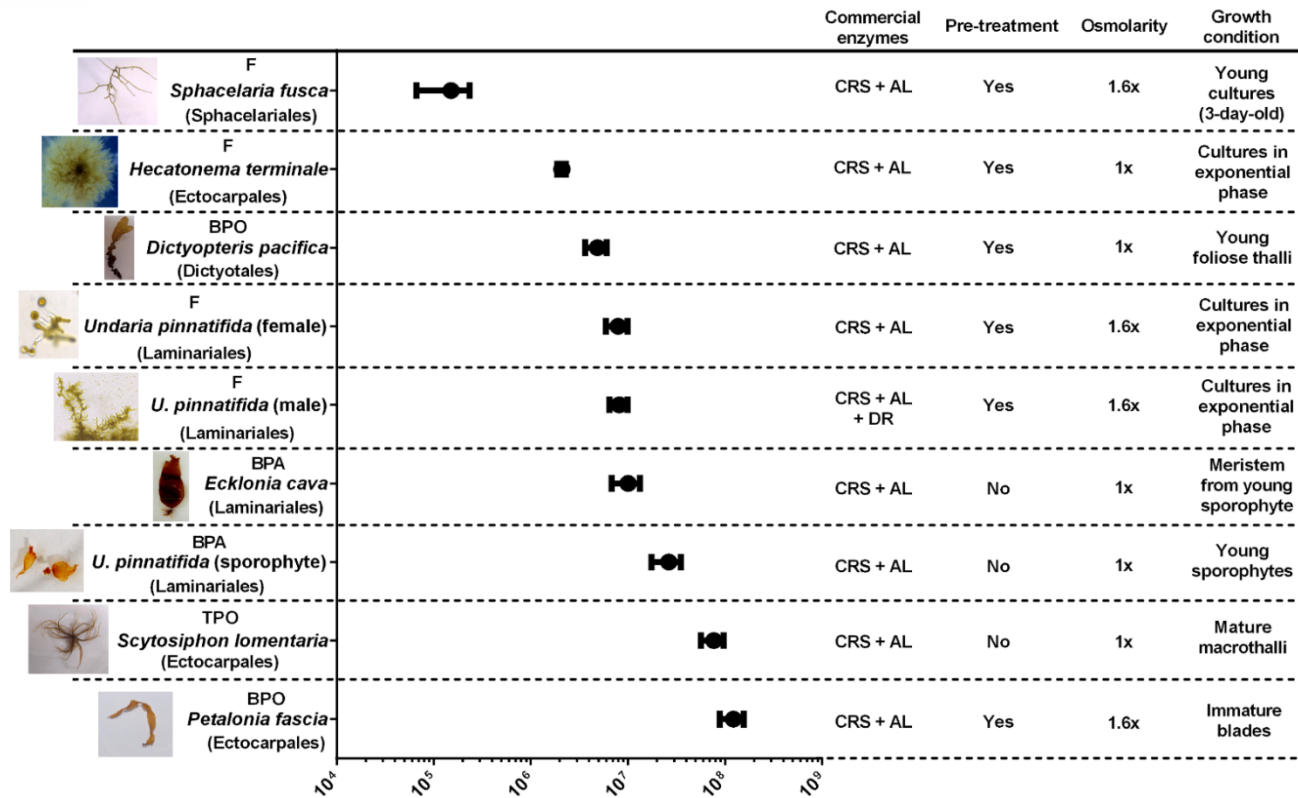
Fig. 37. Summary of protoplast yields and optimal protoplast isolation conditions for the species tested in this work. Black circles represent averages of the maximum protoplast yield reported for each species ($n \geq 3$). In all cases an incubation temperature of 20 °C, pH 6 and digestion time of 4-7 h were used. Error bars represent 95% confidence intervals. F, filamentous form; BPO, blade with polystichous growth; BPA, blade with parenchymatous growth; TPO, terete thallus with polystichous growth; CRS, cellulase RS; AL, alginate lyase; DR, driselase. $1\times$, 1570 mOsm L^{-1} H$_2$O; $1.6\times$, 2512 mOsm L^{-1} H$_2$O.

Optimal conditions for protoplast culture and regeneration

The optimal conditions for protoplast culture and regeneration from *Hecatonema terminale*, male and female gametophytes of *Undaria pinnatifida*, *Sphacelaria fusca* and *Petalonia fascia* are presented in Fig. 38-43.

An optimum plating density of 2.4×10^3 protoplasts mL^{-1} was selected for protoplast cultures based on experiments in *Hecatonema terminale*. A wide range of plating densities have been reported in brown algae (1×10^2 to 5×10^5 protoplasts mL^{-1}); however, its effect had never been explored (Ducreux & Kloareg 1988; Mejjad et al. 1992; Kuhlenkamp & Müller 1994; Benet et al. 1997). Low plating densities avoid fast depletion of nutrients (Davey et al. 2005a) or diminish the detrimental effect of toxins secreted by cells undergoing necrosis (Yeong et al. 2008). We suggest to use low plating densities when culturing protoplast from brown algae.

Six regeneration media have been used in protoplast culture from brown algae (Ducreux & Kloareg 1988; Mejjad et al. 1992; Cheng and Shyu 1994a; Benet et al. 1997; Matsumura et al. 2001; Coelho et al. 2012). Among them, the one reported by Mejjad et al. (1992) and composed of PES, NaCl and $CaCl_2$ was selected for protoplast culture based on experiments in *Hecatonema terminale* and *Undaria pinnatifida*. Besides its simplicity, this regeneration medium contains calcium, which is known as a crucial regulator in plant growth and development (Hepler 2005). Also, it lacked sugars, which can help to control bacterial growth. We recommend PES, NaCl and $CaCl_2$ as regeneration medium for protoplast cultures from brown algae.

An antibiotic mixture of Penicillin G (50 mg L^{-1}), Streptomycin (25 mg L^{-1}), and Chloramphenicol (5 mg L^{-1}) was chosen for protoplast cultures based on experiments in *Undaria pinnatifida* sporophyte. In this species, antibiotics were crucial for successful protoplast regeneration, supporting the idea that protoplasts from Laminariales are very sensitive to microbial contamination (Benet et al. 1997). In *Sphacelaria fusca*, bacterial overgrowth was detrimental for protoplast survival and division, hence the necessity of adding antibiotics into the culture medium. On the other hand, *Hecatonema terminale* and *Petalonia fascia* protoplasts tolerated better bacterial contamination as they underwent whole plant regeneration without adding antibiotics. The same trend was found in protoplasts from *U. pinnatifida* gametophytes; however, we decided to include antibiotics for further experiments as they could greatly reduce

bacterial overgrowth allowing better characterization of their regeneration patterns. Antibiotics have been also used in protoplast cultures of female gametophytes from *Macrocystis pyrifera* (Varvarigos et al. 2004). We suggest adding antibiotics for improving protoplast survival and/or allowing better characterization of protoplast regeneration process.

Temperature was crucial only for protoplast regeneration of *Undaria pinnatifida* sporophyte, affecting the regeneration pattern as previously described (Matsumura et al. 2001). Only cultures at 20 °C could ultimately develop normal sporophytes from PDAFs. Protoplasts from *Hecatonema terminale*, *U. pinnatifida* gametophytes and *Sphacelaria fusca* cultured at 20 °C could regenerate normally. In *Petalonia fascia*, whole plant regeneration was observed at 10 °C, 16 °C and 20 °C without any statistical difference. Temperatures for protoplast cultures in microalgae, red seaweeds and fungi usually match with those ones used for culturing the donor material (Waaland et al. 1990; Yan & Wang 1993; Zhao et al. 2004; Huddy et al. 2015; Levin et al. 2017; Jin et al. 2020). As all the culture samples were maintained at 20 °C, this temperature was chosen as the optimal for protoplast cultures. We recommend to choose the ideal temperature based on the temperatures in which cultures are kept.

Our results showed that protoplasts from different brown algal species presented variable responses to light intensity. In *Sphacelaria fusca* and *Undaria pinnatifida* sporophyte, protoplasts did not survive to a final exposure to 20-40 µmol photons m^{-2} s^{-1}. In contrast, protoplasts from *Hecatonema terminale*, *U. pinnatifida* gametophytes and *Petalonia fascia* tolerated better these light intensities. Usually, high light intensities applied from the beginning of the culture can inhibit protoplast growth and induce photoinhibition (Benet et al. 1994; Chawla 2019). In plants, protoplast-derived callus showed wide range of optimal light intensities (dark to 40.5 µmol m^{-2} s^{-1}) depending on the species (Ochatt & Power 1992). We suggest to initially expose cultures to low light intensity (10 µmol photons m^{-2} s^{-1}), and then evaluate protoplast tolerance to higher intensities (20-40 µmol photons m^{-2} s^{-1}) in each brown algal species.

Start time for osmolarity reduction range from 1 to 4 days in culture. Usually, the earlier cell wall regeneration took place, the sooner the osmolarity reduction started. For instance, *Sphacelaria fusca* protoplasts completed cell wall regeneration after 1 to 3 days in culture, which led us to start the osmolarity reduction after 1 day in culture. Although initially protoplast cultures are kept in high osmotic media, maintaining protoplasts in this condition can hamper cell division

and further growth (Reddy et al. 2008). As the protoplasts start to resynthesize their cell walls, osmotic pressure can be reduced by adding osmotic-free medium (Chawla 2019). We suggest that the start time of osmolarity reduction must be in accordance with the cell wall regeneration period, which has to be established for each brown algal species.

The time of first cell division increased as the anatomy of the samples became more complex. The same trend was found for the complexity of the regeneration patterns. In filamentous forms, protoplasts underwent first cell division after 1 week in culture. In *Petalonia fascia*, first cell division was observed after 2 weeks in culture. In *Undaria pinnatifida* sporophyte, cells did not divide before 2 months in culture. Excepting *Sphacelaria fusca*, protoplasts from all the species mainly underwent asymmetric first cell division. Whole plant regeneration was completed after 3-4 weeks in culture, with exception of *U. pinnatifida* sporophyte, which regenerated normal sporophytes after 3 months in culture.

The regeneration pattern of filamentous forms was direct and simple, which is similar to what was reported in the literature (Ducreux & Kloareg 1988; Mejjad et al. 1992; Varvarigos et al. 2004). *Petalonia fascia* showed prostrate and discoid thalli during protoplast regeneration, stages that have been reported in the life cycle of this species previously (Hsiao 1969; Kogame 1997; Lee et al. 2003). *Undaria pinnatifida* sporophyte presented indirect regeneration, with PDAFs as intermediate stage. This pattern has been already described by Matsumura et al. (2001). It is worth to note that, during initial stages, protoplasts from *U. pinnatifida* male gametophyte and sporophyte show similar regeneration patterns (asymmetric cell division with formation of rhizoid-like protrusions), even though the resulting PDAFs presented later male and female structures.

Fig. 38. Protoplast culture and regeneration of Hecatonema terminale. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl$_2$) at low protoplast density (2.4 - 9 x 10^3 protoplasts mL^{-1}) in the dark. Cultures were maintained at 20 °C.

Fig. 39. Protoplast culture and regeneration of *Undaria pinnatifida* male gametophyte. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl₂) at low protoplast density (2.4 - 9 x 10³ protoplasts mL⁻¹) in the dark. Cultures were maintained at 20 °C. PSC, Penicillin G (50 mg L⁻¹), streptomycin (25 mg L⁻¹), and chloramphenicol (5 mg L⁻¹); AN, antheridia.
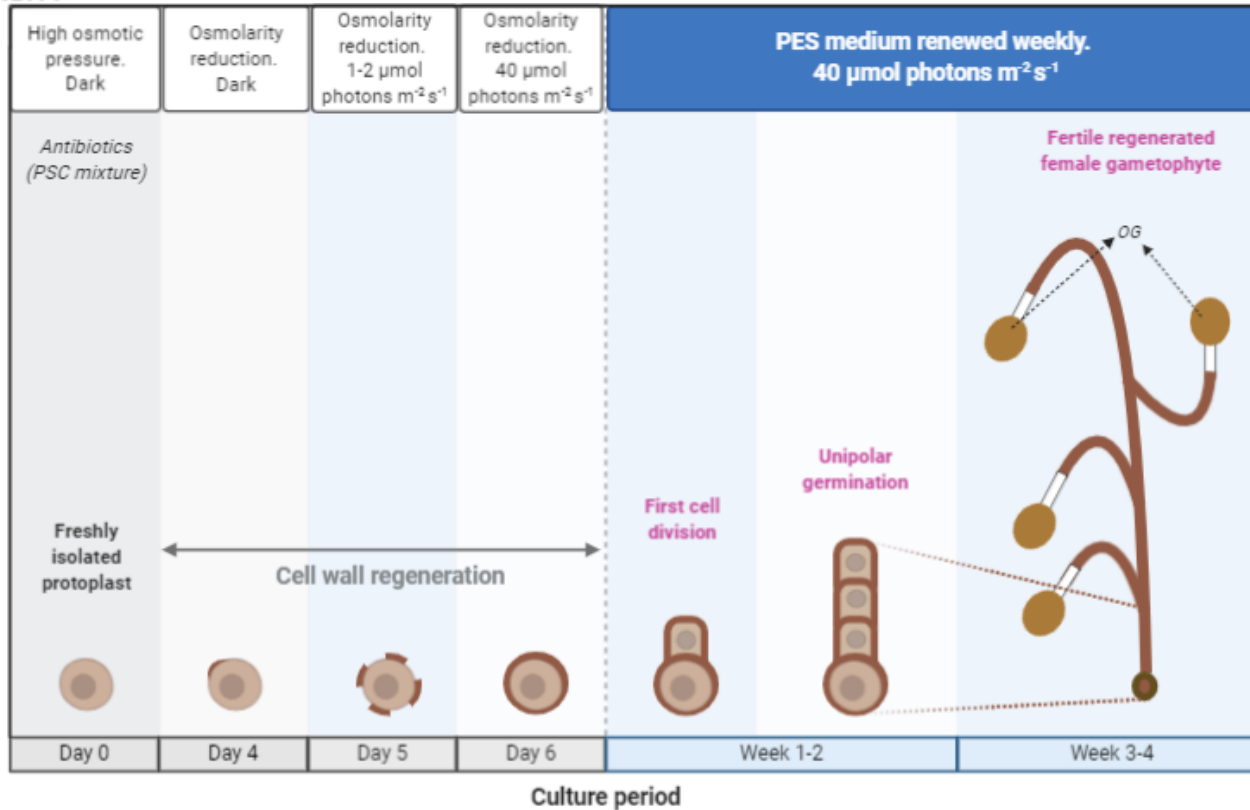
Fig. 40. Protoplast culture and regeneration of *Undaria pinnatifida* female gametophyte. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl$_2$) at low protoplast density (2.4 - 9 x 10^3 protoplasts mL^{-1}) in the dark. Cultures were maintained at 20 °C. PSC, Penicillin G (50 mg L^{-1}), streptomycin (25 mg L^{-1}), and chloramphenicol (5 mg L^{-1}); OG, oogonia.
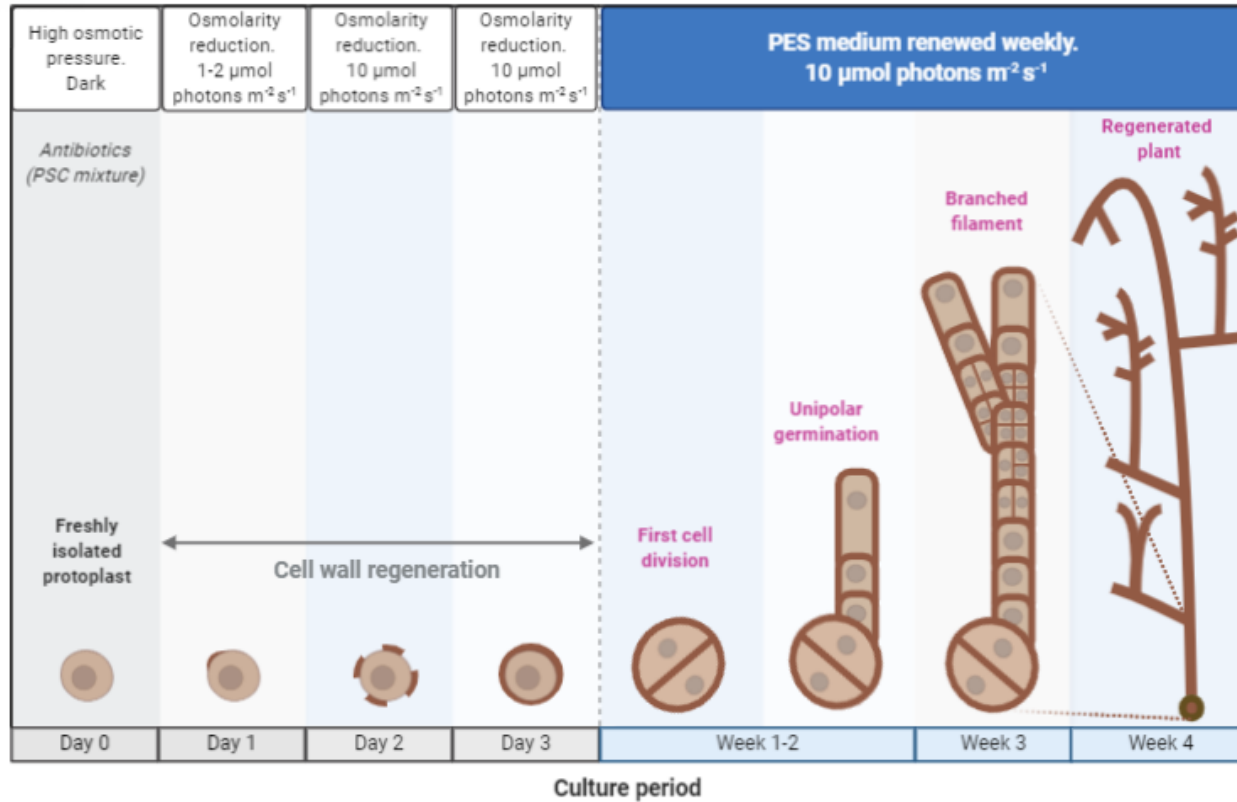
Fig. 41. Protoplast culture and regeneration of *Sphacelaria fusca*. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl$_2$) at low protoplast density (2.4 - 9 x 10^3 protoplasts mL^{-1}) in the dark. Cultures were maintained at 20 °C. PSC, Penicillin G (50 mg L^{-1}), streptomycin (25 mg L^{-1}), and chloramphenicol (5 mg L^{-1}).
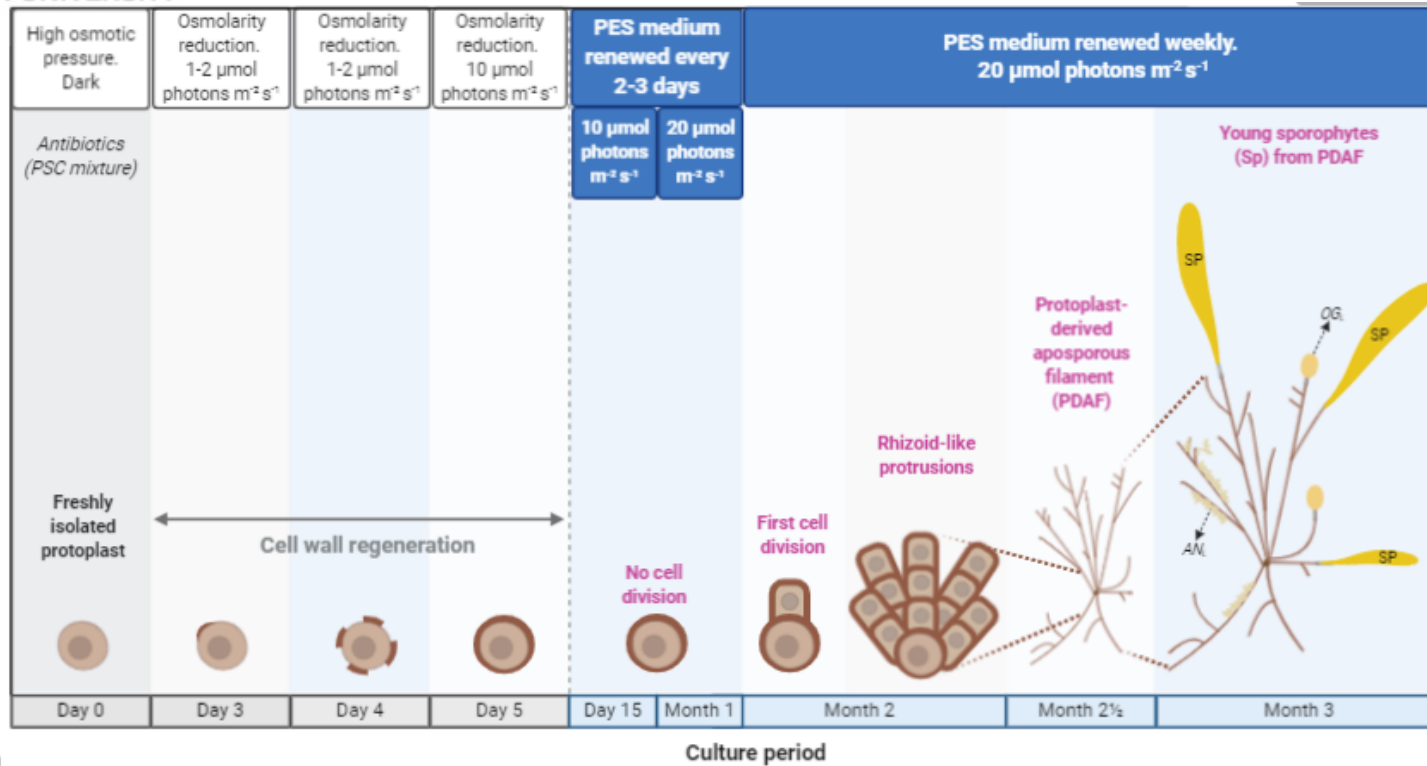
Fig. 42. Protoplast culture and regeneration of *Undaria pinnatifida* sporophyte. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl$_2$) at low protoplast density (2.4 - 9 x 10^3 protoplasts mL^{-1}) in the dark. Cultures were maintained at 20 °C. PSC, Penicillin G (50 mg L^{-1}), streptomycin (25 mg L^{-1}), and chloramphenicol (5 mg L^{-1}); AN$_L$, antheridium-like structure; OG$_L$, oogonium-like structure; SP, young sporophyte.
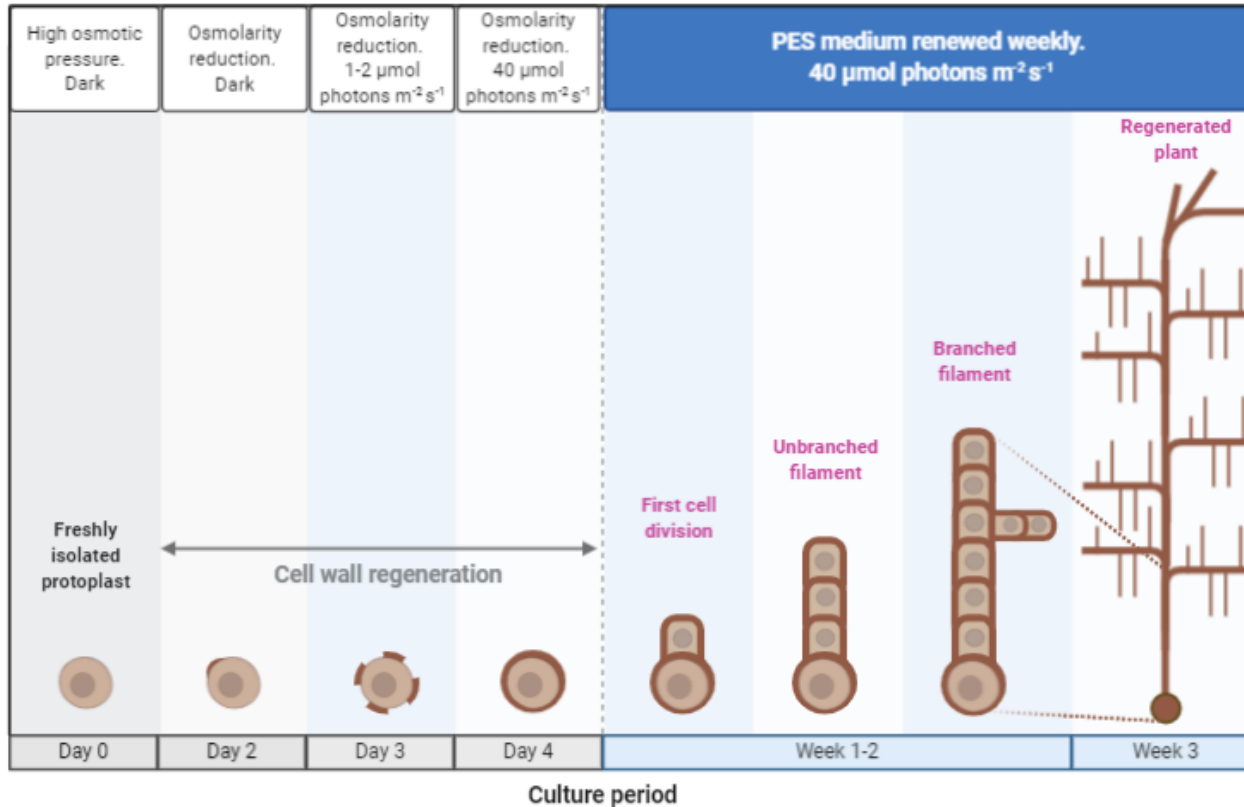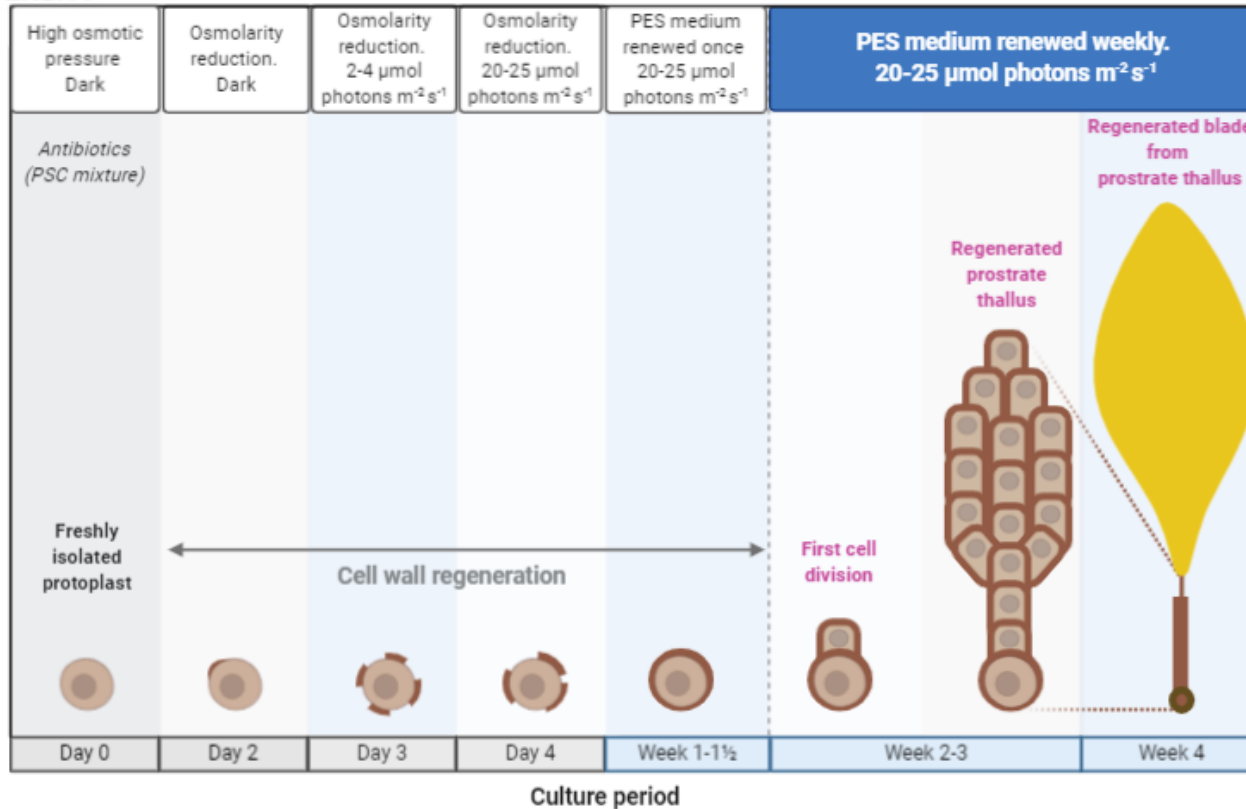
Fig. 43. Protoplast culture and regeneration of *Petalonia fascia*. Optimal conditions for light intensity and osmolarity reduction are shown in the upper part. Protoplasts were initially dispensed in regeneration medium (PES + 285 mM NaCl + 5 mM CaCl$_2$) at low protoplast density (2.4 - 9 x 10^3 protoplasts mL$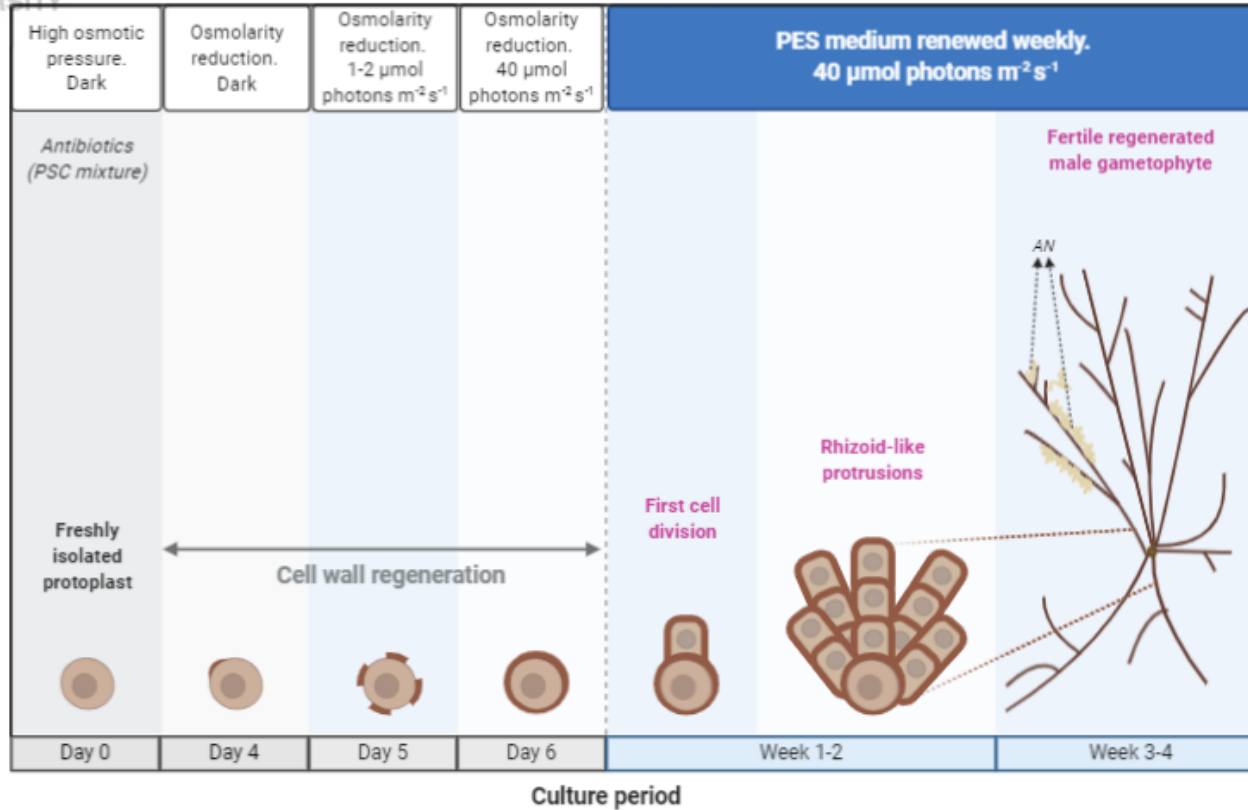^{-1}$) in the dark. Cultures were maintained at 20 °C. PSC, Penicillin G (50 mg L^{-1}), streptomycin (25 mg L^{-1}), and chloramphenicol (5 mg L^{-1}); AN$_L$, antheridium-like structure.

PART 2. EFFECTS OF LIGHT-EMITTING DIODES (LEDS) ON PROTOPLAST REGENERATION FROM THE ECONOMIC BROWN ALGA *UNDARIA PINNATIFIDA*

1. Introduction

Light-emitting-diodes (LEDs) are a rapidly developing lighting source consisting of electronic diodes that produce light when an electric current passes through them. They come in all the three primary light spectra (colors), allowing also the use of dichromatic, trichromatic and white light. Numerous advantages are associated with LEDs over other conventional lights. Among them, the light emission at specific wavelengths allows the precise evaluation of light quality effect on biological systems, especially on the field of photosynthesis research (Gupta & Jatothu 2013; Dayani et al. 2016).

Protoplasts regeneration is the process in which a protoplast ("naked" plant cell) re-synthesizes its cell wall, undergoes cell division, elongation (growth) and differentiation, resulting in a new plant (Warren 1992; Wang & Ruan 2013; Goldman 2014). This ability is the ultimate test of protoplast viability and essential for stablishing protoplast systems (Bhojwani & Razdan 1996; Chawla 2009).

Several factors are known to affect protoplast regeneration ability, such as osmoticum, plating density, culture method and light (Bhojwani & Razdan 1996). Moreover, light spectra play important roles on cell division and regeneration. White light is often best for inducing shoot formation from protoplasts in land plants (Compton et al. 2000). Blue and red lights have shown to increase the number of asymmetric cells on protoplasts of the moss *Physcomitrella* (Jenkins & Cove 1983), whereas the germination of protoplasts from the unicellular marine green alga *Boergesenia forbesii* was shown to be promoted by red light (Ishizawa et al. 1979).

In seaweeds, protoplast regeneration has been accomplished in 38 species (Matsumura et al. 2001; Reddy et al. 2008; Yeong et al. 2008, Lafontaine et al. 2011; Wang et al. 2014; Huddy et al. 2015). Although factors affecting the regeneration process have been studied (Reddy et al. 2008), the effect of light spectra has not yet been determined on protoplasts from multicellular marine algae. In addition, the use of LEDs on seaweed culture has just recently received attention and is still limited to few species (Murase et al. 2014; Kim et al. 2015; Miki et al. 2017; Le et al. 2019).

Undaria pinnatifida (Harvey) Suringar is one of the most important farmed seaweeds worldwide (FAO 2020). It presents a heteromorphic life cycle, with microscopic filamentous

male and female gametophytes, and a macroscopic sporophyte. The gametophytes have the potential for producing useful bioactive compounds (Dwiranti et al. 2012; SEPPIC 2020) and being cultured in cell bioreactors (Rorrer & Cheney 2004; Gao et al. 2005). The sporophyte is popular as food in Korea and Japan, and is also used in cosmetic, medicine and pharmacological applications (Yamanaka & Akiyama 1993; Sanjeewa & Jeon 2018; Wang et al. 2018). Thus, both phases of *U. pinnatifida* life cycle have features that make them suitable for cellular biotechnology techniques, many of them rely on protoplasts (Reddy et al. 2008).

In this study, we aimed to determine the effects of pure primary colors (red, blue and green), dichromatic (red plus blue, RB) and white LEDs on protoplast regeneration from the gametophytes and sporophyte of the economic brown alga *Undaria pinnatifida* during early (cell division) and late (cell elongation/growth) stages. Furthermore, we investigated the effect of different light intensities under the best light color on the regeneration process.

2. Materials and methods

Material

The gametophyte clones of *Undaria pinnatifida* were stablished from matured sporophytes sampled at Geoje and Jindo islands, Korea, in March 1, 2013 and May 5, 2016, respectively. Sporulation, isolation and culture were performed as described in chapter 2 of part 1. Female (from Jindo Island) and male (from Geoje Island) vegetative gametophyte cultures at early and mid-exponential phase, respectively, were used for subsequent experiments.

Young sporophytes (0.5-1 cm in length) of *Undaria pinnatifida* were obtained after 2-3 months in culture at 16 °C by crossing male and female gametophyte strains sampled at Heuksan (May 19, 2016) and Jindo islands (May 5, 2016), Korea, respectively. Crosses were performed following the method described by Shan et al. (2016).

Protoplast isolation

Protoplast isolation was performed as described in "General Materials and Methods" with about 100-200 mg of gametophytes, or explants of 4 mm^2 from cultured sporophytes of (0.5-1 cm in length). For gametophytes, protoplasts were isolated with 1 % cellulase RS (Yakult Co. Ltd,

Japan), 4 U mL^{-1} alginate lyase and 1% driselase from *Basidiomycetes* sp. (Sigma-Aldrich, USA) at 20°C with shaking at 70 rpm for 4 (female) or 6 (male) h in the dark and chelation pre-treatment. For sporophytes, protoplast were obtained using 1 % cellulase RS and 4 U mL^{-1} alginate lyase at 20°C with shaking at 70 rpm for 4 h in the dark without chelation pre-treatment. In gametophytes, osmolarity of the enzymatic solution was raised 1.6× times (2512 mOsm L^{-1} H$_2$O) for improving protoplast yields.

LEDs

The effects of LED wavelength on protoplast regeneration were investigated using a DYLED 44V system (DyneBio Co. Korea). Five LED lights were installed in the growth chambers (Fig. 44A), namely: 1) white (mixed wavelength); 2) red (peak wavelength: 660 nm); 3) blue (peak wavelength: 460 nm); 4) red plus blue (1:2, RB); and 5) green (peak wavelength: 530 nm). Fig. 45 shows the emission spectra of LEDs. Spectral wavelengths were measured using an USB2000+ UV-VIS spectrometer (Ocean Optics Inc.,USA). The photoperiod of LEDs was set as 14:10-h light/dark. LED irradiation intensities were measured using a quantum meter (MQ-500, Apogee Instruments, USA).

Protoplast regeneration under different LEDs

Protoplasts were dispensed into at least 4 wells of 12-well tissue culture plates containing 2 mL of regeneration medium (PES with 285 mM NaCl and 5 mM CaCl$_2$). They were cultured at 20°C in the dark and at initial protoplast density of 9 x 10^3 protoplasts mL^{-1} plus antibiotic mix (50 mg L^{-1} penicillin G, 25 mg L^{-1} streptomycin and 5 mg L^{-1} chloramphenicol). In protoplast cultures from gametophytes, osmotic pressure was reduced slowly after 4 days in the dark using PES medium. Cultures were gradually exposed to a final intensity of 40 µmol photons m^{-2} s^{-1}. In protoplast cultures from sporophyte, osmotic pressure was reduced slowly after 3 days in the dark using PES medium. Protoplats were exposed to 10 µmol photons m^{-2} s^{-1} by the end of osmolarity reduction. Ten days later, light intensity was increased to 20 µmol photons m^{-2} s^{-1}. Well plates were separately irradiated using white (control), red, blue, RB or green LEDs. In the case of gametophytes, the culture medium was renewed weekly throughout the experiments. In the case of sporophytes, PES medium was renewed every 2-3 days during the first month in culture, and then once a week.

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Responses of cultured protoplasts

The responses of cultured protoplasts from gametophytes and sporophytes were assessed at early and late stages of regeneration.

Protoplast cultures from gametophytes: on early stages, the percentage of dividing cells was calculated at 8 (female) and 11 (male) days in culture. In addition, the percentage of asymmetric cells (from dividing ones) was determined for all conditions as it has been reported that LED lighting influences the formation of asymmetric cells in protoplasts of the moss *Physcomitrella* (Jenkins and Cove 1983). On late stages, the percentage of branched filaments (BFs, female) or multiple rhizoid-like protrusions (MRLPs, male) was determined at 15 or 18 days in culture, respectively. Final growth was represented as the gametophyte areas at the end of the experiment (female = 22 days in culture; male = 26 days in culture), which were analyzed using image analysis software (ImageJ, US National Institutes of Health). For percentage calculations, at least 100 cells per well were counted. For area measurements, at least 30 gametophytes per well were analyzed.

Protoplast cultures from sporophyte: on early stages, the percentage of alive cells was calculated at 1 month in culture. In the addition, the percentage of dividing cells, considering only alive ones, was calculated at 1 and 2 months in culture. On late stages, the total number of regenerated protoplasts per well was determined at 3 months in culture. The percentages of callus-like masses (CLM) and PDAFs were calculated based on the number of regenerated protoplasts. The total number of sporophytes per well was assessed at the end of the experiment (4 months in culture). The percentage of normal sporophytes (i.e. sporophytes without outgrowths in the margins consisting of undifferentiated cells) was calculated based on the number of regenerated sporophytes. For percentage calculations on early stages, at least 100 cells per well were counted.

Protoplasts regeneration under different LED intensities

The effect of LED light intensities (from the best treatment) on protoplast regeneration of male and female gametophytes was evaluated. Protoplasts were cultured as described above in at least 4 wells of 12-well plates. They were put at different distances from the LED unit in order to

achieve four different conditions: 20, 40, 60, and 80 µmol photons $m^{-2} s^{-1}$ (Fig. 44B). Intensities were measured with a quantum meter. The responses of cultured protoplasts were assessed as previously mentioned. Intensities were not assessed in protoplasts from sporophytes as previous experiments showed that their viability was reduced significantly at light conditions above 20 µmol photons $m^{-2} s^{-1}$.

Statistical analysis

Percentages were analyzed as proportions with beta regressions, since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari & Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto & Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson & Verkuilen 2006).

The total number of regenerated protoplasts and sporophytes per well was analyzed using either negative binomial or Poisson regression model. Likelihood ratio test was used for deciding which count regression model to use. If zeros were present, Voung test for non-nested data was carried out to check if a zero-inflated regression was needed (Elhai et al. 2008). The pseudo-R^2 was chosen as effect size in case of significant results. The analyses were performed using "pscl" (Jackman 2015) and "MASS" (Venables & Ripley 2002) packages in R.

To evaluate the difference in gametophyte area, one-way ANOVA test was used. Normality and homoscedasticity were checked with Shapiro-Wilk and Levene tests, respectively, prior to conduct ANOVA. Kruskal-Wallis or Welch's ANOVA tests were used when data did not meet normality or homoscedasticity assumptions, respectively. Effect sizes (Sullivan & Feinn 2012) were presented as ω^2 (Field 2009, Lakens 2013) in case of significant results. All these analyses were performed using "car" (Fox & Weisberg 2019) and "userfriendlyscience" (Peters 2018) packages in R

Tukey's post hoc test was used when the results were significant. In case of heteroscedastic data, Games-Howell test was performed instead. Post-hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.
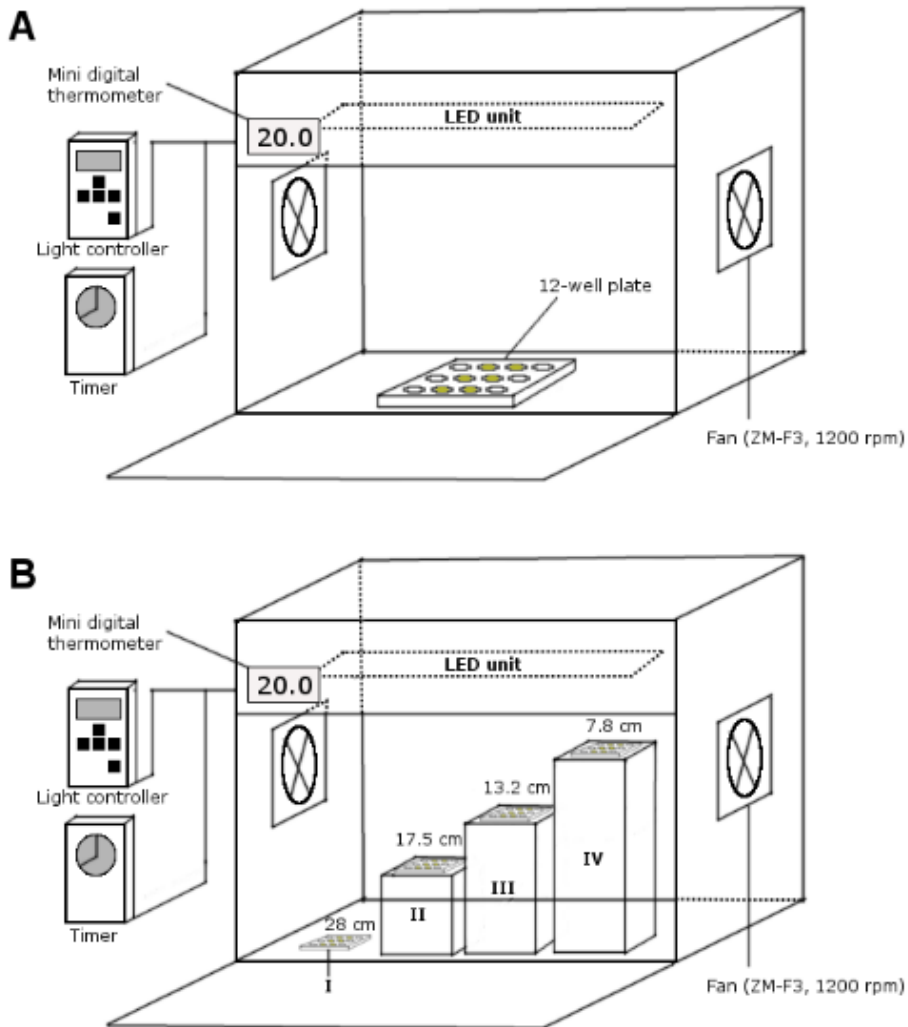
Fig. 44. Experimental conditions for assessing the effect of LED lighting on protoplast regeneration of *Undaria pinnatifida* gametophytes and sporophyte. (A) Growth chamber for culturing protoplasts from *Undaria pinnatifida* at different light spectra. (B) Different light intensities (I = 20 µmol photons m^{-2} s^{-1}, II= 40 µmol photons m^{-2} s^{-1}, III = 60 µmol photons m^{-2} s^{-1}, IV = 80 µmol photons m^{-2} s^{-1}) with their respective distances from the LED unit.
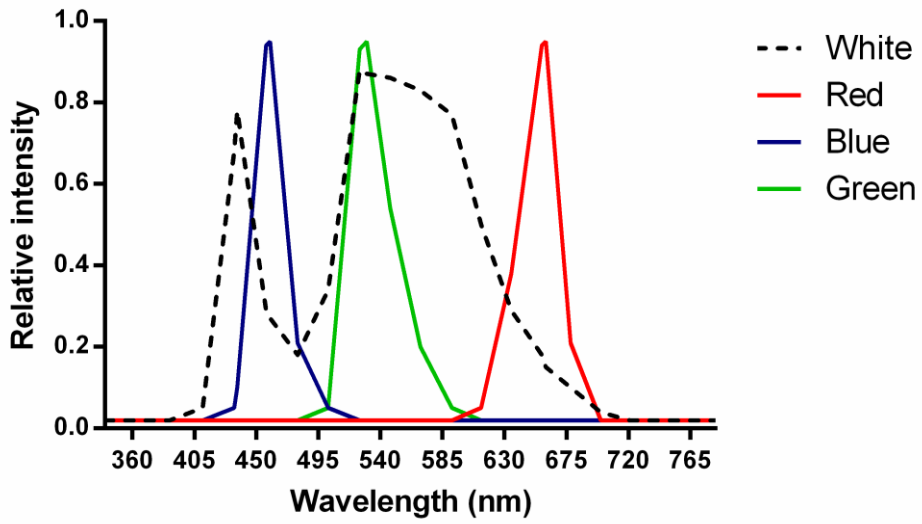
Fig. 45. Relative emission spectra of red, blue, green and white light-emitting diodes (LEDs). Dichromatic LED corresponds to red plus blue (1:2) LED.

3. Results

Comparison of the effects of LED lights on protoplast regeneration from female gametophyte

Cells were able to divide in all LED conditions. On early stages, LED lights affected the percentage of cells in division ($p < 0.001$, PRE= 0.133). Blue and RB increased significantly these values by 1.5 and 1.7 times, respectively, compared to the control (Fig. 46A). Light treatments influenced the amount of asymmetric cells ($p < 0.001$, PRE= 0.088). Significantly more asymmetric cells were found in blue and RB conditions, however they represented a small increase (1.1-1.2 times) compared to the control (Fig. 46B). On late stages, LED treatments did not affect the percentage of BL ($p > 0.01$) (Fig. 46C). However, RB showed a significant increment of final gametophyte area by 3 times compared to white LED ($p < 0.001$, $\omega^2 = 0.78$) (Fig. 46D). Final morphology was affected by LEDs. Under red LED, gametophytes showed longer filaments and more branches than those ones in blue LED, which only presented 1 or none. RB treatment produced gametophytes with longer filaments and more branches than blue but slightly shorter than red condition. Gametophytes under green LED showed a morphology that resembled white LED conditions (Fig. 48A).

Comparison of the effects of LED lights on protoplast regeneration from male gametophyte

All LED conditions allowed cell division. On early stages, LED lights affected the percentage of cells in division ($p < 0.001$, PRE = 0.255). Red, blue and RB increased significantly these values by 2-2.5 times compared to the control (Fig. 47A). Light treatments influenced the amount of asymmetric cells ($p < 0.001$, PRE = 0.041). Significantly more asymmetric cells were found in green condition, however this represented a small increase (1.1 times) compared to the control (Fig. 47B). On late stages, only RB increased the percentage of MRLP ($p < 0.001$, PRE = 0.138) and final gametophyte area ($p < 0.001$, $\omega^2 = 0.87$) by 1.2 and 3.6 times, respectively, compared to white LED (Figs. 47C, D). Final morphology was affected by LEDs. Under red LED, gametophytes showed longer filaments with less profuse branching than those ones in blue LED. An "intermediate" morphology was found on gametophytes under RB treatment, with longer

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filaments than blue but showing more profuse branching than red. Gametophytes under green LED showed a morphology that resembled white and red LED conditions (Fig. 48B).

Comparison of the effects of LED lights on protoplast regeneration from sporophyte

Survival was enhanced in all LED treatments compared to the white LED (control; $p < 0.001$, PRE = 0.046). Treatments did not show significant differences among them (Fig. 49A). All LED conditions allowed cell division. On early stages, LED lights affected the percentage of cells in division after 1 ($p < 0.001$, PRE = 0.067) and 2 months in culture ($p < 0.001$, PRE = 0.052). At first, blue and RB significantly these values by 3.5-4.1 times compared to the control (Fig. 49B). However, during the second month in culture, only blue increased cell division by 4.6 times compared to the control (Fig. 49C). On late stages, blue, RB and green increased the total number of regenerated protoplasts ($p < 0.001$, pseudo-$R^2 = 0.210$), with no differences among them (Fig. 49D). The formation of CLM and PDAFs was greatly affected by light treatments. Red and green conditions inhibited the formation of CLM. The percentages of CLM and PDAFs were similar among white, blue and RB treatments ($p > 0.01$). PDAFs accounted for, at least, 74% of regenerated protoplasts (Fig. 49E). At the end of the experiment, sporophytes were only produced in white, blue and RB LEDs. The number of sporophytes were similar among blue and RB conditions, which in turn were statistically higher than the control (Fig. 50A). RB condition favoured the formation of normal sporophytes compared to blue LED (Fig. 50B).

Comparison of the effects of LED light intensities on protoplast regeneration from gametophytes

RB LED was chosen for testing different light intensities on protoplast regeneration from gametophytes. On early stages, percentage of cells in division was higher under 40 and 60 µmol photons m^{-2} s^{-1} than in 20 or 80 µmol photons m^{-2} s^{-1} for both gametophytes ($p < 0.001$; PRE (female) = 0.134, PRE (male) = 0.236) (Figs. 51A, D). On late stages, the percentage of BL increased at 80 µmol photons m^{-2} s^{-1} than in the other light intensities ($p = 0.002$; PRE = 0.118) (Fig. 51B). In contrast, the percentage of MLRP was higher at 40 and 60 µmol photons m^{-2} s^{-1} than in 20 or 80 µmol photons m^{-2} s^{-1} ($p < 0.001$; PRE = 0.814) (Fig. 51E). Final female gametophyte area showed its peak at 80 µmol photons m^{-2} s^{-1} ($p = 0.002$; $\omega^2 = 0.46$), representing

a 1.8 times increase compared the lowest value at 20 µmol photons m^{-2} s^{-1} (Fig. 51C). Final male gametophyte area showed its highest value at 60 µmol photons m^{-2} s^{-1} ($p < 0.001$; $\omega^2 = 0.87$), representing a 2.9 times increase compared the lowest value at 20 µmol photons m^{-2} s^{-1} (Fig. 51F).

Fig. 46. Effects of light-emitting diodes (LEDs) on protoplast regeneration from female gametophytes of *Undaria pinnatifida*. (A) Effect of LEDs on percentage of dividing cells in early stages. (B) Effect of LEDs on percentage of asymmetric cells in early stages. (C) Effect of LEDs on percentage of branched filaments (BFs) in late stages. (D) Effect of LEDs on final gametophyte area (um^2) at the end of the experiment. Independent data points and averages (horizontal lines) are shown (n ≥ 5) in (A), (C) and (D). Bars in (B) represent averages (n =6). Error bars indicate 95% confidence intervals. Asterisks (*) and double asterisks (**) denote significant differences between treatments and control (white) at $p < 0.01$ and p < 0.001, respectively. Different letters indicate significant differences ($p < 0.01$) between treatments that showed higher values than control. RB, dichromatic LED (red plus blue, 1:2).

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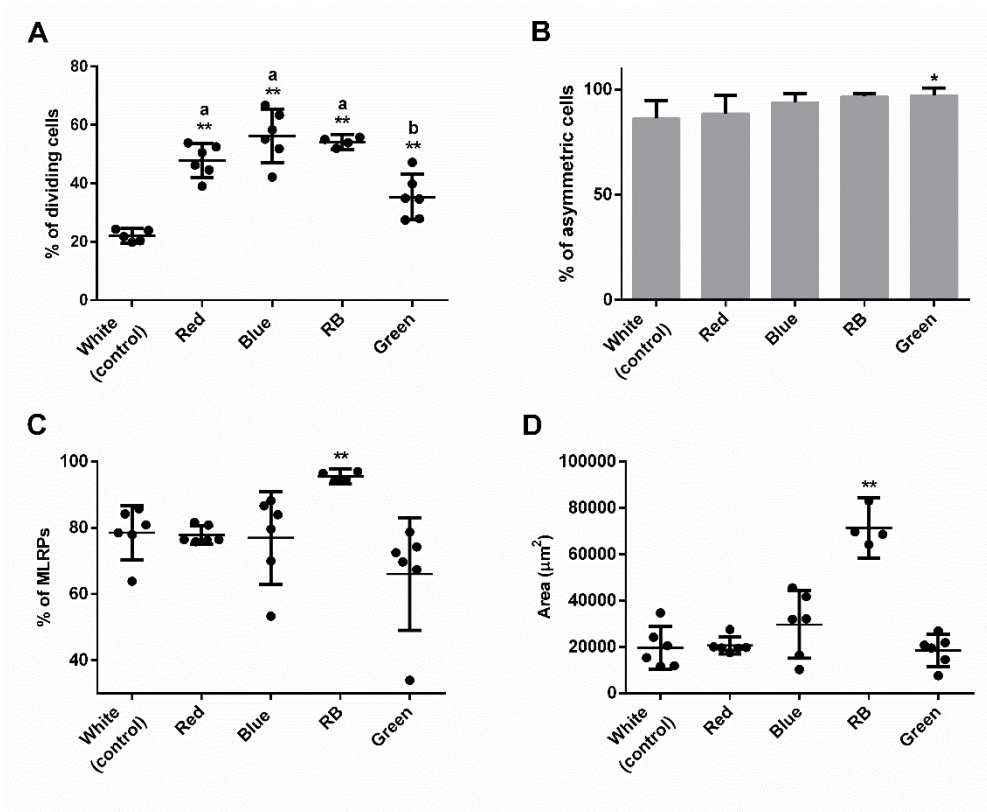
Fig. 47. Effects of light-emitting diodes (LEDs) on protoplast regeneration from male gametophytes of *Undaria pinnatifida*. (A) Effect of LEDs on percentage of dividing cells in early stages. (B) Effect of LEDs on percentage of asymmetric cells in early stages. (C) Effect of LEDs on percentage of multiple rhizoid-like protrusions (MLRPs) in late stages. (D) Effect of LEDs on final gametophyte area (um^2) at the end of the experiment. Independent data points and averages (horizontal lines) are shown (n ≥ 5) in (A), (C) and (D). Bars in (B) represent averages (n = 6). Error bars indicate 95% confidence intervals. Asterisks (*) and double asterisks (**) denote significant differences between treatments and control (white) at $p < 0.01$ and $p < 0.001$, respectively. Different letters indicate significant differences ($p < 0.01$) between treatments that showed higher values than control. RB, dichromatic LED (red plus blue, 1:2).

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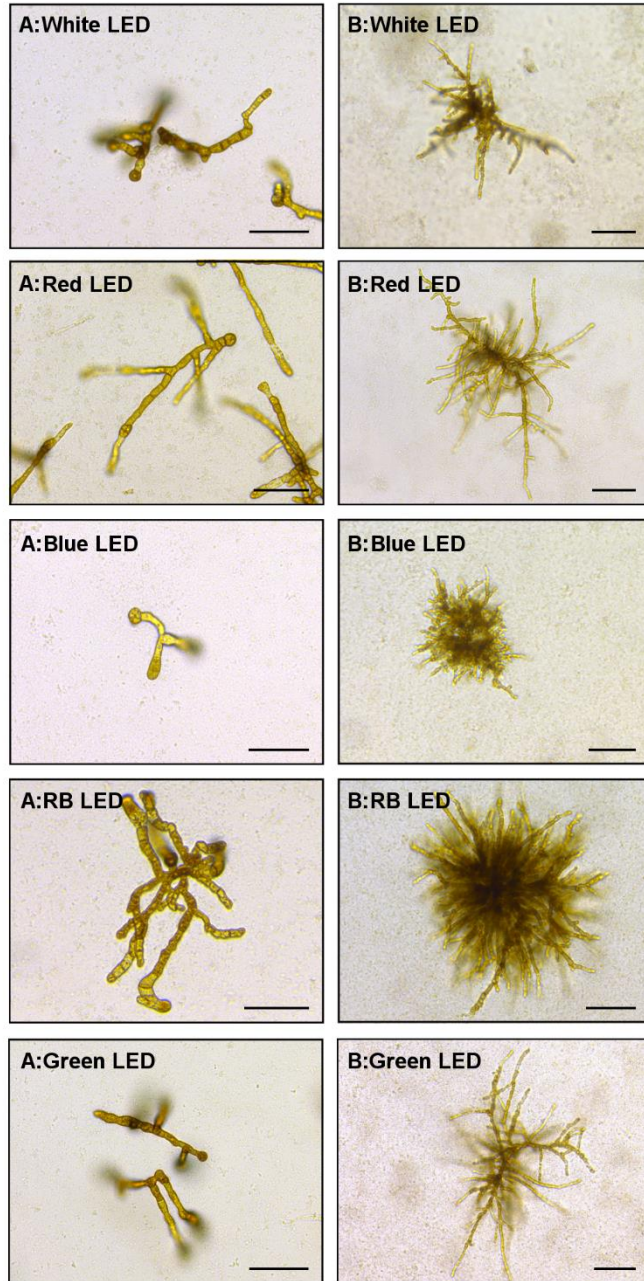
Fig. 48. Comparison of the final morphology from regenerated gametophytes of *Undaria pinnatifida* cultured for 22 (female) and 26 (male) days under different light-emitting diode (LED) conditions (A: female gametophyte, B: male gametophyte). The scales in (A) and (B) are 100 µm.
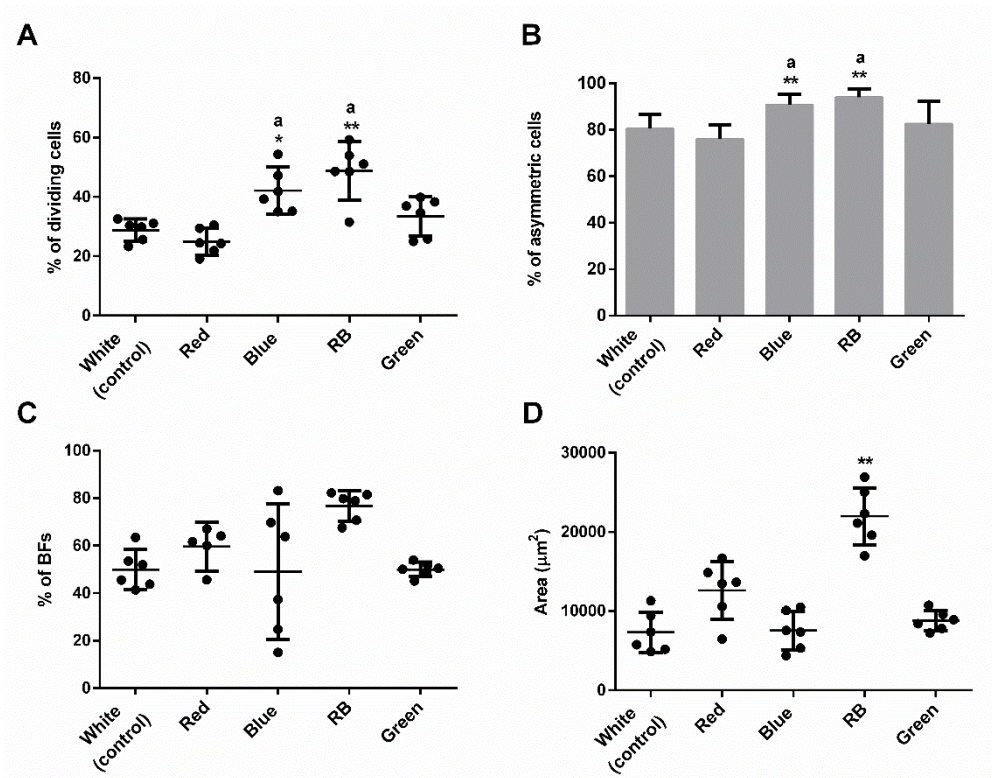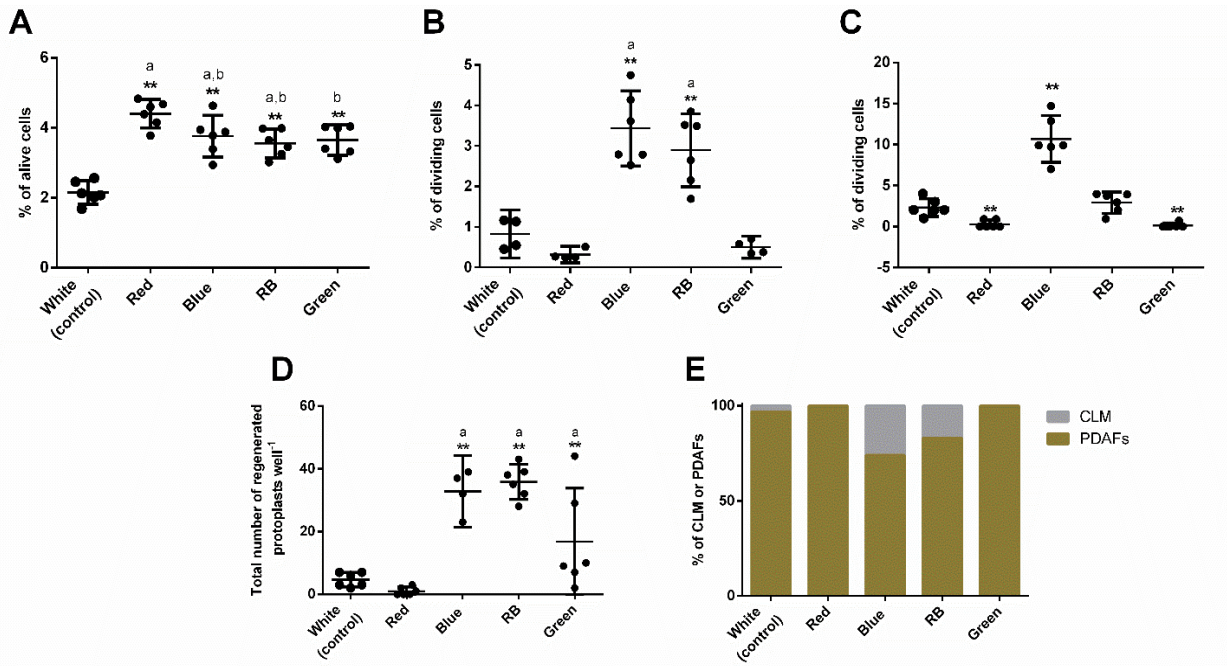
Fig. 49. Effects of light-emitting diodes (LEDs) on protoplast regeneration from sporophyte of *Undaria pinnatifida*. (A) Effect of LEDs on percentage of alive cells in early stages. (B) Effect of LEDs on percentage of diving cells in early stages after 1 month in culture. (C) Effect of LEDs on percentage of diving cells in early stages after 2 months in culture. (D) Effect of LEDs on the number of regenerated sporophytes in late stages. (E) Effect of LEDs on the percentages of callus-like masses (CLM) and protoplast-derived aposporous filaments (PDAFs). Independent data points and averages (horizontal lines) are shown ($n \geq 4$) in (A), (B), (C) and (D). Bars in (E) represent averages ($n = 5$). Error bars indicate 95% confidence intervals. Double asterisks (**) denote significant differences between treatments and control (white) at $p < 0.001$. Different letters indicate significant differences ($p < 0.01$) between treatments that showed higher values than control. RB, dichromatic LED (red plus blue, 1:2).

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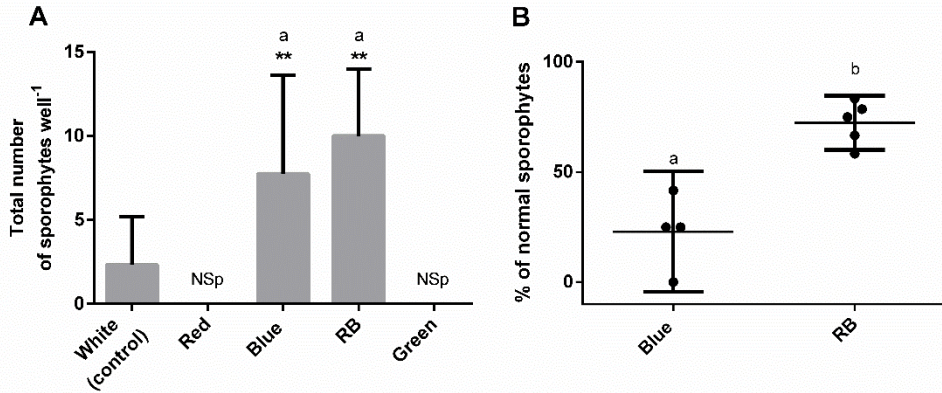
Fig. 50. Effects of light-emitting diodes (LEDs) on sporophyte production from protoplast-derived aposporous filaments of *Undaria pinnatifida*. (A) Effect of LED on total number of sporophytes. (B) Effect of blue and dichromatic light (RB) on the percentage of normal sporophytes. Bars in (A) represent averages (n ≥ 4). Independent data points and averages (horizontal lines) are shown (n = 4) in (B). Error bars indicate 95% confidence intervals. Double asterisks (**) denote significant differences between treatments and control (white) at $p < 0.001$. Different letters indicate significant differences ($p < 0.01$) between treatments that showed higher values than control in (A) or among LED conditions in (B). NSp, no sporophyte production. RB, dichromatic LED (red plus blue, 1:2)

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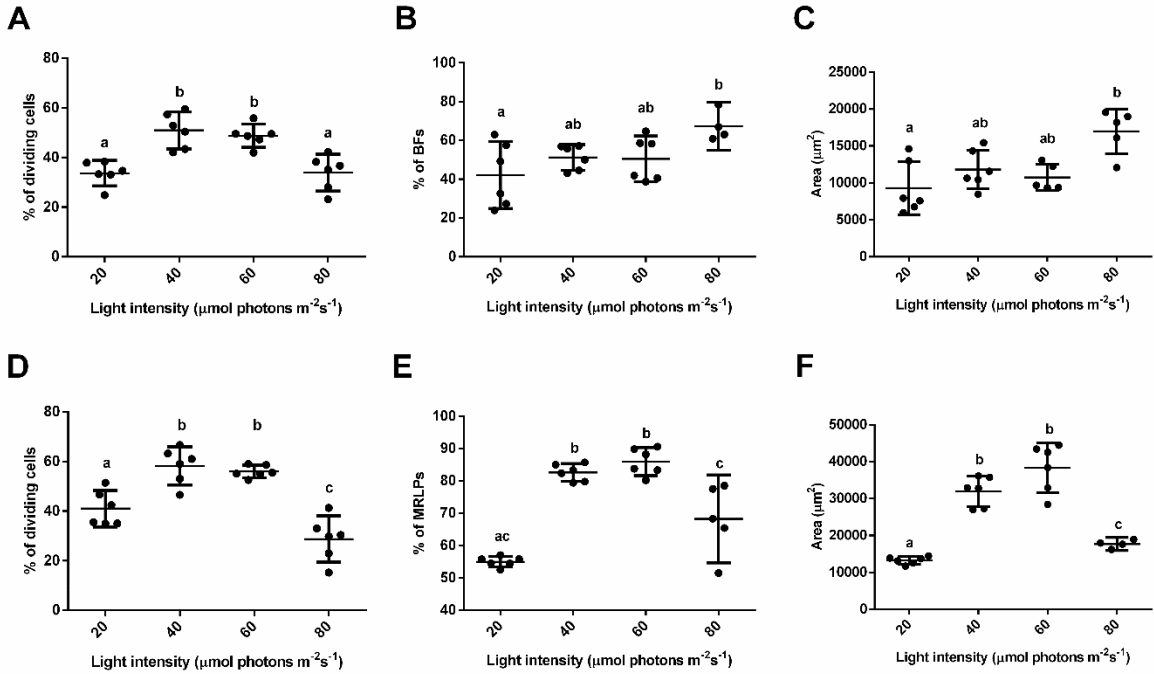
Fig. 51. Effect of different light intensities of dichromatic (red plus blue, 1:2) light-emitting diode (LED) on protoplast regeneration from female (A-C) and male (D-F) *Undaria pinnatifida* gametophytes. (A) and (D) Effect of light intensities on percentage of dividing cells in early stages. (B) and (E) Effect of light intensities on percentage of branched filaments (BFs) and multiple rhizoid-like protrusions (MRLPs), respectively, in late stages. (C) and (F) Effect of light intensities on final gametophyte area (um^2) at the end of the experiment. Independent data points and averages (horizontal lines) are shown (n $\geq$ 4). Error bars indicate 95% confidence intervals. Different letters indicate significant differences at $p < 0.01$.

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4. Discussion

Color light has been tested for enhancing growth of different seaweed species. In brown algae, Xu et al. (2005) and Miki et al. (2017) showed that blue LED induced higher growth in female gametophytes from *Undaria pinnatifida*, and germlines and immature stages of *Sargassum horneri*, respectively. Although our experiments in gametophytes also showed a positive effect of blue LED on early stages, dichromatic condition was more effective in protoplast regeneration than white LED (mixed wavelength) and pure primary color lights. This was also evident in the final morphology of both gametophytes. A similar result was obtained for the red alga *Gracilaria tikvahiae*, which showed higher growth under dichromatic (red+green or green+blue) and trichromatic (red+green+blue) LEDs (Kim et al. 2015). Morphology was also affected in young germlines of *Ulva compressa* under different LED conditions (Kuwano et al. 2014).

In protoplast cultures from sporophytes, blue condition also showed positive effects throughout the experiment, including sporophyte regeneration from PDAFs. Asensi et al. (2001) reported that blue light favored sporophyte regeneration of *Laminaria digitata* from callus-like cell suspension cultures. However, the highest percentage of normal sporophytes in our experiments was observed under RB LED. In this regard, Takahide et al. (2017) showed that *Undaria pinnatifida* sporophytes grown under only in red or blue LED withered considerably. They concluded that both red and blue wavelength bands were necessary to grow healthy sporophytes.

Blue light plays a major role on brown algae growth. It can be efficiently captured by the accessory pigment fucoxanthin and stimulate photosynthetic capacity by upregulating genes of photosystem components, F-type H^+-ATPase, cytochrome b6/f complex and ferredoxin (Foster & Dring 1994, Wang et al. 2013). The perception of blue light in Phaeophyceae may be mediated by cryptochromes and a set of aureochromes (Takahashi et al. 2007, Wang et al. 2013, Deng et al. 2012). Interestingly, promotion of metabolism and growth in kelps might not only depend on increase of blue light illumination but also on reduction of red light proportion (Wang et al. 2013). This interaction between both lights could explain our results with RB LED, where red light was present but in lower proportion than blue.

Red and green conditions suppressed sporophyte formation from PDAFs. Red light is well known for inhibiting the formation of reproductive structures in gametophytes from Laminariales

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(Forbord et al. 2018). Also, Asensi et al. (2001) showed that red condition diminished the formation of sporophytes from *Laminaria digitata* cell-filamentous cultures. In contrast, the effects of green light are less known in Laminariales. Matsui et al. (1992) reported that sporophytes cultured under green light had abnormal shape. In fact, *Undaria pinnatifida* blades do not absorb the green wavelength band (Calogero et al. 2014). Thus, the inhibition of sporophyte regeneration under green LED is reported for first time. In the orchid *Cymbidium hybridium*, higher levels of methylation were found in continuous proliferating protocorm-like bodies (PLB) compared to those ones that spontaneously differentiate into seedlings (Chen et al. 2009). Whether methylation or other epigenetic factors are involved or not in the inhibition of sporophyte regeneration, further studies are needed for clarifying the mechanism behind this effect.

Studies on protoplast cultures from *Ulva* and *Pyropia* showed little effect of light intensity on regeneration (Reddy & Fujita 1991, Gall et al. 1993). In our experiments, whole plant regeneration of gametophytes was observed in all light intensities under RB LED, confirming the stronger resistance of protoplasts from gametophytes of Laminariales towards high illumination (Benet et al. 1997). Although this effect was similar for both gametophytes in early stages, the optimum light intensity varied between them in late stages. Protoplasts from female gametophytes showed improved regeneration at 80 µmol photons m^{-2} s^{-1}, while their male counterparts showed better results at lower intensities (40-60 µmol photons m^{-2} s^{-1}). Additionally, light intensity showed higher size effects on male than female gametophytes. Ideal light intensities for growth of *Undaria pinnatifida* gametophytes are between 40 and 60 µmol photons m^{-2} s^{-1} (Kim & Nam 1997; Xu et al. 2005; Choi et al. 2005; Deng et al. 2009), which is similar to our findings for male gametophytes. Xu et al. (2005) found no difference in optimal light intensities of male and female *U. pinnatifida* gametophytes based on photosynthetic rate. However, our results showed that optimal light intensities depended on gametophyte sex. Destombe and Oppliger (2011) probed that growth rate was strongly influenced by sex in gametophytes of *Laminaria digitata* due to the development of different reproductive strategies between them. It should be noted that, in the previous studies, growth was not estimated by calculating the gametophyte area and that all of them used white or monochromatic lights.

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In conclusion, this is the first report of the effect of LEDs on protoplast regeneration from multicellular marine algae. The combination of red and blue LED (1:2) had the most significant effect on cell division (early stages) and elongation/growth (late stages) in protoplast from *Undaria pinnatifida* gametophytes. This light combination was also ideal for normal sporophyte production from PDAFs. In addition, red and green LED inhibited the formation of sporophytes in protoplast cultures. Under dichromatic light, optimal light intensity was 80 µmol photons m^{-2} s^{-1}, for female gametophytes, and 40-60 µmol photons m^{-2} s^{-1}, for male gametophytes. Further studies with different light ratios are needed in order to clarify the effect of dichromatic light on brown seaweed growth and development.

PART 3. CLONAL PROPAGATION OF SPOROPHYTES FROM *UNDARIA PINNATIFIDA* THROUGH PROTOPLAST-DERIVED APOSPOROUS FILAMENTS (PDAFs)

1. Introduction

Undaria pinnatifida (Harvey) Suringar is one of the most important farmed seaweeds worldwide (FAO 2020). It belongs to the Laminariales, a group of brown algae commonly known as "kelps". As other members of this order, it presents a heteromorphic life cycle, with microscopic filamentous male and female gametophytes, and a macroscopic sporophyte (Goecke et al. 2020).

Undaria pinnatifida is mainly cultivated in China, Japan and Korea (Yamanaka & Akiyama 1993), although there are some efforts to establish cultures outside of Asia (e.g. Spain; Peteiro et al. 2016). Cultivation of *U. pinnatifida*, as well as production and maintenance of strains, rely on sexual reproduction, i.e. fertilization of gametes that can be produced from vegetatively propagated gametophyte clones (Shan & Pang 2009)

Clonal propagation is the multiplication of genetically identical individuals by asexual methods of regeneration from somatic tissues or organs. It has been widely applied in higher plants, where is used for preserving desirable characters of selected genotypes or varieties (Bhojwani & Dantu 2013). In kelps, apospory, which is the production of sporophytes without formation of meiotic spores, represents a potential way for producing clonal plants. To date, this has been only successfully achieved in *Laminaria digitata* (Asensi et al. 2001).

In *Undaria pinnatifida*, clonal propagation through apospory has not been reported. Matsumura et al. (2001) mentioned that gametophytes-like filaments derived from protoplasts could be propagated indefinitely by subculturing, representing a suitable way for the mass production of clonal *Undaria* sporophytes. However, no further research has been conducted to test this novel way of propagation in *U. pinnatifida.*

In previous experiments, we established protoplast-derived aposporous filaments (PDAFs) strains from *Undaria pinnatifida* sporophytes which could be propagated without forming sporophytes. In this work, we aimed to determine the best conditions for sustained clonal propagation of *U. pinnatifida* from PDAFs. We tested the effect of light conditions for maintenance and induction, initial filament density and subculturing on sporophyte production. Also, we determine the ploidy level and the genotypes of PDAFs and regenerated sporophytes. Finally, we assessed the feasibility of long term storage by cryopreserving PDAFs and testing the effect of an antioxidant (2-mercaptoethanol) and antibiotics on post-thaw viability.

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2. Materials and methods

Material

Protoplast-derived aposporous filaments (PDAFs) were produced from protoplasts cultures of *Undaria pinnatifida* sporophytes under white fluorescent (PDAFs-W), and red (PDAFs-R) and green (PDAFs-G) LED lights. Conditions for protoplast culture were as described in chapter 4 of part 1, and part 2. PDAFs were transferred to 100×40 mm Petri dishes and maintained under the same light conditions at 20 °C. In the case of PDAFs-W, they were subcultured 5 times over the course of one year until they lost their capacity for producing sporophytes. The 5th subculture was used for experiments. In the case of PDAFs-R and PDAFs-G, they did not produce sporophytes when maintained in these light conditions. They were subcultured once and then used for experiments after 1 month of regrowth.

Experiment 1: Sporophyte induction under different LED conditions

PDAFs-W, PDAFs-R and PDAFs-G (maintenance light conditions) were cut into small filaments (up to 10 cells long) by using a razor blade and cultured at 500 filaments mL^{-1} in at least 4 wells of 12-well tissue culture containing PES medium. Well plates were separately irradiated using white, blue or red plus blue (1:2, RB) LEDs for sporophyte induction. Culture medium was renewed weekly. The LED system used and the emission spectra of each condition is detailed in part 2. The total number of sporophytes well^{-1} was counted after 1 month of induction. For next experiments, PDAFs under the best light condition for maintenance and induction were used.

Experiment 2: Effect of initial filament density on sporophyte production

In order to investigate the maximum amount of sporophytes a culture can produce, PDAFs were inoculated at 8 different initial filament densities: 25, 50, 100, 250, 500, 750, 1000 and 1500 filaments mL^{-1}. Culture conditions were as previously described. The total number of sporophytes well^{-1} was counted after 2 weeks of induction. The best initial filament density was chosen for subsequent experiments.

Experiment 3: Effect of subculturing on sporophyte production

In order to determine the best conditions for sustained sporophyte production, the effects of the age of the 1st subculture and of subculturing on sporophyte production were investigated.

For the effect of age, PDAFs from the 1st subculture were induced to form sporophytes after 2, 3, 4 and 5 months in culture. Culture conditions were as previously described. The total number of sporophytes well^{-1} was counted after 2 weeks of induction.

For the effect of subculturing, four more subcultures were established (2nd, 3rd, 4th and 5th subcultures). They were allowed to regrow during 2 or 6 weeks prior induction. Culture conditions were as previously described. The total number of sporophytes well^{-1} was counted after 2 weeks of induction.

Experiment 4: Ploidy analysis using DAPI staining

In order to determine the ploidy levels of PDAFs and regenerated sporophytes, the corrected nuclear fluorescence of the samples stained with DAPI were quantified.

One month-old cultures of PDAFs under best light condition for maintenance and three regenerated sporophytes (about 1.5-2 cm in length) were selected for ploidy analysis. A field-collected sporophyte (diploid) and male and female gametophytes cultures (haploid) were used as references. For better imaging, sections of the intercalary meristem were embedded in a matrix (O.C.T., CellPath, Ltd., Newtown, Wales, UK) and sectioned (10-12 μm thickness) using a freezing microtome (Shandon Cryotome FSE, Thermo Shandon, Ltd., Loughborough, UK). Samples were stained with 4′,6-diamidino-2- phenylindole (DAPI) at a concentration of 0.5 μg ml^{-1} in distillated water for 50 minutes (Sheahan et al. 2004). Observation and image acquisition were made with a Leica inverted microscope (DMi8; Leica, Germany) equipped with a Leica DFC450C camera and Leica EL6000 external light source for fluorescence. DAPI stained nuclei were observed at 360/40 nm laser wavelength excitation with emission filter at 425 nm. Chloroplast autofluorescence was observed with a 470/40 nm emission filter and a 515 nm suppression filter. Images in bright field were also captured, as a reference.

Ploidy quantification was performed using the ImageJ software (release 1.50). Images of nuclear fluorescence were digitized and reduced to 300 kB with 8 bits to facilitate the quantification of fluorescent dots. They were captured in only one plane to avoid duplicity of nuclei fluorescence (Zitta et al. 2011). Corrected nuclear fluorescence was calculated according McCloy et al. (2014). For each sample, three technical replications were performed and 100 nuclear areas per replication were measured. Values were presented as averages with 95% confidence interval.

Experiment 5: Genotype analysis using microsatellites

In order to verify the clonal fidelity of the cultures, the genotypes of the PDAF culture and of 10 individual sporophytes regenerated from this culture were investigated using *Undaria pinnatifida* polymorphic microsatellites (Daguin et al. 2005). DNA extraction was performed according to Bustamante et al. (2016). Six of the 20 microsatellite markers developed by Daguin et al. 2005 (Up-AC-1B2, -1B5, -1C1, -1C9, -1G2, -2B2) were used for genotyping. These markers were chosen because they have been used to analyze the genetic identity of sporophytic offspring of cultured and wild populations of *U. pinnatifida* (Shan & Pang 2009).

Experiment 6: Cryopreservation of PDAFs

PDFAs were cryopreserved in order to test the feasibility of long term storage. The cryopreservation protocol used in this experiment is routinely applied to gametophytes of *Undaria pinnatifida* in our laboratory.

About 200 mg of PDAF clumps were selected from the 1st subculture after 2 months in culture under the best light condition for maintenance. Clumps were inoculated into 1.8 mL cryogenic vials (Nunc™ CryoTube™, Thermo Fisher Scientific, Denmark) containing 750 uL of sterile seawater. Cryoprotectant (CPA) solution was applied in three steps, adding 250 uL over a period of 15-min in each step. The solution was prepared at twice the final concentration. It was composed by 20% (w/v) glycerol and 20% (w/v) proline dissolved in sterile seawater. Vials were put in a Cryo 1° C freezing container (Nalgene, Belgium), and the container was placed in a deep freezer at -60 °C during 4 hours. In this system, the vials were cooled at a cooling rate of 1 °C

min^{-1}. After this step, vials were placed in a cryobox (Simport, Canada) and immediately immersed in liquid nitrogen. They were stored for 15 days.

The stored vials were quickly thawed by agitation in water bath at 40 °C during 3 minutes. After clumps settled in the bottom of the vials, the cryoprotectant solution was removed by pipetting. PDAFs clumps were washed twice with seawater before being inoculated into 100×40 mm Petri dishes containing PES medium. For recovery, cultures were maintained in the dark during the first day in culture, and then exposed to the best light condition for maintenance.

Post-thaw viability (%) was measured after 2 weeks of recovery. The viability of the PDAF cells was assessed by staining with erythrosine according to Saga et al. (1989) and Kuwano et al. (2004). More than 300 cells per vial were examined for viability under a light microscope.

Recently, the use of 2-mercaptoethanol and antibiotics has shown positive effects on microalgae post-thaw viability (Kumari et al. 2016; Stock et al. 2018). For this reason, the effects of two different concentrations (50 and 100 uM) of 2-ME, and two different antibiotic mixtures (ES and PSC, see chapter 4 of part 1) on post-thaw viability were tested. 2-ME was added to the cryoprotectant solution before cryopreservation, while antibiotics were included in the culture medium where post-thawed samples were inoculated. In each combinations of treatments, cryopreservation was performed in triplicate. Samples cryopreserved without CPAs were used as controls.

Cryopreserved PDAFs were induced to form sporophytes after 1 and half months of recovery. Induction was carried out in 3 wells of 12-well tissue culture as previously described. The total number of sporophytes well^{-1} was counted after 2 weeks of induction. Sporophytes produced from unfrozen PDAFs were used as reference.

Statistical analysis

The total number of sporophytes per well in experiments 1, 2 and 3 was analyzed using either negative binomial or Poisson regression model. Likelihood ratio test was used for deciding which count regression model to use. If zeros were present, Voung test for non-nested data was carried out to check if a zero-inflated regression was needed (Elhai et al. 2008). The pseudo-R^2 was

chosen as effect size in case of significant results. The analyses were performed using "pscl" (Jackman 2015) and "MASS" (Venables & Ripley 2002) packages in R.

Post-thaw viability (%) in experiment 6 was analyzed as proportions with beta regressions, since beta distribution provides a flexible model for continuous variables restricted to the interval (0, 1) (Ferrari & Cribari-Neto 2004). The analyses were performed using "betareg" package in R (Cribari-Neto & Zeileis 2010). The proportional reduction of error (PRE) statistic was used as the overall model effect size (Smithson & Verkuilen 2006).

Tukey's post hoc test was used when the results were significant. Post-hoc comparisons were conducted using "multcomp" (Hothorn et al. 2008) or "userfriendlyscience" (Peters 2018) packages in R.

3. Results

Experiment 1

Sporophytes were induced in all conditions, with exception of PDAFs-W under white LED. The production of sporophytes was preceded by the formation of male structures (antheridia), which reduced filament growth. In conditions where sporophytes were not observed, PDAFs continued growing without forming antheridia. The formation of oogonia did not seem to be necessary for induction, as sporophytes could be also observed arising from vegetative cells (Fig. 52). Light conditions for maintenance and induction, as well as their interaction, affected significantly the total number of sporophytes ($p < 0.001$; pseudo-$R^2 = 0.31$). There was a tendency toward higher amount of sporophytes in blue LED from PDAFs-G (91 ± 19) compared to RB LED from PDAFs-G; however, this was not statistically significant ($p = 0.95$). In overall, PDAFs-G under blue and RB LED produced, 3.4 and 6.2 times higher than their counterparts from PDAFs-W and PDAFs-R, respectively (Fig. 53). Additional parameters were examined for blue and RB conditions from PDAFs-G. Both lights did not affect the final sporophyte length or the formation of normal sporophytes ($p > 0.01$; Fig. 54A, B); however, a statistically higher amount of blade-like plantlets without polarity was found in blue LED than in RB LED ($p < 0.001$; pseudo-$R^2 = 0.49$; Fig. 54C). For next experiments, PDAFs-G and RB LED were used for maintenance and induction, respectively.

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Experiment 2

The initial filament density affected significatively the amount of sporophytes produced ($p <$ 0.001, pseudo-R^2 = 0.58). The total number of sporophytes initially increased as the filament density increased from 25 to 500 filaments mL^{-1}. Further increment of filament density did not lead to higher amounts of sporophytes. 500-750 filaments mL^{-1} were chosen as the best densities because they presented less variable values compared to higher ones (1000-1500 filaments mL^{-1}) without having statistical differences on the amount of sporophytes produced (Fig. 55).

Experiment 3

The age of the 1st subculture affected negatively the amount of sporophytes produced ($p < 0.001$; pseudo-R^2 = 0.26). Although 2 and 3-month-old cultures produced the same number of sporophytes, 4 and 5-month-old cultures showed 2.3 and 4.7 times less sporophytes, respectively (Fig. 56). Sporophyte production could not be sustained when using subcultures with 2 weeks of regrowth ($p < 0.001$; pseudo-R^2 = 0.52; Fig. 57A). However, when subcultures were allowed to regrow 6 weeks prior induction, sporophyte numbers remained similar ($p = 0.04$; Fig. 57B). Thus, subcultures with 6 weeks of re-growth and less than 3 month-old are recommended for sustained sporophyte production.

Sporophyte development under best conditions for maintenance (green LED), induction (RB LED) and sustained clonal propagation (subcultures with 6 weeks of re-growth and less than 3 months-old) is shown in Fig. 58.

Experiment 4

Differences in the correct nuclear fluorescence were observed among the studied samples. The male and female gametophytes had close values of fluorescence, which were 49-51% of the value reported for the field-collected sporophyte. The results in the reference samples are consistent with a haploid ploidy level (N) in the gametophytes and a diploid ploidy level in the sporophyte (2N). Gametophytes showed 49-51% and 44-49% of the nuclear fluorescence values from PDAFs and regenerated sporophytes, which were also similar to the value reported for the field-collected sporophyte (Fig. 59). The results for PDAFs and regenerated sporophytes showed that both present a diploid ploidy level.

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Experiment 5

Six of the 20 microsatellite loci reported for by Daguin et al. (2005) for *Undaria pinnatifida* were used to genotype the PDAF culture, as well as 10 sporophytes regenerated from this culture. One microsatellite (UP-AC-1G2) failed to amplify or showed very weak amplification products. All loci showed the same band patterns upon electrophoresis of the amplified products. The PCR amplification patterns are presented in Fig. 60 for two of the loci under investigation. Based on these two loci, as well as on the three other markers analyzed, all the regenerated sporophytes displayed the same microsatellite profile, which was identical to that of the mother PDAF culture.

Experiment 6

PDAFs survived cryopreservation in all treatments, but not the controls. 2-ME concentrations and antibiotics, as well as their interaction, affected significantly the post-thaw viability ($p < 0.001$; PRE= 0.28). PDAF cryopreservation without 2-ME and with ES mixture during the recovery period showed the highest viability ($60.62 \pm 8.56\%$). The addition of 2-ME in the ES mixture treatment did not improved the recovery of cryopreserved PDAFs ($p > 0.01$; Fig. 61).

Sporophyte could be successfully induced from cryopreserved samples upon RB LED exposure (Fig. 62). After 2 weeks of induction, cryopreserved PDAFs showed 3 ± 1 sporophytes well^{-1}, which was statistically lower ($p < 0.001$; pseudo-$R^2 = 0.37$) than the values reported for unfrozen PDAFs (11 ± 2 sporophytes well^{-1}).
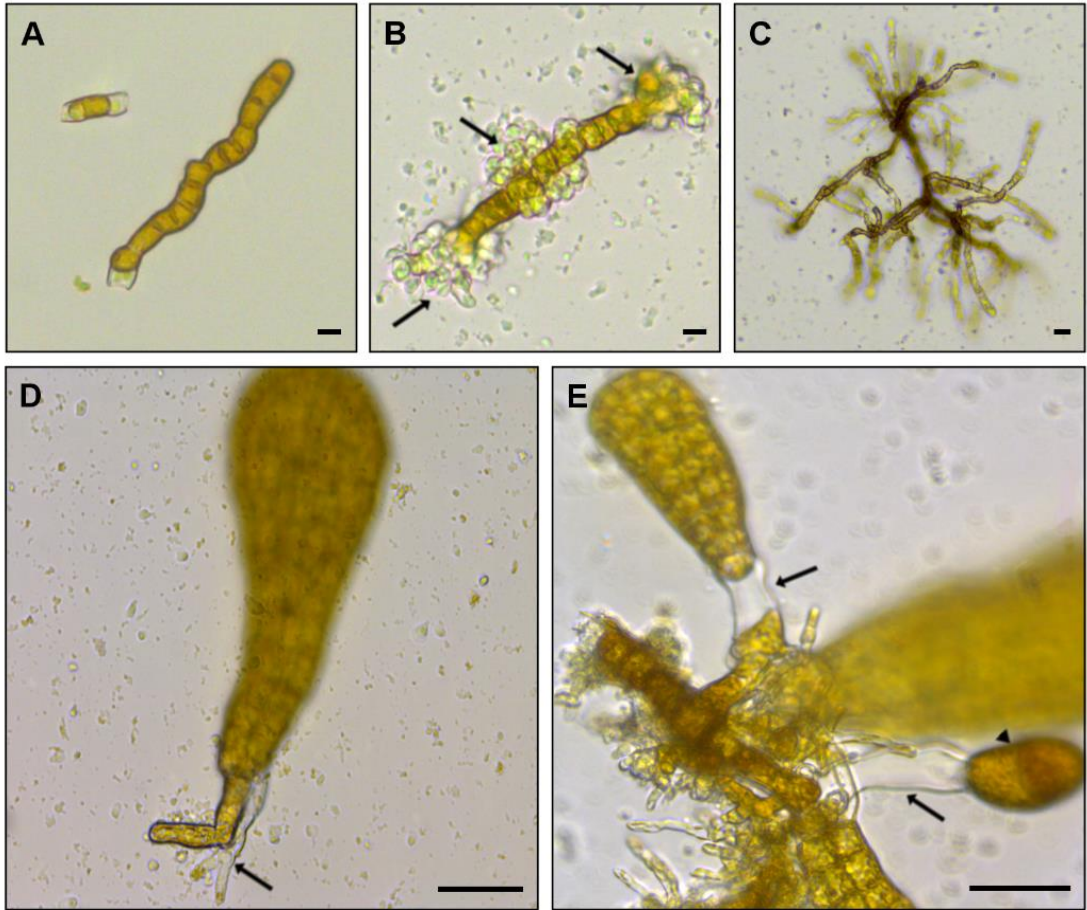
Fig. 52. Sporophyte induction from protoplast-derived aposporous filaments (PDAFs) of *Undaria pinnatifida*. (A) Filament before induction. (B) Filament maintained in green LED showing male structures (antheridia, arrows) after 10 days of induction under RB LED (red plus blue, 1:2). (C) Filaments maintained in white fluorescent light in non-induction conditions (white LED) after 15 days in culture. Note the absence of male structures. (D) Young sporophyte with rhizoids (arrow) produced from a vegetative cell of a filament. (E) Young sporophyte produced from a female structure (oogonium). An egg cell (arrowhead) prior the formation of a sporophyte is also shown. Note that the sporophyte and the egg cell are attached to empty oogonial tubes (arrows). The scales in (A) and (B) are 10 um; the scale in (C) is 20 um; the scales in (D) and (E) are 50 um.
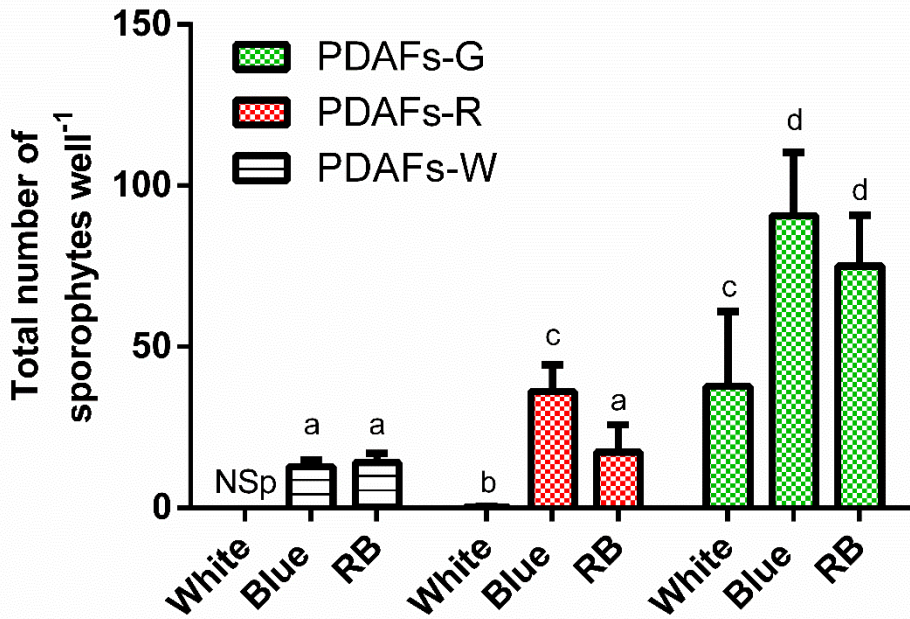
Fig. 53. Effect of light conditions for maintenance (PDAFs-G, PDAFs-R and PDAFs-W) and induction (white, blue and RB LED) on sporophyte production. Bars represent averages (n ≥ 4). Error bars indicate 95% confidence intervals. Different letters indicate significant differences (*p* < 0.01) between treatments. PDAFs-G, protoplast-derived aposporous filaments produced and maintained in green LED. PDAFs-R, protoplast-derived aposporous filaments produced and maintained in red LED. PDAFs-R, protoplast-derived aposporous filaments produced and maintained in white fluorescent light. NSp, no sporophyte production; RB, dichromatic LED (red plus blue, 1:2).

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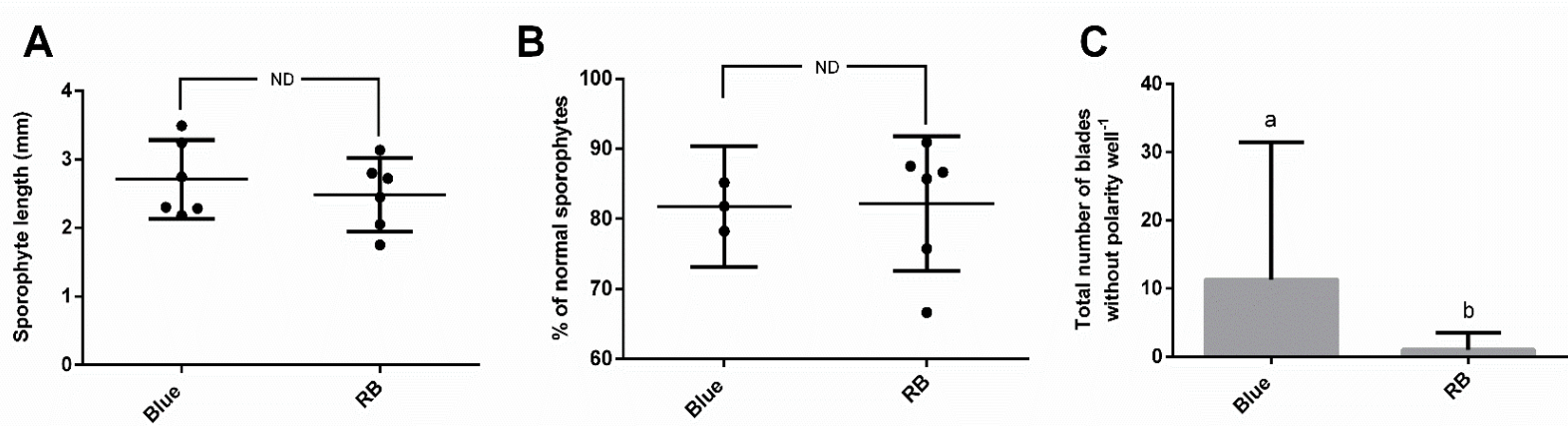
Fig. 54. Effect of blue and RB LEDs on the production of sporophytes from protoplast-derived aposporous filaments from *Undaria pinnatifida* maintained in green LED. (A) Effect of blue and RB LEDs on sporophyte length (mm) after 1 month of induction. (B) Effect of blue and RB LEDs on the percentage of normal sporophytes after 1 month of induction. (C) Effect of blue and RB LEDs on the total number of blade-like plantlets without polarity after 1 month of induction. Independent data points and averages (horizontal lines) are shown (n ≥ 3) in (A) and (B). Bars in (C) represent averages (n = 4). Error bars indicate 95% confidence intervals. Different letters indicate significant differences ($p < 0.01$) between treatments. RB, dichromatic LED (red plus blue, 1:2); ND, so significant difference ($p > 0.01$).

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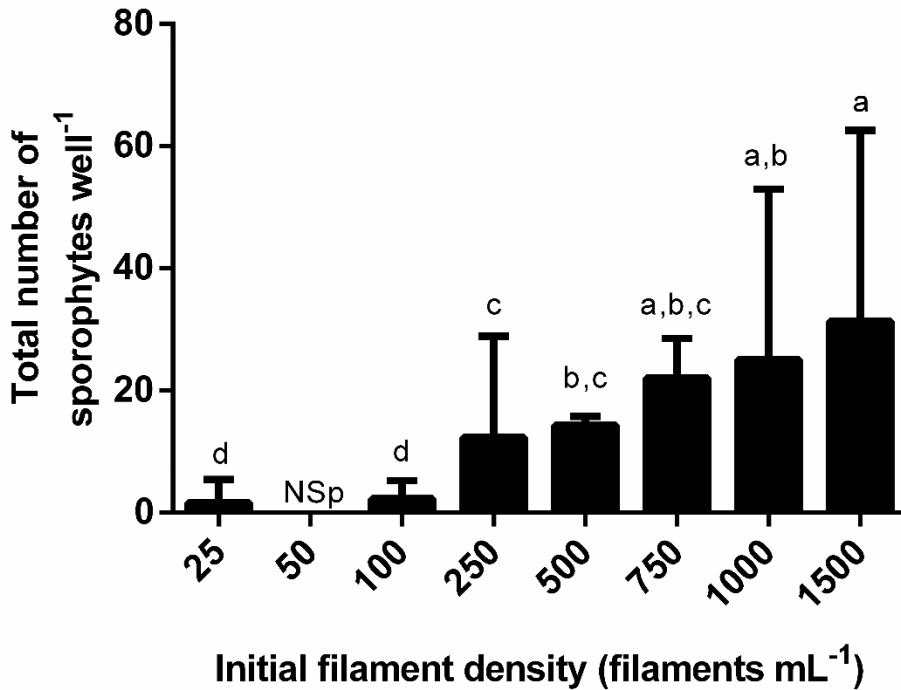
Fig. 55. Effect of initial filament density (filaments mL^{-1}) on sporophyte production from protoplast-derived aposporous filaments (PDAFs) of *Undaria pinnatifida*. PDAFs maintained in green LED were used. Induction was carried out under RB LED (red plus blue, 1:2). Bars represent averages (n = 3). Error bars indicate 95% confidence intervals. Different letters indicate significant differences ($p < 0.01$) between treatments.

Fig. 56. Effect of the age of the 1st subculture on sporophyte production from protoplast-derived aposporous filaments (PDAFs) of *Undaria pinnatifida*. PDAFs maintained in green LED were used. Induction was carried out under RB LED (red plus blue, 1:2). Bars represent averages (n = 3). Error bars indicate 95% confidence intervals. Different letters indicate significant differences ($p < 0.01$) between treatments.

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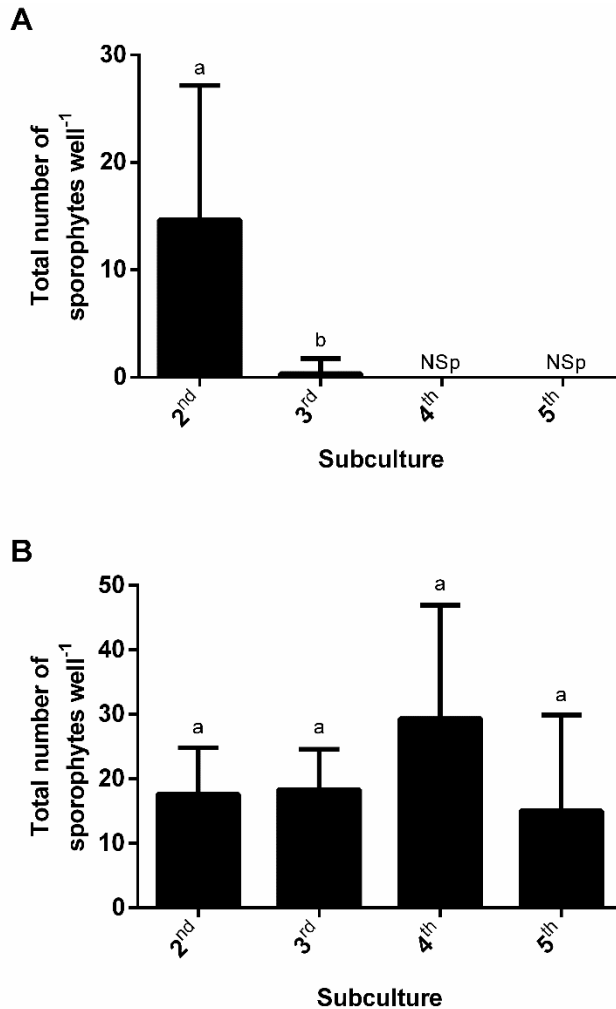
Fig. 57. Effect of subculturing on sporophyte production from protoplast-derived aposporous filaments (PDAFs) of *Undaria pinnatifida*. (A) Subcultures with 2 weeks of regrowth in green LED before induction. (B) Subcultures with 6 weeks of regrowth in green LED before induction. PDAFs maintained in green LED were used. Induction was carried out under RB LED (red plus blue, 1:2). Bars represent averages (n = 3). Error bars indicate 95% confidence intervals. Different letters indicate significant differences ($p < 0.01$) between treatments.

231
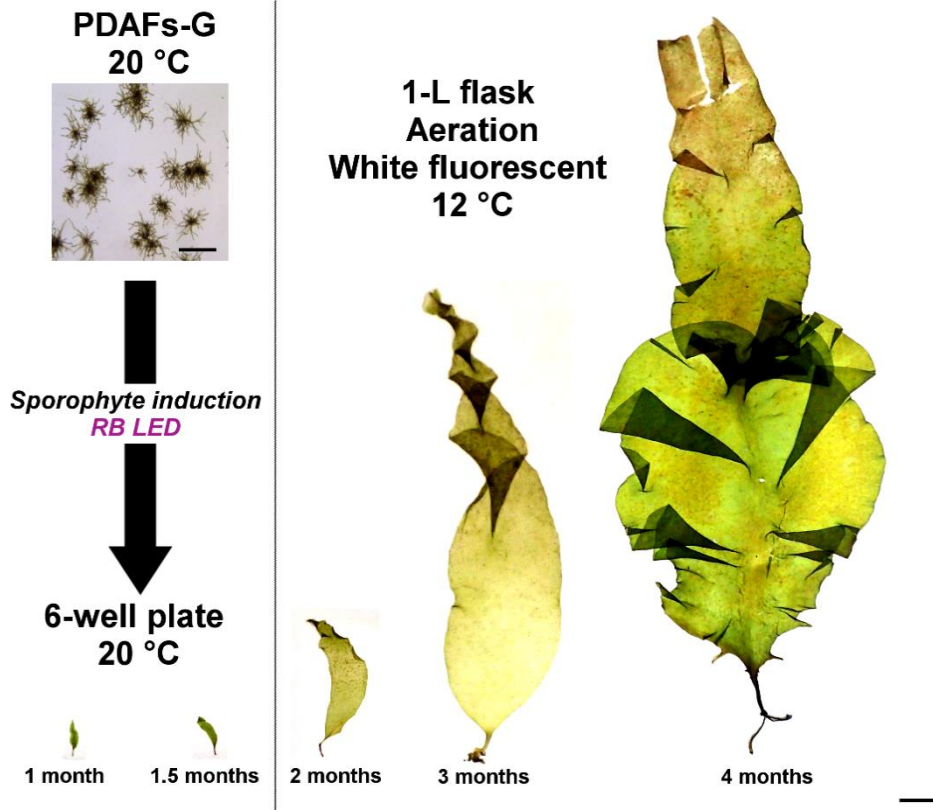
Fig. 58. Sporophyte development from protoplast-derived aposporous filaments (PDAFs) of *Undaria pinnatifida* under best conditions for maintenance (green LED), induction (RB LED) and sustained clonal propagation (subcultures with 6 weeks of re-growth and less than 3 month-old). Sporophytes were maintained in 6-well plates containing PES medium at 20 °C during 1 and half months after induction. They were then transferred to 1-L flasks containing PES medium with constant aeration at 12 °C under white fluorescent light. PDAFs-G, protoplast-derived aposporous filaments produced and maintained in green LED. RB, dichromatic LED (red plus blue, 1:2). The scale is 1 cm; the scale in inset is 500 um.
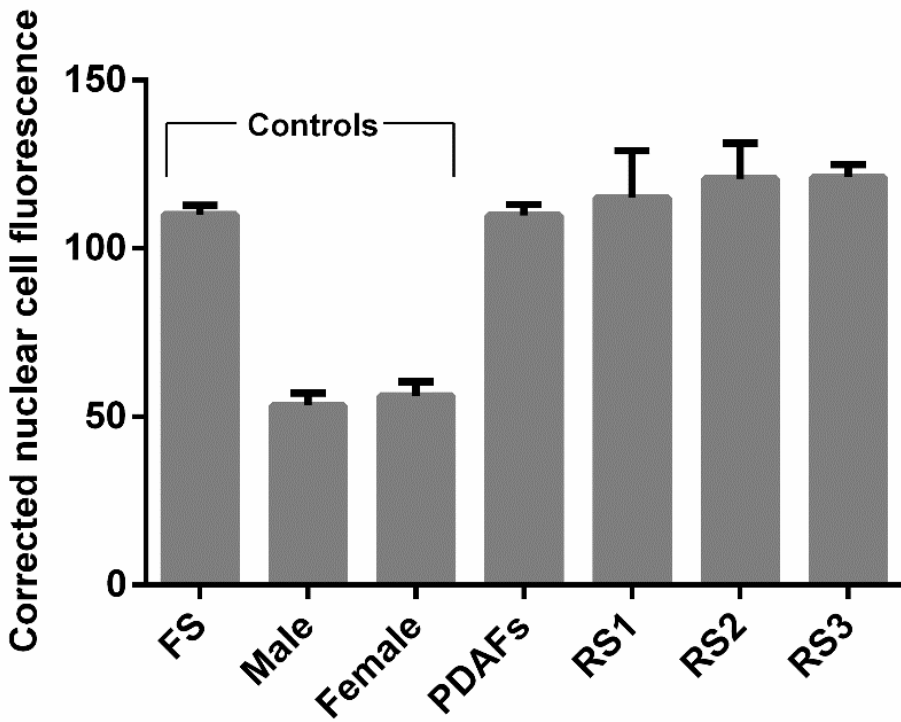
Fig. 59. Corrected nuclear cell fluorescence of a field-collected sporophyte (FS), male and female gametophytes, protoplast-derived aposporous filaments (PDAFs) and three regenerated sporophytes (RS) of *Undaria pinnatifida* obtained through image analysis by ImageJ software. FS and gametophytes were used as controls for diploid and haploid ploidy levels, respectively. Bars represent averages of three technical replications. Error bars indicate standard error of the mean.

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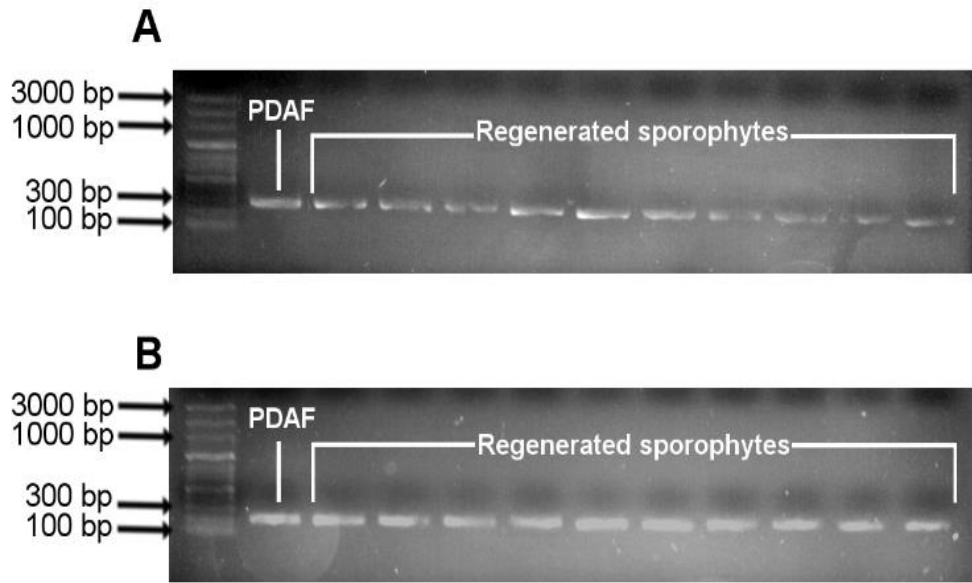
Fig. 60. Genotype analysis of *Undaria pinnatifida* protoplast-derived aposporous filament (PDAF) culture and of 10 regenerated sporophytes using two microsatellite markers. (A) UP-AC-1B5. (B) UP-AC-1C1.
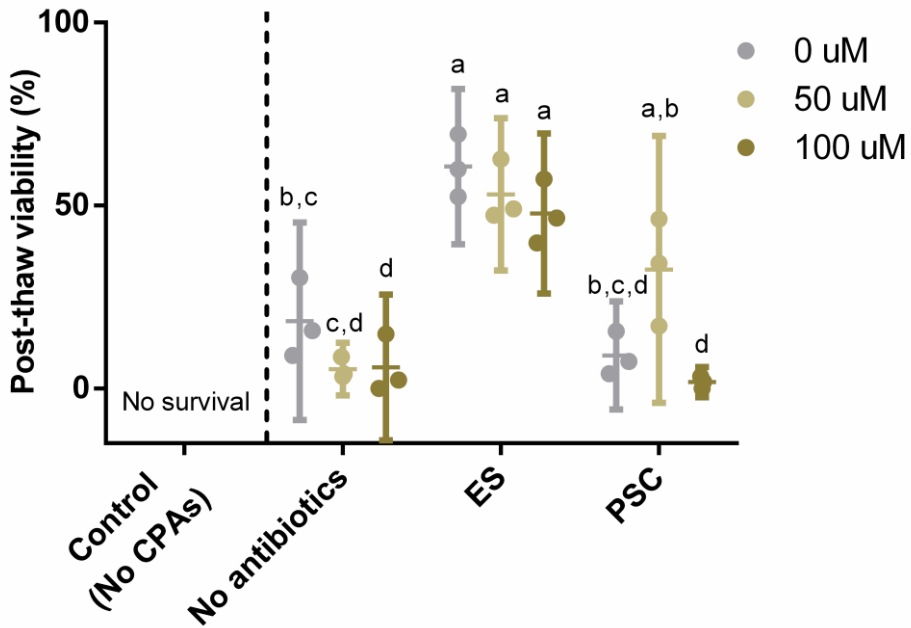
Fig. 61. Post-thaw viability (%) of cryopreserved protoplast-derived aposporous filaments (PDAFs) from *Undaria pinnatifida* after 2 weeks of recovery. Two different concentrations (50 and 100 uM) of 2-mercaptoethanol (2-ME) and two different antibiotic mixtures (ES and PSC) were assessed. Treatments without 2-ME and antibiotics were included for reference. 2-ME was added to the cryoprotectant solution before cryopreservation, while antibiotics were included in the culture medium where post-thawed samples were inoculated. Samples cryopreserved without cryoprotectants (CPAs) were used as controls. The viability of the PDAF cells was assessed by staining with erythrosine. PDAFs from the 5th subculture maintained in green LED for 2 months were used. Independent data points and averages (horizontal lines) are shown (n = 3). Error bars indicate 95% confidence intervals. Different letters indicate significant differences ($p < 0.01$) between treatments. PSC, Penicillin G + Streptomycin + Chloramphenicol; ES, Erythromycin + Streptomycin.
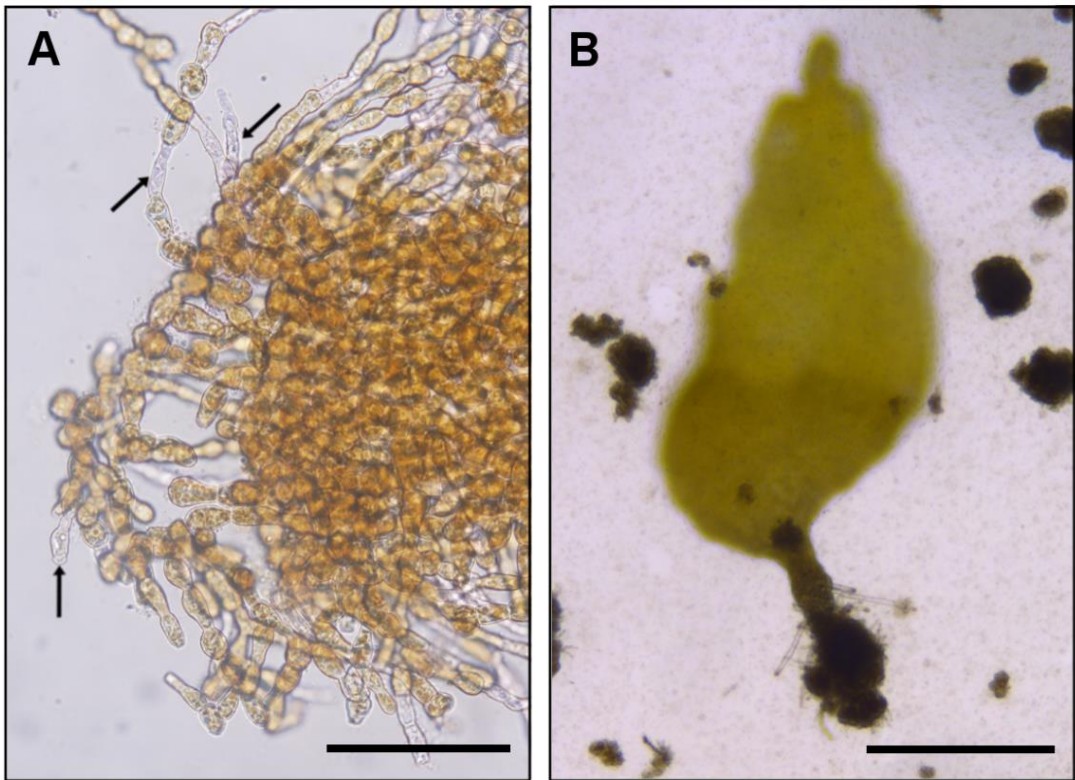
Fig. 62. Recovery of cryopreserved protoplast-derived aposporous filaments (PDFAs) from *Undaria pinnatifida*. (A) PDAFs after 2 weeks of recovery in PES medium containing ES (Erythromycin + Streptomycin) mixture. Recovery was performed under green LED. Few dead cells are shown (arrows). (B) Young sporophyte produced from cryopreserved PDAFs after 1 month of induction under RB LED (red plus blue, 1:2). The scale in A is 100 um; the scale in (B) is 500 um.

4. Discussion

This is the first report of controlled and sustained clonal propagation of *Undaria pinnatifida* through PDAFs. Successful propagation through apospory has been only reported in *Laminaria digitata*, although the filaments obtained were derived from explant cultures instead (Asensi et al. 2001). As shown in Table 34, in terms of production time, control of sporophyte induction and ploidy level of regenerated sporophytes, aposporous filaments obtained from protoplasts are more suitable for clonal propagation than those ones from explants cultures.

Color light during production and maintenance of PDAFs was a key factor for sporophyte induction. When using red LED or white fluorescent light, PDAFs showed diminished capacity for sporophyte production. In this sense, green LED was the best light condition for production and maintenance of PDAFs. The effect of green light has not been widely explored in kelps and it has only shown negative effects on the shape of cultured *Undaria pinnatifida* blades (Matsui et al. 1992). Thus, further research is needed for understanding the way green wavelength band benefits sporophyte induction.

Blue LED usually shows positive effect on growth of different brown algae species (Xu et al. 2005; Miki et al. 2017). Blue light also induces gametogenesis in male and female gametophytes of Laminariales, such as in *Saccharina latissima* (Forbord et al. 2018). In our experiments, blue and dichromatic (red+blue) conditions showed the highest sporophyte production. As culture maintained in red did not develop sporophytes, our findings support the hypothesis mentioned by Asensi et al. (2001) that blue light activates the formation of polystichous structures in aposporous filaments from Laminariales. Although both, blue and dichromatic conditions, presented similar capacity for sporophyte induction, the later presented low amounts of blade-like plantlets without polarity, an abnormal outcome in PDAFs cultures. Takahide et al. (2017) mentioned on this matter that both red and blue wavelength bands were necessary to grow healthy sporophytes from *Undaria pinnatifida*.

Medium values of initial filament density (500-750 filaments mL^{-1}) were ideal for sporophyte production. In *Saccharina latissima*, high initial gametophyte density halted reproduction (Ebbing et al. 2020), while in *Macrocystis pyrifera* and *Pterigophora californica*, high spore densities resulted in elevated sporophyte mortality (Reed 1990). High density cultures of

protoplasts have also shown negative effects (e. g. reduced regeneration ability; Schween et al. 2002; Davey et al. 2005). Although we did not observe sporophyte mortality after induction, high filament density cultures (1000-1500 filaments mL^{-1}) did not increased sporophyte numbers.

Subculturing was a key factor for sustained clonal propagation. Cultures with 6 week of regrowth could maintain sporophyte numbers throughout the subcultures; however, these ones should be used during the first 2 months, as cultures older than 3 months showed diminished sporophyte regeneration capacity. In higher plants, the regeneration potential of callus and cell suspension cultures declines with time (Murashige & Nakano 1962; Vasil & Vasil 1985; Boissot et al. 1990; Zaghmout & Torello 1992). This is attributed to the increase of polyploid and/or aneuploid cells, or physiological changes, such as accumulation of the polyamine Putrescine and high Putrescine/Spermidine ratio in long-term cultures (Nakano et al. 2000; Sundararajan et al. 2020). On the other hand, young cultures have sometimes showed reduced regeneration capacity as they are too small or fragile to survive (Raja et al. 2009). In our experiments, young subcultures (2 weeks of regrowth) also showed reduced regeneration capacity, although they did not present extensive cell death.

PDAFs as well as regenerated sporophytes showed diploid ploidy levels. In explant cultures of Laminariales, regenerated sporophytes were either diploid or tetraploid, whereas those ones arising from aposporous filaments were only tetraploid (Gall et al. 1996; Asensi et al. 2001). Our results suggest that the production of aposporous filaments via protoplasts and/or the conditions for sporophyte induction does not affect the ploidy level of the resulting cultures, which is desirable as only diploid sporophytes were found to be fertile in *Saccharina latissima* (Gall et al. 1996). This also support the idea that fertilization was not involved on the production of sporophytes, even though male and female structures were observed. In addition, microsatellite markers confirmed the clonal fidelity of *Undaria pinnatifida* sporophytes produced from PDAFs. This, together with the findings of Asensi et al. (2001), might indicate the genetic stability of sporophytes obtained through apospory.

In cryopreservation, antibiotic mixtures have shown positive effects in freshwater and marine diatoms by controlling bacterial growth during the recovery period (Stock et al. 2018). Similarly, the addition of antioxidants have increased the post-thaw viability in the green microalgae *Oocystis* sp. and the cyanobacteria *Anabaena* sp. probably through improvement of cryotolerance

(Kumari et al. 2016). In our work, the use of antibiotics but not the addition of the antioxidant 2-ME improved post-thaw viability of cryopreserved PDAFs. This suggest that recovery conditions, especially the inhibition of bacterial overgrowth, might play a major role on successful cryopreservation of PDAFs.

In conclusion, our results showed that sustained and controlled clonal propagation of *Undaria pinnatifida* via PDAFs was feasible. A graphical summary of this propagation system is shown in Fig. 63. The best conditions were: 1) PDAFs produced and maintained in green LED; 2) RB LED for sporophyte induction; 3) initial filament density of 500-750 filaments mL^{-1}; 4) subcultures with 6 week of regrowth under green LED before induction and less than 3-month-old. These conditions allowed the production of diploid sporophytes, which were genetically identical to the mother PDAF culture according to microsatellite analysis. Additionally, PDAF cryopreservation was successfully achieved without losing the sporophyte production capacity. Inclusion of antibiotics during recovery time increased post-thaw viability.

Table 34. Comparison of aposporous filament production and subsequent sporophyte regeneration from explants (Asensi et al. 2001) and protoplast cultures (this work).

	Asensi et al. 2001	This work
Species	*Laminaria digitata*	*Undaria pinnatifida*
Origin of AF	Explant cultures	Protoplast cultures
Time for obtaining AF	1 year	3 months
Conditions for sporophyte induction	Almost all culture conditions, especially in blue light.	Only under blue or dichromatic (red+blue) LED
Ploidy level of sporophytes	Tetraploid	Diploid
Clonal fidelity	Yes	Yes
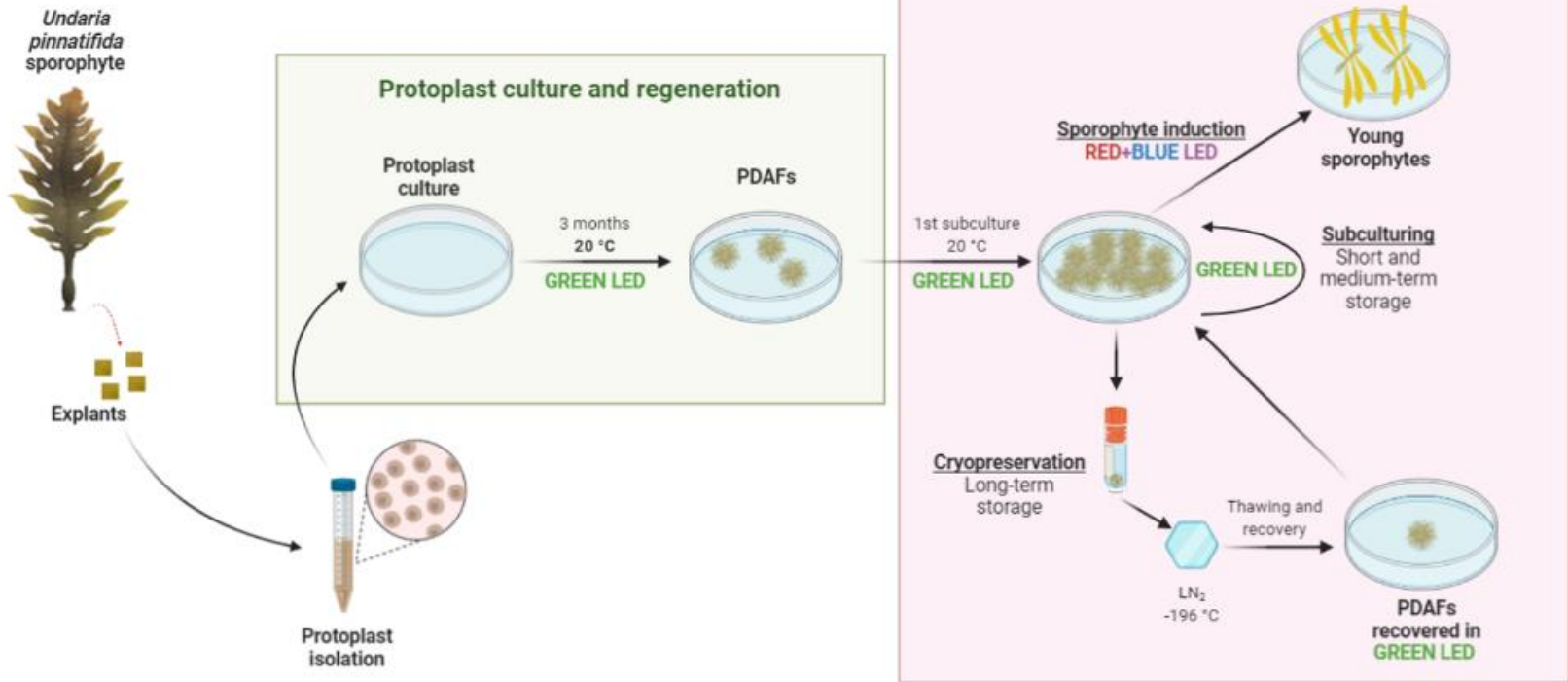
Fig. 63. Main steps for controlled and sustained clonal propagation of *Undaria pinnatifida* through protoplast-derived aposporous filaments (PDAFs).

IV.   CONCLUDING REMARKS

Protoplast studies in multicellular algae have made major accomplishments in green and red seaweeds (Dipakkore et al. 2005; Wang et al. 2014; Huddy et al. 2015; Chen et al. 2018; Gupta et al. 2018; Reddy & Gupta 2018). In contrast, studies in brown algae still lag far behind despite their economic importance, as well as their key roles in marine ecosystems and in understanding developmental and evolutionary processes. One of the main disadvantages of protoplast technology in brown algae is the use of non-commercial enzymes and/or crude extracts for protoplast isolation, which make this process time consuming, expensive and low reproducible (Cocking 1972; Gupta et al. 2011; Inoue et al. 2011). This has hampered the full development of protoplast technology in brown algal species, together with the wide variety of applications it can offer. In our study, we developed protocols for isolating high yields of protoplast from 7 species of brown algae (*Dictyopteris pacifica*, *Ecklonia cava, Hecatonema terminale*, *Petalonia fascia*, *Scytosiphon lomentaria*, *Sphacelaria fusca*, and *Undaria pinnatifida*) using a simple mix of commercial enzymes (cellulase "Onozuka" RS and alginate lyase). Protoplast isolation was reported for first time in *D. pacifica*, *E. cava*, *H. terminale* and *Sp. fusca*. Additionally, protoplast culture and successful regeneration was first accomplished in *H. terminale*, *P. fascia,* and *Sp. fusca*. An improved method for culture and regeneration was also developed for the economic brown alga *U. pinnatifida*. The establishment of these protocols not only proved that protoplast technology in brown algae can rely only on commercial enzymes, but also allowed us to explore in detail the factors affecting isolation, culture and regeneration, as well as the potential of being used in clonal propagation.

During our protoplast isolation experiments, we found out that that cell walls from filamentous brown algae were usually difficult to digest compared to more complex forms, such as *Ecklonia cava* or *Undaria pinnatifida*. The same tendency has been also observed in other filamentous species (Ducreux & Kloareg 1988; Mejjad et al. 1992; Varvarigos et al. 2004). However, this was not impediment to obtain true and viable protoplasts from filamentous species.

Among the factors explored during protoplast isolation, the positive effect of chelation pre-treatment and increased osmolarity varied according to the species. Young cultures as well as explants from meristematic zones were recommended for obtaining high yields of protoplasts. Incubation temperature of 20 °C and pH 6 proved to be ideal for all the species tested. Incubation

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times of 6-7 h were suitable for most of the species, and it could be reduced to 4 h in *Undaria pinnatifida* female gametophyte and sporophyte, and in *Petalonia fascia*.

Our experiments suggested that low initial protoplast densities and the regeneration media proposed by Mejjad et al. (1992), composed by PES medium supplemented with NaCl and CaCl$_2$, were ideal for culture and regeneration in all the species tested. Antibiotics (Penicillin G + Streptomycin + Chloramphenicol) were crucial for protoplast survival and division only in *Undaria pinnatifida*, but they also helped controlling bacterial overgrowth in the rest of the species, allowing a better characterization of protoplast regeneration process. Temperature only affected regeneration patterns in *U. pinnatifida* as previously reported (Matsumura et al. 2001). It was maintained at 20 °C for all the species as this temperature matched with those one used for our stock cultures. Light exposure and the start time of osmolarity reduction were crucial for protoplast survival, and they needed to be set for each species according to the cell wall regeneration period of each one. The regeneration patterns became more complex as the anatomical complexity increased. Filamentous forms showed direct regeneration, while in *U. pinnatifida* sporophytes, this was indirect via aposporous filaments.

We also reported, for first time in multicellular algae, the effect of color light in protoplast regeneration of the economic brown alga *Undaria pinnatifida.* Our findings showed that a combination of red and blue LED (dichromatic condition) was better than blue LED (monochromatic condition) in both protoplast cultures of gametophytes and sporophyte. Despite the positive effects of blue light on growth and development of brown algae (Xu et al. 2005; Miki et al. 2017), the dichromatic conditions seemed to give better responses in protoplasts cultures, reinforcing the idea that red wavelength band is also necessary for proper growth and development of brown algae (Takahide et al. (2017; Wang et al. 2013). Interestingly, red and green conditions totally inhibited sporophyte regeneration from PDAFs, an outcome that was expected for red LED, as this has also inhibit gametogenesis in Laminariales (Forbord et al. 2018), but not for green LED, whose effects are not well study in brown algae.

We explored the possibility of using PDAFs for controlled and sustained clonal propagation of *Undaria pinnatifida* sporophytes. Among the conditions tested, only those PDAFs that were obtained and maintained in green LED were ideal for producing sporophytes upon induction with dichromatic LED, as they did not showed diminished sporophyte regeneration capacity

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compared to those ones obtained and maintained in red LED and white fluorescent light. An initial filament density of 500-750 mL^{-1} were enough for maximizing the sporophyte production. Subculturing was necessary for keep propagating PDAFs. 6 weeks of regrowth before induction and less than 3-month-old cultures were ideal for sustained sporophyte production. Under these conditions, diploid sporophytes were obtained which also showed clonal fidelity according to microsatellite analysis. PDAFs could be successfully cryopreserved and the addition of antibiotics improved the recovery of thawed samples. After recovery, PDAFs could be induced to regenerate sporophytes, meaning that this capacity was not affected by the cryopreservation process.

The feasibility of using aposporous filaments for clonal propagation in Laminariales has been successfully tested also in explant cultures of *Laminaria digitata* (Asensi et al. 2001). Nevertheless, our experiments showed that obtaining aposporous filaments via protoplast involves less time (3 months) than previously reported for explant cultures (1 year). In addition, Asensi's work could not precisely control the production of sporophytes, and these ones were tetraploid. In our method, sporophyte regeneration could be tightly controlled by color light and the resulting sporophytes showed diploid ploidy level. Thus, using PDAFs for clonal propagation seems to be a better option than explant cultures. Propagation via protoplast cultures has been explored only in green and red marine algae. Protoplasts-based plantlets have been used for propagation of the green seaweed *Ulva lactuca* (Gupta et al. 2018), and protoplasts themselves have served as artificial seeds for aquaculture of the red seaweed *Porphyra* (Dipakkore et al. 2005). Our results suggest that PDAFs could be used in a similar way in brown algae, opening a new possibility of protoplasts uses in this algal group.

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