Overcoming Propagule Supply Bottlenecks for Seaweed Production

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Acknowledgments

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Executive Summary

The global seaweed market is expected to grow to US$ 14.3 billion by 2028, up from US$ 7.5 billion in 2022 (IMARC Group Report, 2023). The potential for sustainable seaweed resources to be converted into novel goods such as food, feed, cosmetics, pharmaceutics, biomaterials, and bioenergy has raised their global demand. Therefore, managing a seaweed biomass supply chain to meet the rising market demand is the primary challenge in the seaweed aquaculture sector. Vegetative fragments or reproductive cells (spores) are commonly used to grow seaweed for large-scale farming. However, significant challenges include loss of vigour, vulnerability to various stresses and diseases, reliance on fertile female plants, and complicated life cycles. It is crucial to tackle these bottlenecks promptly to take advantage of the growing commercial demand for seaweed production.

Protoplast technology has the potential to revolutionize the seaweed aquaculture industry. The protoplast is a living plant cell without a cell wall, protoplasts offer opportunities for mass propagation and plant regeneration. The remarkable potential of protoplast technology in the seaweed aquaculture industry lies in its ability to generate an astounding number of protoplasts and develop countless plants from mere milligrams of tissue. This transformative capability sets it apart from conventional spore-based or vegetative farming methods. Phycologists can unlock many advantages by harnessing protoplasts beyond whole plant generation. This technique enables advancements in breeding, hybridization, genetic engineering, and transformation, presenting the opportunity to revolutionize the seaweed industry by introducing desirable traits. These traits include accelerated growth rate, increased biomass production, enhanced stress tolerance, disease resistance, and improved nutritional composition.

While seaweed protoplasts offer numerous benefits, as mentioned above, their practical application in commercial settings faces technological and methodological challenges. These challenges include the isolation of viable protoplasts, the successful seeding of protoplasts, and their subsequent regeneration. These complexities possibly have hindered the widespread adoption of seaweed protoplast technology for commercial purposes.

The University of Technology Sydney (UTS) and the Department of Primary Industries (DPI, NSW) together have developed a ground-breaking seaweed farming tool – "Protoplast-based seedling production." This innovative method offers a scalable and independent approach to producing high-quality seedlings, eliminating the reliance on spores and vegetative fragments cultivation methods.

A highly efficient and rapid tool was specifically developed using diverse green seaweeds (*Ulva* species) to isolate a large number of viable protoplasts with high regeneration potential and their seeding on twine. The project also investigated the effects of vacuum infiltration and exogenous supplementation of polyamines on protoplast yield, development, and regeneration potential.

The process involved physically disrupting the thalli by chopping the tissue into <1mm^2 size, followed by vacuum infiltration (low pressure, 10 min) in a cell wall-digesting enzyme solution. This ensured optimal enzyme access to the cells, significantly increasing protoplast yield (9-14-fold; 1.62 x 10^8 protoplasts g^{-1} fresh weight). The protoplast isolation and purification process was completed in less than an hour. Further, protoplast culturing in a nutrient media supplemented with polyamines (spermidine – Spd, and spermine – Spm; each 50µM) exhibited rapid cell differentiation and enhanced growth rate with higher branching patterns. To ensure the economic viability of the protoplast technology, we investigated the potential reusability of the enzymes. The results revealed that the enzyme mixture could be reused for up to four cycles without compromising protoplast yield or viability. Additionally, under laboratory conditions, it was observed that scraping off two-week-old, regenerated protoplasts from Petri dishes and subsequently seeding them on twines resulted in superior attachment and regeneration into complete thalli within 4 weeks compared to directly seeding protoplasts onto nylon twine.

Using this protoplast technology, various *Ulva* species' biomass (0.5 – 0.8 kg fresh weight, FW) was successfully generated under laboratory conditionsby culturing protoplast generated seedlings in

photobioreactors (PBRs). This biomass was obtained from an initial tissue size of 100 mg FW within three months. The effectiveness of the protoplast technique was initially tested at the DPI-Port Stephens Fisheries Institute (DPI-PSFI) hatchery and has since been successfully replicated by hatchery technicians on multiple occasions. In collaboration, UTS and DPI-PSFI developed a prototype of protoplast airlift suspension technology (AST) to enhance protoplast seeding and germination on vertical frames. Protoplast seeding on these frames, which were wrapped with twines, was achieved successfully; however, the protoplast attachment and subsequent germination rate was relatively low. To improve the protoplast germination rate, growth, and development, further optimization is required in areas such as airflow control, light settings, and the extent of angular rotation of frames. On the other hand, when comparing it to the direct seeding of protoplasts onto twines, a more successful approach involved seeding seedlings generated from microscopic protoplasts onto the twines. These seedlings had been cultured in petri dishes for three weeks before being carefully scraped off from the petri dishes. Importantly, the intact natural binder present in their rhizoidal structure was retained. This modified method led to significantly improved rates of attachment and successful regeneration.

By establishing such protoplast-based seaweed farming tools in other red and brown seaweeds, the industry could benefit from improved productivity, enhanced genetic traits, disease management, and scalability. These advancements foster a more resilient, sustainable, and economically viable seaweed industry, resulting in higher-quality seaweed products and capitalizing on the current commercial interest in seaweed production.

Keywords: Seaweed, protoplast, seeding, polyamines, elite strains, micropropagation, hatchery, Ulva sp.

Introduction

Background

Australian seaweed aquaculture is a nascent industry, making up less than 1% of the $15 billion global seaweed market. Currently valued at AUD$ 3 million, the Australian industry is based upon the collection and export of storm-cast kelp, compared to the annual production of over 30 million tonnes of seaweed primarily from China (48%), Indonesia (39%) and the Philippines (5%) (Kelly, 2020). As a result, Australia imports an estimated $40 million worth of seaweed a year. Interest is growing in seaweed farming (land and ocean-based) for the production of high-value bioproducts for use in animal feed, agricultural fertiliser and functional foods. A recent Agrifutures report (Kelly, 2020) suggests that with appropriate research and investment, an opportunity exists to establish an Australian seaweed industry that could generate over $100 million GVP and create 1,200 direct jobs in regional areas by 2025.

The livestock sector is Australia's third largest source of domestic greenhouse gas (GHG) emissions, accounting for 11% of our total GHG emissions (DPIRD, Government of Western Australia). This is mainly due to the release of potent greenhouse gases, methane (CH_4) and nitrous oxide (N_2O), which account for 56% and 73% of agriculture's emissions footprint. Due to this impact, Meat and Livestock Australia (MLA) has identified the development of methane reduction technologies for the livestock sector as a critical research area in their Carbon Neutral 2030 (CN30) Roadmap. One approach, recommended in the Roadmap, involves screening novel feed supplements that inhibit enteric methane production and can be easily and cheaply integrated into existing feeding systems.

Research has recently sparked commercial interest demonstrating the effectiveness of cattle feed supplementation with native Australian seaweed. Studies have shown that supplementing as little as 2% of livestock feed with seaweed from the *Asparagopsis* spp. group, can reduce the methane emissions of cattle by 99% (Kinley et al., 2016; Machado et al., 2016). *Asparagopsis* produces a bioactive compound called bromoform, which prevents methane formation by inhibiting fermentation within the cow's gut. Asparagopsis supplements reduce methane emissions and may restore the 2-10% metabolic conversion efficiency lost during methane production, thereby improving the cattle's productivity (Kinley et al., 2016; Machado et al., 2016).

According to the Agrifutures report (Kelly, 2020), "the greatest opportunity to grow the Australian seaweed industry is in producing cattle feed for methane reduction. Despite some technical gaps in realising the cultivation of this seaweed at scale, the demand from the meat and dairy sectors to reduce carbon emissions will mean this novel solution has a ready market and can attract significant investment. However, the breeding and cultivation techniques for this seaweed need to be developed and this requires significant RD&E to realise".

Need

If an Australian seaweed industry is to develop and a sector such as livestock is to reduce its emissions footprint, and achieve its promised carbon neutrality by 2030, handpicked seaweed will not be enough to create a commercial seaweed industry for cattle feed. For example, achieving a 1% DW supplement of *Asparagopsis* to reduce the methane output for the NSW dairy herd alone (approximately 15% of Australia's dairy herd and < 1% of Australia's total cattle herd) would require annual production of approximately 8,000 tonnes (dry weight) of *Asparagopsis*. This would necessitate over 400 hectares of seaweed farms alone. Therefore, there is an urgent need, and significant commercial incentive, to rapidly develop next-generation ways to propagate, farm, and process *Asparagopsis* at scale.

The existing seaweed farming techniques (vegetative fragments and spore/gametes based) create several major bottlenecks to large-scale seaweed farming including: the requirement for large amounts of biomass and fertile sporophyte plants, dependencies on complex life cycles, loss of vigour, and high susceptibility to a variety of pest and diseases. Addressing these bottlenecks immediately is a top priority to capitalise upon the significant current commercial interest in seaweed production and the projected demand for this innovative animal feed additive.

We propose that protoplasts-based seedstock generation for seaweed farming to provide a sustainable and scalable solution, resulting in higher yields of quality plantlets production independent of spores and vegetative fragments. Further, developing seaweed protoplasts isolation tools will also aid in vitro genetic manipulation techniques for developing genetically improved strains of seaweed crops.

This protpplast based micropropagation tool addresses four of the barriers to developing the seaweed aquaculture industry in Australia. These include 1) propagation and control of complexities in the life cycle, 2) reliable quality of seedstock supply, 3) commercial hatchery development, 4) farming and harvest technology. This approach will overcome the industry's reliance on wild harvest for parental stocks and the seasonality of mature sporophyte plants.

Developing micropropagation tools for the seaweed aquaculture industry will:

1. Make a sizeable contribution to achieving the National Aquaculture Strategy and can support Australia's economic recovery post-COVID 19.
2. Support NSW's developing seaweed industry
3. Increase the red meat industry's capability to achieve MLA's commitment to carbon neutrality by 2030.
4. Promote finfish aquaculture by growing seaweeds in integrated multi-trophic aquaculture systems by removing and utilizing excess nutrients.

Objectives

1. Establish micropropagation tools for year-round seedstock supply of seaweeds.
2. Demonstrate the commercial practicality of workflow for micropropagation.

Method

Objective 1: Establish micropropagation techniques for year-round seedstock supply of seaweeds.

1.1 Sample collection and maintenance in laboratory conditions:

During the period from August 2021 to December 2022, various seaweed species, including five *Ulva* sp. (green seaweed), *Pterocladia* sp. (red seaweed), *Ecklonia* and *Rugulopteryx* sp. (brown seaweed), were collected from the Coogee and Maroubra coastlines in New South Wales (NSW). These thalli were carefully transported to the UTS-C3 laboratory under cool conditions. The species were identified based on their distinct morpho-anatomic characteristics. To establish and maintain axenic cultures of these seaweed species, they were grown in sterilized seawater with a salinity of 30 psu, supplemented with germanium dioxide (5 mg/L) and half-strength MPI media (Reddy and Seth, 2018). To ensure proper culture maintenance, the seawater was replenished once a week. The cultures were maintained under white LED lights with an irradiance ranging from 50 (red and brown seaweed) to 100 (green seaweed) µmol photon m^{-2} s^{-1}, following a 12:12-hour light-dark photoperiod.

1.2 Protoplast Isolation and purification for seaweed hatchery development:

To isolate protoplasts, axenic seaweed cultures were utilized, and various commercially available cell wall lytic enzymes were tested individually or in combination. Different concentrations of mannitol in a MES buffer-based enzyme solution were also investigated. Optimization was performed for the pH of the enzymatic mixture, incubation temperature, and duration to achieve a high yield of protoplasts. For protoplast isolation, healthy growing tissue (in the case of green seaweed) and growing apical tips (for red and brown seaweeds) from axenic cultures of each species were finely chopped into small pieces (≤1 mm thin) and thoroughly rinsed with sterile seawater to remove debris. The clean chopped tissues were then subjected to different enzyme mixtures and incubated on a rotary shaker (at 55 rpm) in the dark at 26°C. The incubation duration ranged from 1 to 6 hours, depending on the specific seaweed species being studied for protoplast isolation. Microscopic observations were periodically conducted during the incubation period to monitor protoplast release. Before examining the yield and viability of the

protoplasts, a gentle purification step was carried out using a Pluriselect filter with a pore size of 20-30 µm.

Furthermore, to enhance the protoplast yield, vacuum infiltration was explored as a pre-treatment method to ensure maximal penetration of the enzyme solution into the intercellular spaces of the tissue and facilitate the action of cell wall digestive enzymes, and other components of the cell wall polysaccharides.

1.3 Protoplast yield, viability, and purity analysis:

The estimation of protoplast yield was conducted using a hemocytometer, while the viability was assessed by measuring the autofluorescence of red chlorophyll. To confirm the purity of the protoplasts, which indicates the complete removal of the cell wall, the protoplasts were stained with calcofluor white M2R and observed under an inverted microscope equipped with appropriate excitation and emission filters. Additionally, to enhance the protoplast isolation process's cost-effectiveness, the enzyme mixture's potential re-usability was evaluated to determine if it could release protoplasts again from newly chopped thalli tissue.

1.4 Seeding of protoplast seedstocks onto vertical frames using airlift pumping spore re-suspension technology:

The protoplast suspension, obtained in large quantities using the optimized protoplast isolation method as described previously, was utilized to assess its potential for seeding on twines. For this purpose, an innovative protoplast seeding technology known as "airlift spore re-suspension technology" (AST) was developed. This technology aims to maximize the settlement of protoplasts onto double screen vertical frames composed of tightly twined nylon ropes or fibreglass sheets. The ASR technology utilizes air bubbles to mix the spore suspension from the bottom, causing the spores to be uplifted and re-suspended within the system, facilitating their settlement onto the frames. A similar approach, involving protoplast re-suspension supported by airlift technology, was also developed to evaluate its effectiveness in promoting protoplast settlement, germination efficiency, and overall productivity.

Objective 2: Demonstrate the commercial practicality of workflow for micropropagation – from seedstocks to seeding to grow-out in ponds.

2.1 To demonstrate the commercial practicability of protoplast technology, biomass generation from single cell protoplast stage to full-grown plantlets by growing protoplast seedstocks initially in round flasks (2 L capacity) and later in cylindrical PBRs (10 L capacity) was tested under laboratory conditions. Further, the biomass generated under the laboratory conditions at UTS was transferred to hatchery facility of Port Stephens Fisheries Institute for their further growth and biomass generation in 500 L PBRs.

Results and Discussion

Protoplasts – naked plant cells with the potential to develop into complete plants, play a versatile role in biotechnological research. Economic cultivars enable in vitro manipulation and crop improvement, bypassing sexual reproduction. Large-scale seaweed farming, including *Ulva*, *Kappaphycus* and Kelps can benefits from protoplasts as artificial seeds, ensuring a continuous plantlet supply without waiting periods. However, challenges like microbial contamination, uncontrolled growth/regeneration, and limited algal group-specific protocols have hindered commercial implementation. Additionally, using non-commercial enzymes in studies has increased isolation process costs and time, impeding the establishment of standardized protocols.

This project explored ways to isolate protoplast less laboriously and cost-effectively with high seeding efficiency on twine suitable for land-based or open-sea seaweed farming practices.

1. Establishment of seaweed micropropagation tool: Young and healthy thalli of seaweed including *Ulva intestinalis*, *U. compressa*, *U. fasciata*, *U. linza and U. australis (green seaweeds)*; *Pterocladia* sp. (red seaweed), and *Rugulopteryx* sp. (brown seaweed) collected from Sydney coastline, cultured and maintained under defined laboratory conditions (see material and method section) were used for protoplast isolation. Due to unprecedented rainfall that affected the seaweed populations, we could not collect *Asparagopsis* sp. and other seaweeds mentioned in the original proposal from the coastlines in NSW. We were also unsuccessful in culturing vegetative thali of *Ecklonia sp.* under laboratory conditions. Therefore, we focused on different *Ulva* species, *Pterocladia* and *Rugulopteryx* species only to develop protoplast tools.

To begin with, explants of seaweeds to be used for protoplast isolation were pre-treated with Povidone–iodine (Betadine®; 1%, 2 min prepared in seawater) to minimize the bacterial load on the explants and in subsequent protoplast cultures. Reddy et al. (2008) emphasized the crucial role of preparing axenic explants for successful seaweed micropropagation. By subjecting the explants to sequential treatments with detergent, chemical disinfectant (betadine), and broad-spectrum antibiotics, researchers achieved a relatively high frequency of viable axenic material, initiating micropropagation in different seaweed species.

We only preferred to pre-treat the explant with betadine as we didn't aim to obtain a completely axenic plant. The presence of some ecto-symbiotic marine bacteria is suggested to be essential, allowing the seaweed to form its characteristic vegetative body (thallus formation). Without bacteria, *Ulva* only develops into undifferentiated cell clusters (callus) (Wichard et al., 2023).

Various compositions of cell wall digestive enzyme buffer solution (Table 1) were examined to maximize protoplast yield in green, red, and brown seaweeds by using –

1. different enzymes combinations (cellulase, macerozymes (0.5-2%); agarose, alginate lyase, driselase, and pectinases; 5-50 units/mL),
2. different osmoticum concentrations (mannitol; 0.6-1 M),
3. different pH (5-6.5); incubation time (1-6 h), incubation temperature (20-27^{o}C), and
4. protoplast viability and regeneration assessment.

Table 1. Composition of cell wall digestive enzyme buffer mix used for protoplast isolation in green seaweeds (Reddy and Seth, 2018). Different enzymes namely – (agarose +pectinase for red seaweeds), (alginate lyase + driselase for brown seaweeds) were added in this mixture at a concentration of 5-50 units/mL for protoplast isolation.

Enzyme Buffer components (dissolved in distilled water)	Concentrations
Cellulase Onozuka R-10	2%
Macerozyme*	0.5%
NaCl	1%
Mannitol	0.8M
MES buffer	25 mM
Dextran sulfate	0.5%
pH	6.0

*Macerozyme only needed for tubular *Ulva* species

Pre-chelation treatments to ease protoplast isolation: For brown and red seaweeds, an additional step, **C**alcium/**S**ulphur-**C**helation **P**re-treatment (CSCP) with and without digestive enzymes was examined to improve the protoplast isolation efficiency and yield by chelating sulphur and calcium present in their cell wall polysaccharides.

For brown seaweeds, Ca-chelation pre-treatment (CCP), with a calcium-chelating solution [665 mM NaCl, 30 mM $MgCl_2 \cdot 6H_2O$, 30 mM $MgSO_4$, 20 mM KCl, and 20 mM ethylene glycol-bis(β-amino-ethyl ether)-N,N,N′,N′-ethylene glycol-bis(β-amino-salt (EGTA-Na_4) as the calcium chelator; pH5.5] was investigated for 15-60 min prior to enzymatic digestion (Avila-Peltroch et al., 2022).

For red seaweeds, S- chelation pre-treatment with a sulphur chelation solution [1 M mannitol, 450 mM NaCl, 100 mM $MgCl_2 \cdot 6H2O$, 20 mM KCl, and 40 mM $BaCl_2$ as sulfate chelator] was examined by incubating explants in this solution for 15-60 min before protoplast isolation (Gupta et al., 2011).

The pre-treatment procedure is utilized to shrink protoplasts away from the cell wall before adding the enzyme solution. Its purpose is to safeguard the cell membrane and improve protoplast yield. A study comparing protoplasts from *Undaria pinnatifida* gametophytes, both with and without pre-plasmolysis, found that the pre-treated protoplasts yielded approximately three times more than the non-treated ones (Avila-Peltroch et al., 2022).

However, none of the tested pre-treatments proved effective in our experiments with red and brown seaweeds, regardless of the incubation period. Similarly, using different enzyme concentrations, individually or in combination, failed to release the protoplasts from red and brown seaweeds. One possible explanation for these unsuccessful results could be using mature plants' thalli for treatment instead of spore-generated juveniles, as suggested by Avila-Peltroch et al. (2021, 2022).

In brown seaweeds, the CCP treatment (40 min) resulted in a mild loosening of thalli, revealing cells resembling protoplasts. Nevertheless, these protoplasts did not fully detach from the explants even after incubating the chopped thalli in enzyme mixture for >5 hours (Figure 1 A and B). However, we have recently achieved success obtaining and regenerating spores into germlings (Figure 2 A-F). These germlings hold promise for future protoplast isolation experiments, both with and without CCP pre-treatment.

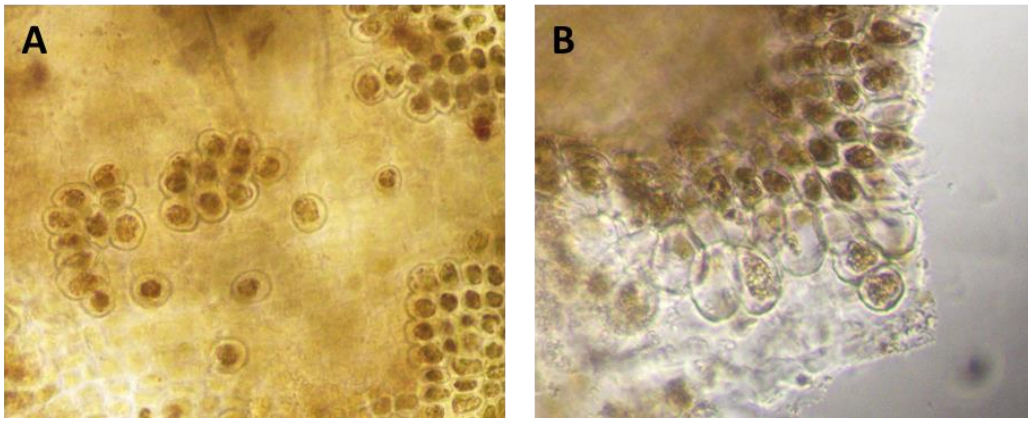

Figure 1: Protoplast cell formation observed in brown seaweed *Rugulopteryx* sp. after Ca-chelation pre-treatment (CCP) for 30 minutes (A); few protoplasts about to release from thalli after CCP and incubation in enzymatic solution for 5 hr (B).

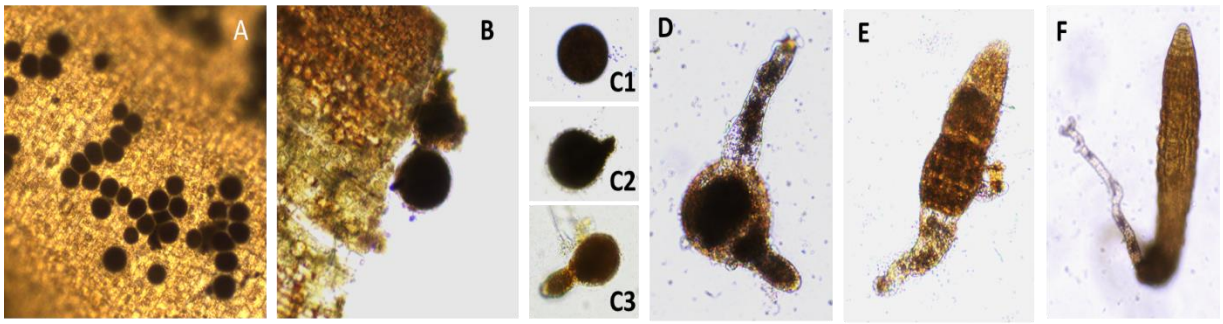

Figure 2: Fertile spore observed under microscope in the thalli surface of brown seaweed *Rugulopteryx* sp. (A), spore release, cell division and their growth to a juvenile plantlet in 2 weeks' time (B-F).

The isolation of protoplasts in red seaweed, specifically *Pterocladia* sp., was attempted both with and without pre-chelation treatment. Unfortunately, these attempts were unsuccessful, despite adding agarase and pectinase at 50 Units/mL concentration into the enzyme mixture used for green seaweeds. However, we successfully achieved plantlet regeneration through vegetative propagation under low light intensity laboratory conditions (30 µmol photons m^{-2} s^{-1}). This was accomplished by culturing small cuttings of *Pterocladia* thalli in sterile seawater supplemented with MP1 media (Figure 3A).

Over 2-3 months, it was observed that some of the *Pterocladia* sp. plantlets were infected with endophytic filamentous red algae known as *Colaconema daviesii* (Figure 3B) We propose culturing these plantlets under high light (100 µmol photons m^{-2} s^{-1}) and/or high salinity conditions to address this issue. This approach could minimize such infections without compromising the growth of *Pterocladia* plantlets.

Although *Colaconema daviesii* has been previously identified as an endophytic contaminant in many red seaweeds (Lam et al., 2016), it also presents an opportunity as a valuable source for extracting the Phycoerythrin (PE) pigment protein. It has been demonstrated that PE from *Colaconema* sp. differentially stimulates the immune response of whiteleg shrimp in vitro and in vivo, making it a potential immunomodulator in shrimp culture (Lee et al., 2021).

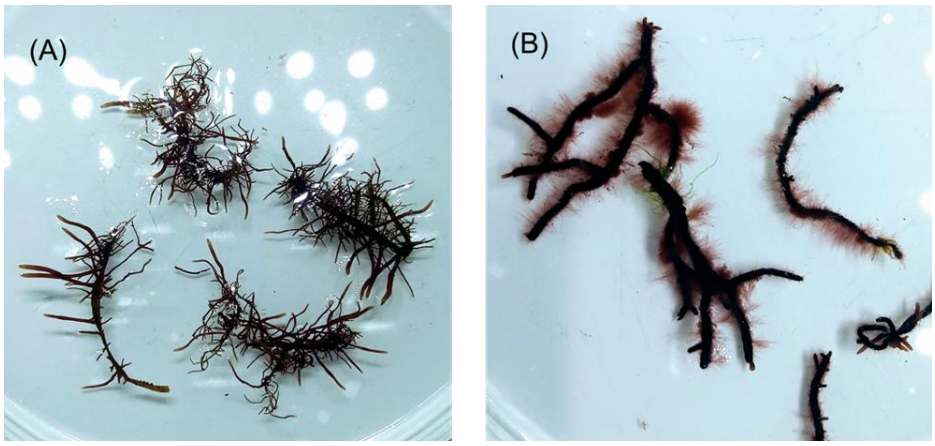

Figure 3: Plantlet regeneration of red seaweed *Pterocladia* sp. under laboratory conditions (A); growth of endophytic filamentous red algae *Colaconema daviesii* on plantlets of *Pterocladia* sp.

In green seaweeds, pre-treatment was deemed unnecessary. Simply chopping the tissue and incubating it in an enzymatic solution for 3 hr at 26°C and a speed of 55 rpm resulted in a high yield of viable protoplasts across all five *Ulva* species tested in this project (Table 2) with a maximum protoplast yield in *U. linza* (1.13 x 10^7/gram fresh weight). Additionally, adding maceroenzyme was not essential for isolating protoplasts from tubular *Ulva* species, including *U. intestinalis* and *U. compressa*.

Vacuum infiltration mediated protoplast isolation: To streamline the protoplast isolation process, reduce time consumption, and make it more cost-effective, cell wall disruption using enzymes under vacuum infiltration treatment was investigated. To achieve this, chopped explants digestion was experimented using a desiccator unit coupled with a vacuum line at various pressure levels (15-100 mbar) for different time periods ranging from 2 to 20 minutes. Vacuum-infiltration mediated protoplast isolation has been successfully tested in higher plants ensuring the proper infiltration of enzyme solution into the intercellular spaces to act on the cellulose, hemicelluloses and other cell wall components (Nanjareddy et al., 2016).

Our study subjected the chopped tissue to a specific protoplast isolation protocol. First, the tissue was allowed to incubate in an enzyme solution containing 2% cellulase and 0.5% macerozyme (if required) under vacuum conditions at a low pressure of 25 mbar. This vacuum treatment was performed in two cycles of 5 minutes each, with a 2-minute interval brake between them.

Next, the tissue was transferred to an enzymatic solution and placed on a temperature-controlled rotatory shaker. The incubation was carried out for 40-50 minutes at a temperature of 26°C and a speed of 55 rpm. This process resulted in a significant yield of viable protoplasts, ranging from 1.6 to 3.3 x 10^{6-8}/gram of fresh tissue in different Ulva species (Table 2 and Figure 4 A and B). Notably, *U. linza* exhibited the highest protoplast yield, reaching an impressive 1.62 x 10^8 per gram of fresh tissue, which is 14-fold higher than the yield obtained without vacuum infiltration. In contrast, previous reports on *Ulva* species have shown relatively lower protoplast yields, ranging from 1.6 x 10^{5-7} per gram of fresh tissue (Gupta et al., 2018).

However, it is worth noting that vacuum infiltration did not significantly affect the protoplast yield in *U. intestinalis* and *U. australis* (Table 2). On the other hand, when vacuum infiltration was applied at high pressure and for longer durations, a considerable number (>50%) of disrupted protoplasts were observed (Figure 5). Vacuum-infiltration has been demonstrated to negatively impact the yield and shape of isolated protoplast in White Lupin cotyledon seedlings (Sinha et al., 2003).

Table 2: Protoplast isolation yield in different *Ulva* sp. with and without vacuum infiltration treatment.

Seaweed	**Protoplast yield (Number of protoplast/gm fresh weight)**	
	With vacuum infiltration	Without vacuum infiltration
Ulva linza	1.62×10^8	1.13×10^7
Ulva intestinalis	3.12×10^6	2.14×10^6
Ulva compressa	1.74×10^6	1.89×10^5
Ulva fasciata	3.36×10^7	2.79×10^6
Ulva australis	2.71×10^6	1.54×10^6

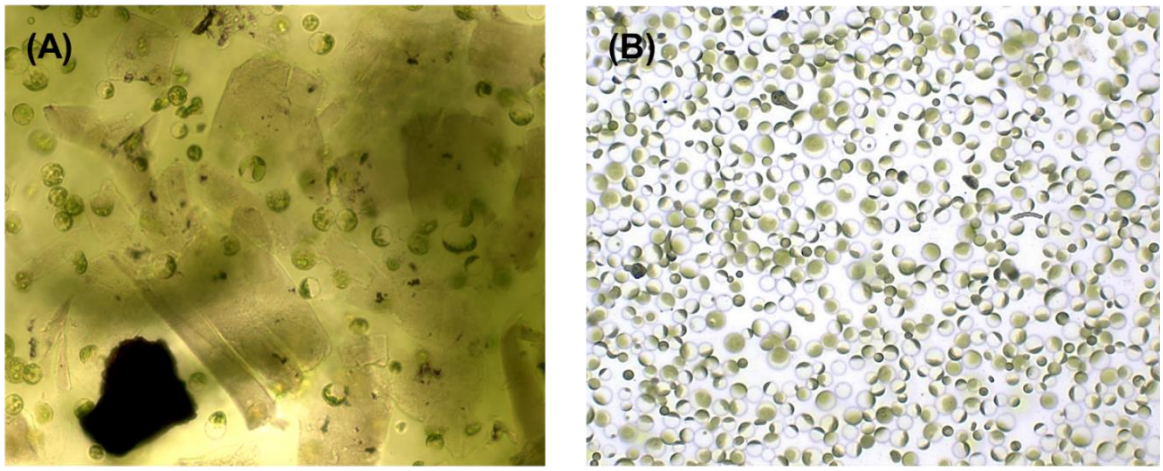

Figure 4: Protoplast isolation in green seaweed Ulva sp. Protoplast start to release from tissue within 50 minutes [under vacuum infiltration (A)]; isolated protoplasts with high quality and yield (B).

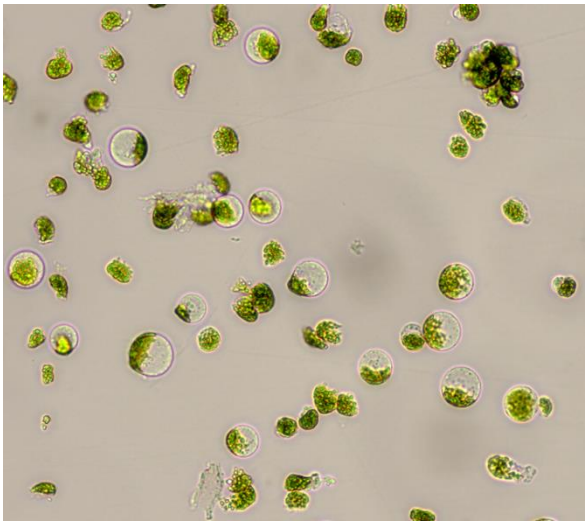

Figure 5: Broken protoplast under high vacuum (50-100 mbar) and prolonged (>10min) vacuum infiltration conditions in *Ulva* sp.

Protoplast viability and purity assessment: Using calcofluor white M2R fluorescence staining, which selectively stains cellulose in the cell wall (Figure 6), the viability of the isolated protoplasts was assessed. Epifluorescent images of the stained protoplasts were observed under a fluorescence microscope equipped with a UV fluorescence filter set (excitation filter: 350 nm; barrier filter: 430 nm). The absence of fluorescence in freshly isolated protoplasts confirmed the complete cell wall removal and, thus the lack of cellulose.

Notably, the vacuum infiltration-mediated optimized protocol proved highly successful and efficient, yielding viable protoplasts within just one hour. This significantly improved compared to the 3-6 hours required for protoplast isolation procedures reported in previous studies in various seaweeds (Reddy et al., 2008; Gupta et al., 2011 and 2018; Avila-Peltroche et al., 2021 and 2022).

Given the remarkable success of vacuum infiltration for protoplast isolation in green seaweeds, our next step will involve optimizing the CSCP protocols using gametophytic germlings generated from sporophytes. Subsequently, we will extend our investigation to explore the application of vacuum infiltration-mediated protoplast isolation techniques in red and brown seaweeds.

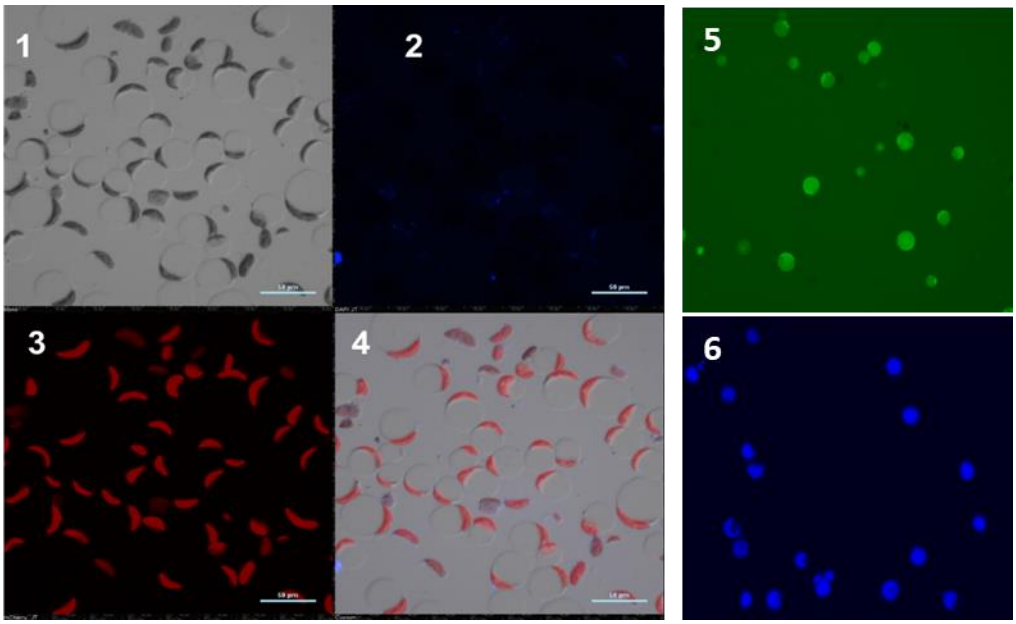

Figure 6: Freshly isolated protoplasts of *Ulva* sp. after calcofluor white M2R staining. 1) under bright field; 2) calcofluor stained protoplasts (indicates complete cell removal and intact protoplasts; 3) chlorophyll auto-fluorescence; 4) overlay image of intact protoplasts, 5-6) protoplast of *U.linza* and *U. intestinalis* stained with CellTracker fluorescent green and blue stain.

Recycling of cell wall digestion enzyme mixture to develop a cost-effective protoplast tool: To assess enzyme re-usability, the protoplast enzyme solution was first centrifuged for 10 minutes at 1000 rpm, and the resulting supernatant was then utilized as an enzyme mix for the next isolation step, involving both field-collected plants and laboratory-generated plants from protoplasts of *U. linza*. After each reuse of the enzyme mix, protoplast yields, viability, and cell wall removal were determined, and the enzyme mix was reused until a significant decrease in protoplast yield was observed. The enzyme mix was stored at −60 °C during the intervals between each reuse. Remarkably, we achieved successful recycling of the enzyme solution for a minimum of either 3 cycles (for field-collected samples) or 5 cycles (for protoplast regenerated young plants) without compromising the high protoplast yield, resulting in a cost-effective tool for industrial applications (Table 3).

Contrary to our research findings, the protoplast isolation method employed in the study of *Ulva lactuca* exhibited a 10-fold reduction in yield after the second cycle of enzymatic mixture reuse (Gupta et al., 2018).

This discrepancy could potentially be attributed to their omission of the vacuum infiltration method, which we suggested and utilized in our present work.

On a positive note, recent research on brown seaweeds, specifically *U*. *pinnatifida*, brought promising results. They successfully demonstrated the re-usability of the enzymatic mixture up to six times by employing gametophytic juveniles generated from sporophytic plants for protoplast isolation (Avila-Peltroche et al., 2022). This approach seems to have contributed significantly to the increased re-usability and subsequent yield improvement.

Considering the potential economic advantages, the reusability of enzymes in protoplast technology can enhance the economic sustainability of the seaweed aquaculture industry, as highlighted by Gupta et al. (2018). These findings underscore the importance of optimizing enzyme reusability for the progress and viability of seaweed aquaculture.

Table 3: Effects of the recycling of enzyme mix on protoplast yield*.

Enzyme Reusability Cycle	Protoplast Yield (10^8/ gram of fresh weight)	
	Field collected Plants	Protoplast regenerated plants (Stage F, Figure 10)
1	1.41± 0.41	1.88± 0.34
2	1.27± 0.19	1.39± 0.21
3	1.02± 0.32	1.62± 0.13
4	0.43± 0.08 (x10^6)	1.41± 0.24
5	0.21± 0.03 (x10^6)	1.11± 0.09
6	0.07± 0.00 (x10^4)	0.31± 0.06 (x10^5)

*Viability of protoplast was >96% in each recycle of enzyme mix in both field collected and protoplast regenerated plants.

Protoplast regeneration and seed stock biomass generation for hatchery development: The growth and development of seaweed protoplasts and subsequent regeneration were examined in relation to phytohormones known as polyamines (PAs). Polyamines are low molecular weight aliphatic nitrogenous compounds involved in various plant physiological processes like cell division, dormancy breaking, plant morphogenesis, and response to environmental stresses (Kumar et al., 2015). Polyamines namely – Putrescine (Put), Spermine (Spm), and Spermidine (Spd) (Figure 7) at a concentration of 50 µM were investigated for their potential role in promoting these cell division, branching and growth.

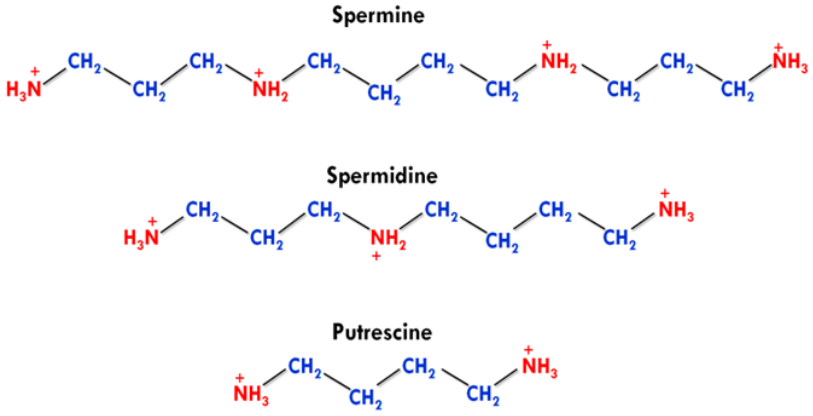

Figure 7: Chemical structure of polyamines.

Isolated and purified protoplasts from *U. linza* were cultured in sterile seawater supplemented with MP1 media, with and without PAs. All explants were obtained from a single photobioreactor (PBR) for protoplast isolation, allowing for at least four experiments (control, Put, Spm, and Spd) to be conducted with a single protoplast isolation. Three extractions were carried out to ensure sufficient biological replicates, providing a minimum of three replicates with an equal number of protoplasts. Each experiment involved culturing protoplasts in 60 mm diameter petri dishes. The PAs concentration was determined based on previous experience (Kumar et al., 2012) and preliminary experiments, ensuring protoplast survivability of >90%. Initially, the protoplasts were incubated in darkness for two days and then gradually shifted to increased light conditions, as indicated in Table 4. Additionally, all protoplast cultures in each experiment were supplemented with germanium dioxide at a concentration of 5 mg/L to inhibit diatom growth.

Table 4: Effect of polyamines phytohormones on the protoplast growth and development in *U. linza*.

Developmental stages	Days (After protoplast isolation)	Control	Putrescine	Spermine	Spermidine	Light conditions (µmol photons m^{-2} s^{-1})
		Protoplasts in different stages (%)				
Stage 1	0	100	100	100	100	Dark
Stage 2	3	36	48	78	63	20
Stage 3	5	40	53	70	58	
Stage 4	7	48	66	75	60	
Stage 5	11	52	58	83	79	50
Stage 6	13	56	64	88	82	
Stage 7	15	50	58	85	75	
Stage 8	17	49	61	89	81	
Stage 9	19	54	60	90	83	80
Stage 10	21	58	66	92	88	

In this study, a total of 100 protoplasts were carefully screened daily across each corner and the middle section of the petri dish to closely monitor their development from stage 1 to stage 10 over a span of 3 weeks (Figure 8). Protoplast cultures supplemented with PAs exhibited a higher percentage of cell division and growth, particularly with Spm and Spd supplementation, when compared to Put and the control plates.

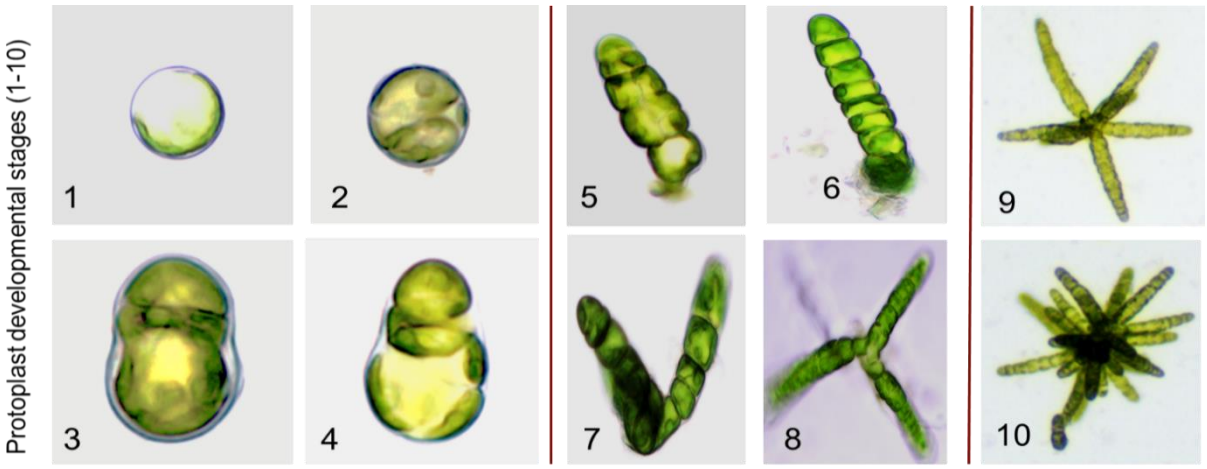

Figure 8: Differential stages of protoplast's growth and development into branched thalli in *U. linza*

By the end of the 21-day observation period (Table 4), more than 90% of the regenerated plantlets under the Spm and Spd treatments reached stage 10, displaying multiple and expanded branches. In contrast, only 60% of the plantlets in the control and Put treatments developed to stage 10. Continuing the experiment, these stage 10 plantlets were continuously cultured for an additional 2 weeks in a PAs-supplemented seawater medium. The results showed a significantly higher growth rate, especially in the Spm (18.3% DGR) and Spd (16.4% DGR) treatments. Plantlets subjected to these treatments developed multiple branches, ranging from 15 to 22, while the control plantlets exhibited 7 to 12 branches with an 8.6% DGR (Figure 9).

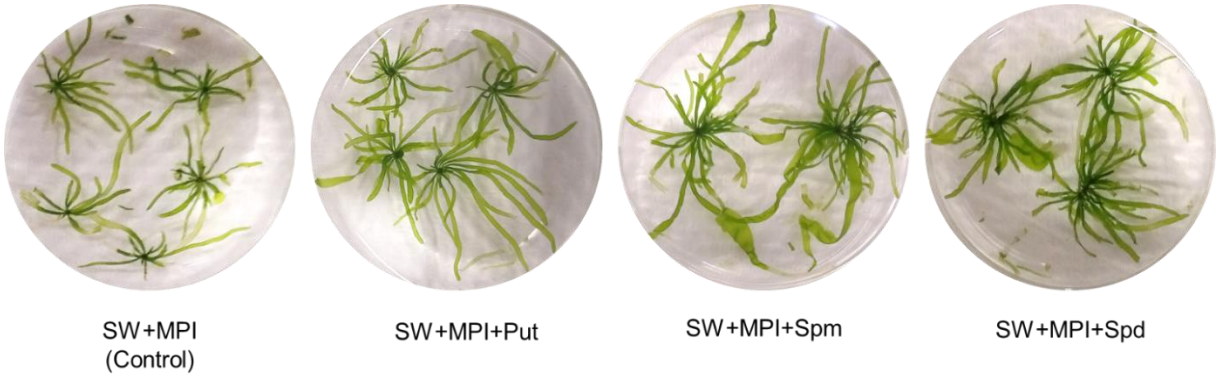

Figure 9: Effect of PAs on branching and daily growth rate of protoplast regenerated plantlets in *U.linza*

PAs have been proposed to regulate plant growth and stress responses by enhancing antioxidant activity and controlling oxidative stresses (Kumar et al., 2015). Our findings align with previous research, as Spm showed the highest plating efficiency on sugar beet cell suspension-derived protoplasts compared to Put and Spd, attributed to its stronger inhibitory effect on ethylene production (Majewska-Sawka et al., 1997). Additionally, in cabbage hypocotyl protoplast culture, exogenous application of PAs, specifically Put, resulted in the highest frequency of shoot organogenesis (Kiełkowska and Adamus, 2021), while no effects were observed in *Nigella damascena* callus protoplasts (Klimek-Chodacka et al., 2020). Considering these findings and our own, further investigations are warranted at both the biochemical and molecular levels to comprehensively unravel the underlying mechanism behind enhanced cell division and branching of protoplasts when supplemented with exogenous PAs in seaweeds.

2. Demonstrate the commercial practicality of micropropagation workflow – from seedstocks to seeding to grow-out in ponds:

After the successful development of the protoplast isolation tool and plantlet generation in Petri dishes, their subsequent culturing with and without seeding on substrate twines for land and open sea cultivation purposes was assessed.

Protoplast seedstocks for biomass generation without seeding onto the substrate: Protoplast cultures (Figure 10A) reaching stage 10 (Figure 10B) were carefully scraped off from Petri dishes using single-edge razor blades and transferred into 2L flat bottom round flasks (Figure 10C). They were maintained under low light (80 µmol photons m^{-2} s^{-1} of white LED light, 12:12 dark and light cycle) with aeration for 4 days or until visible as star-shaped plantlets (Figure 10D). Subsequently, these plantlets were cultured under moderate to high light conditions for several weeks (Figure 10E-F), with regular seawater changes supplemented with MPI media (half strength).

Later, the plantlets were transferred to 10L cylindrical PBRs (tumbling aeration, under 150 µmol photons m^{-2} s^{-1} of white LED light, 12:12 dark and light cycle) for further growth and biomass generation (Figure 10G). During these culture periods the medium was changed once every week.

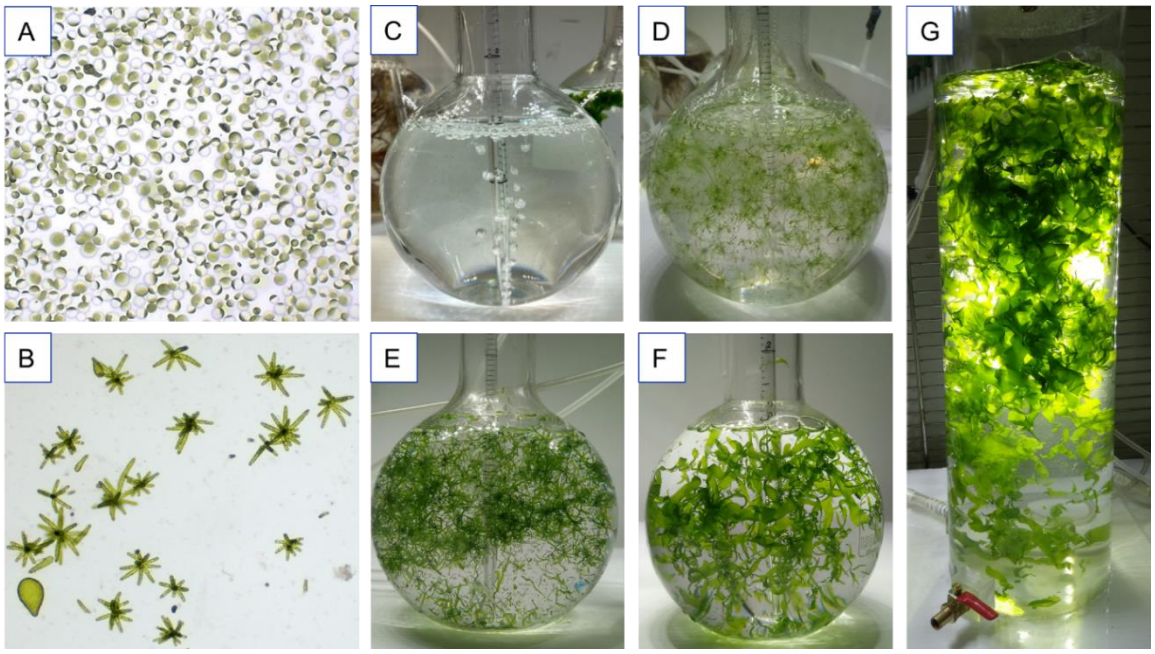

Figure 10: Protoplast based micropropagation workflow for a hatchery establishment of *Ulva* species.

Utilizing this workflow, an impressive biomass of 0.47 kg was obtained from just 100 mg of explant (starting material) within 8 weeks under laboratory conditions (Figure 10). However, a critical factor that can potentially extend the biomass production time from 8 weeks to over 12 weeks is very high if the density of cultures (after stage E) is used in the flasks or PBRs. To achieve a high daily growth rate (>20% DGR in a 7-day culture period), it is recommended to culture 500 mg of biomass in 1 L of enriched seawater medium. Optimizing the stocking density becomes vital for obtaining the best growth outcomes. The observed decline in DGR (%) could be attributed to the higher stocking density, which limits the space and nutrient availability required for sustainable growth.

Our results align with the findings of Gupta et al. (2018), who also achieved high DGR by maintaining a stocking density of 250 mg/L in enriched seawater media, resulting in a 28% DGR in a 10-day culture period. Conversely, Bruhn et al. (2011) reported the highest DGR of 19%, highlighting a negative correlation with increasing stocking density. Furthermore, the DGR of *Ulva* was significantly influenced by the frequency and dosage of nitrogen supplementation achieving a DGR of 12-15% with an initial stocking density of 5 g/L in PBR bags (Zollmann et al., 2023). Additionally, cultivating *Ulva* in eutrophicated waters rich in nitrogen content demonstrated a remarkable DGR of 35% (Nielsen et al., 2012).

One of the key advantages of this protoplast tool is its simplicity and scalability. It enables the production of many high-quality seedlings without relying on fertile female plants for spore-based seedstock production. Additionally, it eliminates the need for protoplast seeding on twines, mainly when used in land-based cultivation systems. As a result, this micropropagation workflow is highly efficient, streamlining the process and ensuring a consistent production of substantial biomass. This makes it a valuable and indispensable tool for seaweed cultivation and propagation.

The protoplast isolation techniques were initially tested in the hatchery at the DPI-Port Stephens Fisheries Institute and have since been successfully replicated on multiple occasions by hatchery technicians. This remarkable success highlights the reliability and consistency of the protoplast tool, further solidifying its practicality and effectiveness.

Protoplast seeding on to substrate for biomass generation:

A crucial step in achieving global industrialization in the seaweed industry is adopting a standardized seedling production method. This involves developing standard seeding procedures that produce quality seedlings with high plant regeneration efficiency, ensuring a sustainable, competitive, and reliable macroalgae cultivation sector. As the industry advances, focusing on standardization will undoubtedly unleash its full global potential.

Commercial seaweed farming is primarily dominated by twelve species worldwide (FAO, 2018). For instance, Kelp farming involves spore seeding on strings in a controlled hatchery environment. Several critical parameters must be carefully managed at hatchery to achieve high plant regeneration efficiency when these strings are transferred to the open ocean. These include seawater velocity, growth conditions (such as light, temperature, salinity, and nutrient media), the use of binder (glue), and twine selection (synthetic vs natural polymer twines) to enhance attachment (Kerrison et al., 2019; Visch et al., 2020). Proper attention to these factors is essential for successful out-planting and cultivation in the open ocean. Pérez Massad et al. (2020) introduced a novel spore re-suspending technology (SRT) for red seaweeds. It improves spore seeding on vertically placed frames by resuspending spores from the bottom of tanks.

When spore seeding tools are developing in the seaweed industry, the set of conditions optimized for sporulation is highly species-specific, varies with the age and physiological background of the plant, and often isn't reproducible. Moreover, these tools are completely dependent on fertile female plant availability. When protoplast technology can address most of these issues, their regeneration efficiencies and seeding tools are underdeveloped. To date nearly 100 species belonging to green, red, and brown seaweeds from which successful protoplast isolation and regeneration have been reported (Reddy et al., 2008). Many of these studies have aimed at developing fundamental techniques for protoplast isolation and possible regeneration, and only a few species have shown success in their seeding to laboratory scale. Recently, Gupta et al (2018) demonstrated the seeding of *Ulva* protoplasts on glass fibre plates (53 × 28 cm) at a laboratory scale, however, they scraped off the regenerated seedlings from plates for biomass production in 2-5L flasks.

We have successfully created a straightforward seeding tool that eliminates the need for binders while ensuring efficient attachment. To achieve this, we conducted experiments using two protoplast seeding methods: direct seeding and indirect seeding. We tested various twine materials in both trials, including natural and synthetic polymers such as nylon, polypropylene, and natural jute.

In the direct seeding method, freshly isolated and filtered protoplasts were directly inoculated onto heat and UV-sterilized twines. These twines were wrapped around an acrylic fiber frame (measuring 8x8 cm) and then placed in a mini aquarium filled with enriched sterile seawater (as shown in Figure 11).

Among the tested seeding substrates in a direct seeding method, only natural jute twine showed some attachment of protoplasts, but development ceased before the induction of cell division and subsequent germination within 48 hours of protoplast inoculation as most of the protoplasts simply burst (Figure 12).

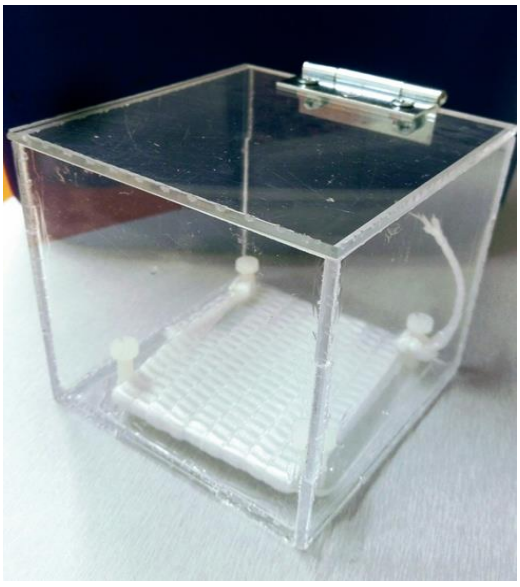

Figure 11: Prototype of protoplast seeding to twines frame placed in a mini-aquarium.

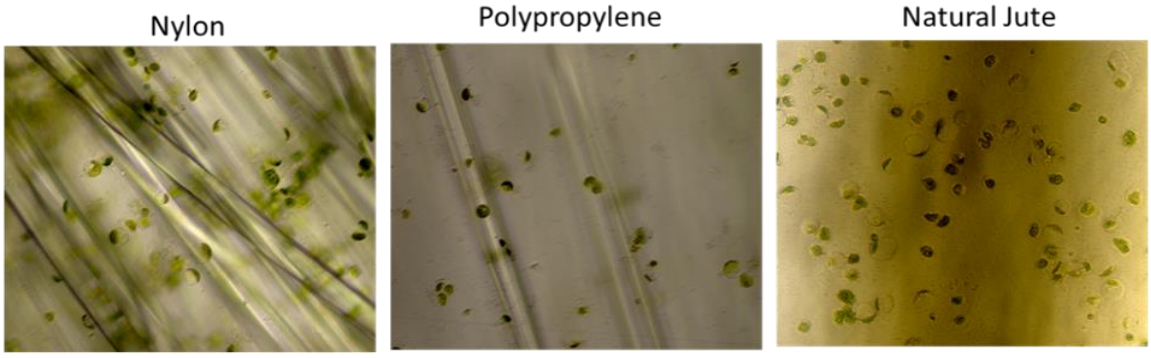

Figure 12: Protoplast seeding on substrate twines (nylon, polypropylene, and natural jute)

In the indirect seeding method, protoplasts cultured in Petri dishes until stage 10 were used for seeding. These seedlings developed natural sticky substances in their holdfast, firmly attaching them to the petri dish surface. The plantlets were gently scraped off and spread onto sterilized frames wrapped with twines in mini aquariums (Figure 13A). The aquarium boxes were slowly filled with enriched seawater media without disturbing the plantlets settlements and left it without aeration for 24 hours. Subsequently, the seedlings were cultured under mild aeration for two weeks with weekly media replacement. Plantlet growth was monitored and photographed under a microscope (Figure 13B and C). Later, the twines were transferred to 50 L tanks for further growth and development under laboratory conditions (Figure 13D). In the indirect seeding method, all kinds of twine (nylon, polypropylene, and natural jute) showed similar results of high seedling attachment and regeneration potential.

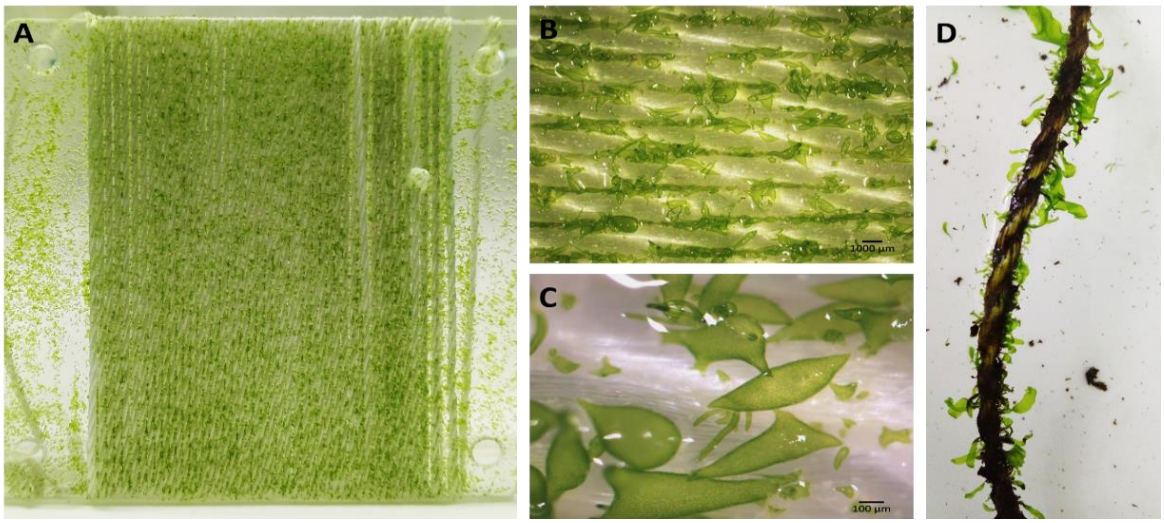

Figure 13: Protoplast seeding to substrate twine wrapped on an acrylic plate (A); successful attachment (B and C); and germination into full plants (D)

The natural sticky substances in each plantlet's holdfast acted as a binder, firmly attaching them to the twine for subsequent growth in the tanks. The liquid mucilaginous substance secreted from spores in brown seaweed species has been reported to be composed of protein and anionic polysaccharides - alginate, fucan polysaccharides, and polyphenols (Petrone et al., 2011; Kerrison et al., 2009 and 2019). Energy dispersive X-ray microanalysis of these bio-adhesives has identified sulfur and phosphorus, as well as calcium and magnesium ions, as key contributors to facilitate the gelation of the anionic polysaccharides in seawater (Petrone et al., 2011). The AtSeaNova BV has designed AlgaeRope and AlgaeRibbon to provide a large, textured surface with suitable chemistry to enhance the attachment of sporophytes during binder-seeding (Kerrison et al., 2020).

However, a complete understanding of the bio-adhesives secreted from the holdfast of most seaweed species including *Ulva* sp. is lacking, warranting further research at both the biochemical and molecular levels to identify the chemical structure of adhesives and their biosynthesis pathways from protoplast to plantlet formation.

Development of Vertical Frames Airlift Suspension Technology (AST) for protoplast seeding: A prototype of AST technology for protoplast seeding was developed at DPI- NSW to maximise protoplast settlement on a double-sided vertical frame made from tightly twined ropes (2-3 mm diameter) of different materials. AST prototype was developed using a 50 L capacity aquarium that can hold 5-10 vertical substrate frames for protoplast seeding and subsequent germination under hatchery conditions. This system was designed to facilitate the suspension and mixing of protoplasts into the system to encourage their settlement on the vertical frames. To assess the performance of this prototype (Figure 14), the protoplast settlement on different substrate twines including nylon is underway. The AST technology is expected to eliminate the need for excessive handling and enable the automation of the seeding process, thereby optimising the use of inoculated protoplasts, and increasing the overall efficiency and productivity of the system.

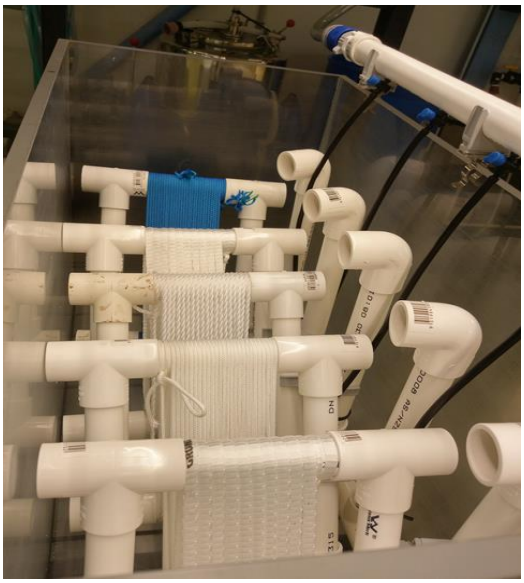

Figure 14: Airlift Suspension Technology (AST) prototype developed at DPI-NSW for protoplast seeding and germination.

OUTPUTS AND OUTCOMES

- Protoplast isolation was successfully achieved in five *Ulva sp*. with high protoplast yields.
- Protoplast culture and regeneration conditions were optimized under laboratory conditions.
- Phytohormone supplementation (PAs) in culture media is suggested to be beneficial for promoting the growth and development of protoplasts.
- Protoplast-based micropropagation workflow was successfully demonstrated for biomass generation using *Ulva sp*. suitable for both land and open sea cultivation settings.
- A simple method of protoplast seeding onto substrate twines with guaranteed attachment and germination was demonstrated.
- A prototype of AST with vertical substrate frames was developed to maximise protoplast seeding under hatchery conditions.

Conclusion

The FAO (2018) report highlights that none of the twelve dominant seaweed species produced worldwide are native to Australia. Therefore, Australian seaweed growers have an opportunity to explore their diverse native species. However, the challenge of developing or adapting culture methods hampers rapid industry growth.

To address the limitations of traditional farming tools, such as loss of vigor, susceptibility to stresses, and reliance on fertile female plants, the University of Technology Sydney (UTS) and the Department of Primary Industries (DPI, NSW) have developed a cost effective, rapid, and a high growth performing seaweed farming tool with efficient seeding using protoplast based micropropagation. This innovative tool features – 1) vacuum infiltration to reduce protoplast isolation time, 2) cost-effective reuse of enzyme mixture for up to 5 cycles, 3) the use of polyamines phytohormones to promote protoplast growth, and 4) the use of natural binders secreted from germinated protoplasts for successful seeding onto substrates. This tool is suitable for generating large-scale biomass in land- and sea-based farming settings. By establishing such protoplast-based seaweed farming tools in other red and brown seaweeds, the industry could benefit from improved productivity, enhanced genetic traits, disease management, and scalability. These advancements foster a more resilient, sustainable, and economically viable seaweed industry, resulting in higher-quality seaweed products and capitalizing on the current commercial interest in seaweed production.

Implications

Developments in seaweed protoplast technology can offer a range of possibilities for crop improvement, genetic engineering, disease resistance, increased crop yield, hybrid seed production, and conservation of genetic resources in the seaweed aquaculture industry. It has the potential to accelerate seaweed farming progress and address various challenges the global food production system faces. Protoplast technology has implications for commercialization and various stakeholders in the seaweed aquaculture sector. It provides plant breeders, seed companies, farmers, and biotechnology companies opportunities to develop and market improved plant varieties. Consumers can benefit from access to enhanced quality products, while regulatory authorities and environmental organizations play vital roles in overseeing safety, regulation, and sustainability in applying protoplast technology.

Recommendations and Further Developments

1. The future of seaweed protoplast regeneration holds great promise in various applications, including gene-editing, hybrid development, and crop trait improvement. We anticipate witnessing more successful examples of its application in the future.

2. Despite its potential, protoplast regeneration faces challenges that need to be addressed. Specialized tissue culture expertise, complex enzyme manipulation, time consumption, and expensive commercial enzymes have affected its success. Our innovative technology will help overcome some of these limitations; however, efforts are needed to make protoplast regeneration methods more universal for wider applicability.

3. Successful seeding relies on attaching viable protoplasts or protoplast-derived seedlings to substrates. Understanding the molecular structure of natural bio-adhesives secreted from the holdfast of seaweed (spore, gametophytes, protoplasts) can aid in developing artificial adhesives to enhance seeding and regeneration success. A system biology approach, exploring adhesion-related genes, proteins, and metabolites expressed during protoplast to plantlet regeneration, can provide valuable insights.

To validate these advancements, adhesion mutants, gene knockouts, and transformants should be created and utilized, such as *Ulva mutabilis* mutants that fail to establish holdfast in the absence of specific bacterial communities on their surface can be used for such studies in a collaborative interdisciplinary research environment.

Extension and Adoption

A demonstration of protoplast isolation and Airlift Suspension Technology (AST) prototype was organised at DPI-Port Stephens Fisheries Institute and showcased to FRDC Board members on 20th April 2022.

Staff of DPI-Port Stephens have provided the NSW Aquaculture Research advisory committee with regular updates on project progress and will reach out to NSW seaweed lease permit holders with the details of this technology.

Project coverage

Findings of this project were presented and discussed at national and international conferences –

1. Kumar Manoj *, Ralph P, O'Connor W, Dove M (2023) Revolutionizing Seaweed Farming: Protoplast-Based Tools for Commercial Success. Aquafarm Conference, International Conference on Aquaculture, Melbourne (10-12 July 2023).

2. Kumar Manoj *, Ralph P, O'Connor W, Dove M (2023) Protoplast Technology: Redefining the Future of Seaweed Aquaculture. Marine BioConnect 23, MBCRC, Adelaide (30-31 August 2023).

Project materials developed

Two manuscripts from this project are in preparation.

1. Manoj Kumar, Peter Ralph, Wayne O'Connor et al. (2023) An improved and cost-effective micropropagation tool for year-round seed stock supply of seaweeds (*Ulva* sp.). Frontiers in Plant Science (In preparation)

2. Manoj Kumar, Peter Ralph, Wayne O'Connor et al. (2023) Polyamines promote the growth, and morphogenesis of *Ulva* protoplasts. Plants (In preparation)

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