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**Research Article**

**Keywords:** Gracilaria salicornia, invitro propagation, biomass multiplication, modified PES medium, vegetative regeneration, seaweed aquaculture

**Posted Date:** November 6th, 2026

**DOI:** https://doi.org/10.21203/rs.3.rs-11336101/v1

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**Additional Declarations:** No competing interests reported.

# In vitro clonal propagation of *Gracilaria salicornia* for rapid biomass multiplication and sustainable seedstock production

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## ABSTRACT

*Gracilaria salicornia* is an economically important agar-producing red seaweed with potential for biomass production and value-added applications. The availability of adequate and consistent planting material remains an important consideration for the expansion of its cultivation. This study evaluated a liquid modified Provasoli's Enriched Seawater (PES)-based in vitro culture system for vegetative biomass multiplication of *G. salicornia*. Healthy apical thallus segments were surface sterilized and cultured under controlled conditions for six weeks. Biomass development was monitored at weekly intervals using relative biomass multiplication, percentage increase in biomass, recorded weekly growth-rate values and qualitative morphological observations. Relative biomass multiplication increased progressively from 1.40-fold after the first week to 5.00-fold after six weeks, corresponding to a 400% increase over the initial biomass. The recorded weekly growth-rate values ranged from 20.3 to 27.4%. Biomass accumulation was accompanied by thallus elongation, new shoot development and lateral branch formation, indicating sustained vegetative growth under the tested culture conditions. These findings demonstrate the potential of liquid modified PES medium for the controlled multiplication of *G. salicornia* from limited starting material and establish a quantitative baseline for further optimization of the propagation protocol. Future studies should evaluate the effects of culture conditions and plant growth regulator concentrations, as well as genetic fidelity, acclimatization and field performance, to determine the suitability of this approach for scalable clonal planting-material production. The study provides a basis for further development of controlled propagation methods to support *G. salicornia* cultivation.

**Keywords:** *Gracilaria salicornia, invitro* propagation, biomass multiplication, modified PES medium, vegetative regeneration, seaweed aquaculture.

## INTRODUCTION

Seaweed cultivation has become an important component of sustainable aquatic biomass production, as seaweeds provide renewable raw materials for food, hydrocolloids, nutraceuticals, pharmaceuticals, agriculture, and various industrial applications (Buschmann et al. 2017; McHugh 2003). Among the commercially important red seaweeds, members of the genus *Gracilaria* are particularly valuable because of their capacity to produce agar and other galactan-rich polysaccharides. The genus is also important in aquaculture because several species can be propagated vegetatively from fragments and cultivated in different production systems, enabling biomass production under suitable environmental conditions (Buschmann et al. 2017).

Consequently, the development of reliable propagation systems capable of producing adequate quantities of healthy and uniform planting material is an important requirement for the expansion and sustainability of *Gracilaria* aquaculture. The commercial importance of *Gracilaria* is closely associated with its use as a source of agar and other seaweed-derived products. Agar-producing species of *Gracilaria* are important sources of commercially valuable hydrocolloids, and their cultivation offers opportunities for sustainable biomass production and reduced dependence on naturally occurring populations (McHugh 2003; Mantri et al. 2022).

*Gracilaria* cultivation has consequently been developed in several countries, with vegetative propagation remaining an important method for the production of planting material. The availability of healthy and productive propagules is particularly important for cultivation systems in which substantial quantities of biomass are required as seedstock for subsequent production cycles. Thus, improving the efficiency of seedstock multiplication is directly relevant to the productivity and sustainability of commercial *Gracilaria* cultivation, as reliable production of planting material supports large-scale farming and reduces dependence on naturally occurring populations (Yeong et al. 2014; Bhushan et al. 2023).

*Gracilaria salicornia* is a commercially relevant tropical red alga that has attracted attention as a source of agar and biomass for industrial applications. In India, the species has been exploited from natural populations, particularly along the southeastern coast. Historical assessments of Indian seaweed resources documented substantial harvesting of *Gracilaria salicornia* from Palk Bay. The depletion of wild stocks associated with overexploitation subsequently encouraged the development of cultivation approaches, including tube-net culture, to support a more sustainable supply of agar-producing biomass (Ganesan et al. 2019).

The species has also been reported as a source of food-grade agar, with commercially useful gel characteristics (Ganesan et al. 2018). More recently, cultivation trials have demonstrated the potential of *Gracilaria salicornia* for biomass production under brackishwater conditions using raft-based culture systems. In a 45-day cultivation trial, biomass increased from an initial stocking weight of 1.2 kg to an average of 9.09 kg per tube net, highlighting the potential of raft-based cultivation for commercial seaweed production in brackishwater environments (Raja et al. 2025). These developments indicate the potential of *G. salicornia* as a cultivated biomass

resource; however, the availability of sufficient and consistent seedstock remains an important consideration for the expansion of cultivation.

Conventional cultivation of *Gracilaria* largely depends on vegetative propagation using thallus fragments. Although this approach is relatively simple and practical, it requires continuous availability of healthy donor biomass and may be constrained by seasonal variation, environmental stress, epiphyte loads and the quantity of material that can be diverted from production as seedstock. The availability of adequate and continuous planting material is an important prerequisite for the commercial cultivation of *Gracilaria* species. Tissue culture and micropropagation offer alternative approaches for producing clonal planting materials when natural populations provide insufficient biomass for large-scale cultivation (Yeong et al. 2006, 2014).

In this context, controlled propagation methods capable of rapidly multiplying selected and healthy explants could provide an alternative means of increasing the availability of planting material and reducing dependence on large quantities of donor biomass.

In vitro tissue culture provides an alternative approach for the controlled multiplication of economically important seaweeds. Tissue-culture techniques enable selected explants to be maintained and regenerated under defined nutritional and environmental conditions and have been investigated in several commercially important red algae. Such approaches can facilitate clonal propagation from relatively small quantities of starting material and may be useful for maintaining selected strains for subsequent cultivation (Baweja et al. 2009). Earlier studies on *Gracilaria* have demonstrated the influence of culture media, irradiance, temperature, salinity and plant growth regulators (PGRs) on growth, callus formation and regeneration (Yokoya et al. 1999; Yokoya et al. 2004; Ramlov et al. 2013).

The response of *Gracilaria* explants to in vitro culture is influenced by both intrinsic and environmental factors. Explant source, physiological condition, developmental stage and tissue position may affect survival and regenerative capacity, whereas culture-medium composition, nutrient availability, salinity, temperature, irradiance, photoperiod, pH and plant growth regulator (PGR) concentrations can influence subsequent growth and morphogenesis (Baweja et al. 2009; Yokoya et al. 1999). In particular, interactions between irradiance and PGRs can influence growth, callus formation and regeneration in *Gracilaria*. Ramlov et al. (2013) demonstrated that irradiance and PGR treatments affected growth and regenerative responses in different colour morphs of *G. domingensis*. These findings highlight the need to optimize multiple interacting culture variables rather than relying on a single culture component.

Plant growth regulators (PGRs) play an important role in regulating morphogenetic responses in algal tissue culture. Auxins and cytokinins have been investigated for their effects on cell proliferation, callus development and regeneration in *Gracilaria* species. Studies have examined compounds such as indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin and 6-benzylaminopurine (BAP), with responses varying according to species, explant type, irradiance and culture conditions (Yokoya et al. 2004; Ramlov et al. 2013). Therefore, species-specific optimization of nutrient media and PGR regimes is essential for developing reproducible propagation protocols.

Previous studies have established the feasibility of tissue culture and clonal propagation in several *Gracilaria* species. PGR-mediated callus formation, growth and regeneration have been investigated in *G. tenuistipitata* and *G. perplexa*, demonstrating species- and tissue-dependent responses to auxins and cytokinins (Yokoya et al. 2004). Similarly, growth, callus formation and regeneration in *G. domingensis* have been shown to vary with irradiance, explant type and PGR treatment (Ramlov et al. 2013). Tissue- and cell-culture approaches have been investigated for *Gracilaria changii*, with light intensity, photoperiod, plant growth regulators, nutrient composition and enrichment media evaluated to optimize explant growth and micropropagation (Yeong et al. 2006, 2014). Collectively, these studies demonstrate the regenerative potential of *Gracilaria* tissues but also emphasize that propagation responses are strongly species- and culture-condition dependent. Consequently, protocols developed for one species cannot necessarily be transferred directly to another species without appropriate optimization.

Recent research has further shifted the emphasis from demonstrating regeneration towards developing systems capable of producing useful quantities of clonal propagules for cultivation. In *Gracilaria dura*, recent experiments have investigated explant priming with BAP and kinetin to enhance regeneration and adventitious lateral shoot formation. BAP treatment enhanced explant regeneration, whereas kinetin promoted adventitious lateral shoot development in *Gracilaria dura*, demonstrating the potential of targeted plant growth regulator treatments to improve propagule production (Bhagiya et al. 2025). Such findings are particularly relevant to the development of nursery and seedstock production systems because regeneration efficiency and lateral shoot formation directly influence the number of propagules that can be generated from limited starting material.

This requirement is particularly relevant to *Gracilaria salicornia*. Recent field-based research has demonstrated the cultivation potential of this species under brackishwater conditions, with substantial biomass production achieved using raft-based culture systems (Raja et al. 2025). Earlier assessments of Indian seaweed resources documented the commercial exploitation of wild *G. salicornia* stocks and the subsequent adoption of tube-net cultivation, highlighting the importance of maintaining an adequate and reliable supply of planting material for sustainable cultivation (Ganesan et al. 2019).

However, compared with several other commercially investigated *Gracilaria* species, comparatively limited information is available on a standardized *in vitro* propagation system specifically designed for rapid biomass multiplication of *G. salicornia*. In particular, there is a need to determine whether controlled liquid culture conditions can sustain continuous vegetative biomass accumulation while simultaneously promoting the formation of new shoots and lateral branches suitable for subsequent propagation.

A propagation system based on rapid biomass multiplication could provide several practical advantages, including increased production of planting material from a limited quantity of donor biomass, maintenance and multiplication of selected healthy strains under controlled conditions, and continuous production of vegetative propagules for subsequent cultivation cycles. Tissue culture has been identified as a potential approach for the clonal propagation of seaweed seed material for mariculture (Baweja et al. 2009). Therefore, the development of a

reproducible propagation protocol for *Gracilaria salicornia* could complement existing cultivation approaches and contribute to a more consistent supply of planting material.

In the present study, we developed and evaluated an *in vitro* clonal propagation system for *Gracilaria salicornia* using liquid modified Provasoli's enriched seawater (PES) medium supplemented with plant growth regulators. The study examined biomass multiplication over a six-week culture period and assessed the development of new shoots and lateral branches as indicators of vegetative regeneration. The specific objectives were to (i) establish an effective culture system for vegetative propagation of *G. salicornia*, (ii) quantify progressive biomass multiplication over six weeks of culture, and (iii) evaluate morphological development associated with biomass accumulation. The resulting protocol provides a basis for further studies on acclimatization, nursery transfer and scale-up of clonal seedstock production for *G. salicornia* cultivation.

## 2 Materials and methods

### 2.1 Collection and preparation of *Gracilaria salicornia*

Healthy, actively growing thalli of *Gracilaria salicornia* were collected from Pazhaverkadu, Tiruvallur District, Tamil Nadu, India, during November and December in 2023, 2024 and 2025. Thalli showing visible mechanical damage, bleaching, necrosis, disease symptoms or extensive epiphyte colonization were excluded from the study. The collected material was transported to the laboratory on the day of collection in clean containers containing seawater to minimize handling stress and maintain the thalli in a hydrated condition.

Upon arrival, the thalli were gently rinsed several times with seawater to remove adhering sediment, debris and loosely attached organisms. Healthy, actively growing portions were selected for subsequent explant preparation. Species identification was based on morphological characteristics and relevant taxonomic descriptions of the genus *Gracilaria* (Wynne 2011). Molecular confirmation of species identity was not performed in the present study.

### 2.2 Explant preparation

Explant preparation was performed aseptically in a laminar airflow cabinet. Healthy apical portions of *Gracilaria salicornia* were rinsed twice with sterile seawater and gently blotted using sterile tissue paper to remove excess surface moisture. The thalli were sectioned into explants approximately 1.0 ± 0.5 cm in length using a sterile No. 3 scalpel handle fitted with a sterile No. 10 surgical blade. A total of 5,000 explants were prepared for the experiment. Care was taken to minimize mechanical damage during excision, and the prepared explants were immediately subjected to surface sterilization. (Figure 1.)

### 2.3 Surface sterilization

The prepared explants were rinsed twice with sterile seawater and immersed in a 0.5–1.0% (v/v) povidone–iodine solution for 5 min. Following treatment, the explants were rinsed three times with sterile seawater to remove residual sterilant, gently blotted with sterile tissue paper and aseptically transferred to the culture medium. Sterilization efficiency was assessed by regularly inspecting the cultures for visible bacterial or fungal contamination throughout the

culture period. Contaminated explants were removed promptly to minimize the risk of cross-contamination among cultures.

### 2.4 Culture medium and plant growth regulator supplementation

The explants were cultured in liquid modified Provasoli's enriched seawater (PES) medium. The medium was prepared using seawater and supplemented with sucrose and urea at 0.2% (w/v) each. The plant growth regulator (PGR) combination comprised 6-benzylaminopurine (BAP, 3.0 mg $L^{-1}$), indole-3-acetic acid (IAA, 0.6 mg $L^{-1}$), α-naphthaleneacetic acid (NAA, 0.5 mg $L^{-1}$) and kinetin (KIN, 1.0 mg $L^{-1}$). The medium pH was adjusted to 8.0 before sterilization. The prepared medium was autoclaved at 121 °C and 15 psi, then allowed to cool to room temperature before inoculation.

The complete composition of the modified PES medium used in the experiment is provided in Table 1, including the nutrient salts, vitamins, trace elements, sucrose, urea and PGRs. The concentrations of the individual medium components were checked against the laboratory stock-solution preparation records to ensure accurate reporting of the final working concentrations.

**Table 1. Composition of the modified PES medium used for *in vitro* propagation of *G. salicornia***

| Component | Concentration/ Quantity | Specification |
|---|---|---|
| Sucrose | 0.2% | Modified PES |
| Urea | 0.2% | Modified PES |
| BAP | 3.0 mg $L^{-1}$ | Plant growth regulator |
| IAA | 0.6 mg $L^{-1}$ | Plant growth regulator |
| NAA | 0.5 mg $L^{-1}$ | Plant growth regulator |
| Kinetin | 1.0 mg $L^{-1}$ | Plant growth regulator |
| pH | 8.0 | Before sterilization |
| $NaNO_3$ | 3.50 g | PES solution I; make to 1000 mL |
| $Na_2$-glycerophosphate | 0.50 g | PES solution I |
| Thiamine-HCl | 0.004 g | PES solution I |
| Cyanocobalamin | 0.004 g | PES solution I |
| Tris buffer | 5.00 g | PES solution I |
| $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ | 0.700 g | PES solution II |
| $Na_2EDTA$ | 0.600 g | PES solution II |
| $Na_2EDTA$ | 1.0 g | PES solution III |
| $H_3BO_3$ | 1.14 g | PES solution III |
| $MnSO_4 \cdot H_2O$ | 0.130 g | PES solution III |
| $FeCl_3 \cdot 6H_2O$ | 0.049 g | PES solution III |
| $ZnSO_4 \cdot 7H_2O$ | 0.022 g | PES solution III |
| $CoSO_4 \cdot 7H_2O$ | 0.005 g | PES solution III |
| Biotin | 0.001 g | PES solution IV |

| | | |
|---|---|---|
| Vitamin B12 | 0.002 g | PES solution IV |
| Deionized water | 25 mL | PES solution IV |

### 2.5 Culture conditions

Approximately 2 L of modified Provasoli's enriched seawater (PES) medium was dispensed into each 2-L culture vessel. Each vessel was inoculated with approximately 75–100 explants of *Gracilaria salicornia*, each measuring approximately 1.0 ± 0.5 cm in length. Three independent culture vessels were maintained for the experimental treatment. Continuous aeration was provided to facilitate gas exchange and promote uniform distribution of nutrients within the culture medium. The cultures were maintained at 22–24 °C, a salinity of 33–35 practical salinity units (approximately 33–35 ppt), and an irradiance of 50–60 µmol photons $m^{-2}$ $s^{-1}$ under a 13 h light:11 h dark photoperiod for six weeks. The culture medium was completely replaced every four days under aseptic conditions to maintain medium quality and nutrient availability throughout the experimental period.

### 2.6 Control treatment

A seawater-maintained control was included for comparative observation. The control thalli were maintained for six weeks and examined at the same sampling intervals as the tissue-cultured material. The experimental records indicated comparatively limited vegetative multiplication in the control. However, complete replicate-level biomass measurements were unavailable for this treatment. Consequently, the control observations were used only as a qualitative reference and were excluded from quantitative statistical comparisons. No statistical significance or quantitative treatment effect was inferred from the control observations.

### 2.7 Experimental design and biomass assessment

Biomass development was monitored at weekly intervals over the six-week culture period. At each sampling point, the cultured material was removed from the culture medium and gently blotted with sterile tissue paper to remove excess surface moisture. Fresh biomass was measured using a calibrated analytical balance. Relative biomass multiplication was calculated as:

$$\textbf{Relative biomass multiplication (fold)} = B_t/B_0$$

where $B_t$ represents the fresh biomass at week $t$ and $B_0$ represents the initial fresh biomass.

The percentage increase in biomass relative to the initial biomass was calculated as:

$$\textbf{Biomass increase (\%)} = [(B_t - B_0)/B_0] \times 100$$

New shoots and lateral branches were recorded throughout the culture period as morphological indicators of vegetative regeneration and multiplication.

### 2.8 Growth-rate assessment

The weekly growth-rate values available in the experimental dataset were recorded as 26.5, 26.8, 20.3, 26.3, 21.4 and 27.4% for weeks 1–6, respectively. Because the original equation used to derive these values was not included in the supplied experimental record, the values

are presented as **recorded growth-rate values** rather than recalculated growth rates. The calculation method should be specified from the original laboratory record before final submission to ensure complete reproducibility.

### 2.9 Morphological assessment

Morphological development was monitored throughout the six-week culture period. Observations included thallus elongation, development of new shoots and formation of lateral branches, together with the general visual condition of the cultured thalli. The formation of multiple shoots and lateral branches was considered a qualitative indication of active vegetative regeneration and multiplication.

### 2.10 Data presentation and statistical considerations

The available experimental dataset contained time-course biomass multiplication values and recorded weekly growth-rate values but did not contain the underlying replicate-level biomass observations required to calculate variability estimates or conduct formal inferential statistical testing. Accordingly, the present results are reported descriptively as observed temporal trends.

The statistical-analysis statement in the previous version referring to factorial ANOVA of explant sections, PGR concentrations, exposure times and commercial biostimulants has been omitted because those factors were not part of the experimental design described in the present study. If the original replicate-level observations are available, mean ± SD or SE and an appropriate repeated-measures or mixed-effects analysis should be added before submission.

## 3 Results

### 3.1 Temporal pattern of biomass multiplication

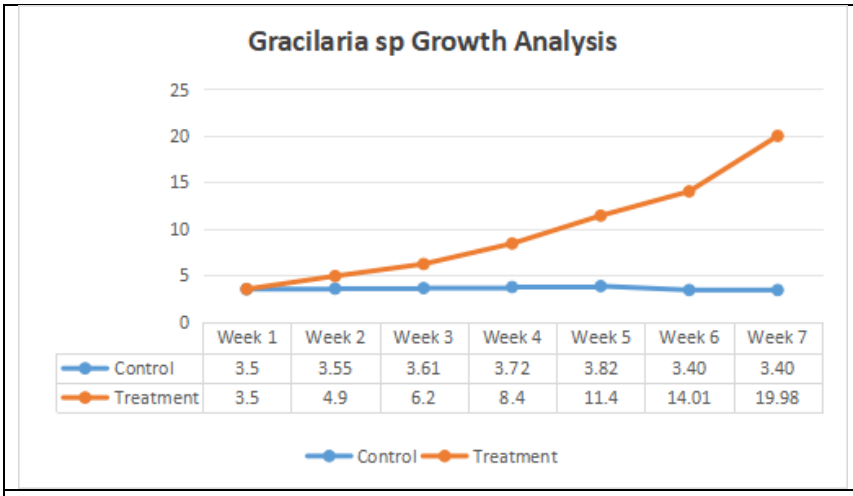
The in vitro-cultured *Gracilaria salicornia* exhibited a progressive increase in relative biomass during the six-week culture period (Table 2; Fig. 4). Relative biomass increased from 1.40-fold of the initial biomass at week 1 to 1.80-fold at week 2 and 2.30-fold at week 3. A further increase was observed at weeks 4 and 5, when biomass reached 3.20-fold and 4.00-fold of the initial value, respectively. The biomass multiplication pattern therefore indicated continued biomass accumulation throughout the recorded culture period.

Based on the relative biomass values, the corresponding increases over the initial biomass were 40, 80, 130, 220 and 300% at weeks 1–5, respectively. The original time-course dataset reports a fivefold relative biomass value at week 6, equivalent to a 400% increase over the initial biomass. This value should be confirmed against the original biomass records because the accompanying figure notes indicate a 5.5-fold increase at week 6. The verified value should be used consistently in **Table 2, Fig. 4** and the manuscript text.

**Table 2. Temporal pattern of relative biomass multiplication and recorded weekly growth-rate percentages of *Gracilaria salicornia* during six weeks of in vitro culture.**

| Culture period | Relative biomass (fold) | Increase relative to initial biomass (%) | Recorded growth rate (%) |
|---|---|---|---|
| Initial | 1.00 | 0 | — |
| Week 1 | 1.40 | 40 | 26.5 |
| Week 2 | 1.80 | 80 | 26.8 |
| Week 3 | 2.30 | 130 | 20.3 |
| Week 4 | 3.20 | 220 | 26.3 |
| Week 5 | 4.00 | 300 | 21.4 |
| Week 6* | 5.00* | 400* | 27.4* |

*The week-6 relative biomass value and percentage increase are provisional and must be verified against the original records. If 5.5-fold is confirmed, the corresponding increase over the initial biomass is 450%, not 400%.




Control – in Seawater
Treatment – in Tissue Culture Media
Weight - in grams

**Biomass increase**
After 1st week : 1.4 times
After 2nd week : 1.8 times
After 3rd week : 2.3 times
After 4th week : 3.2 times
After 5th week : 4.0 times
After 6th week : 5.5 times

**Growth rate**
After 1st week : 26.5%
After 2nd week : 26.8%
After 3rd week : 20.3%
After 4th week : 26.3%
After 5th week : 21.4%
After 6th week : 27.4%

**Figure 4. Comparison of biomass multiplication in Gracilaria maintained in seawater (control) and modified Provasoli's enriched seawater (PES) medium (tissue-culture treatment) during the six-week culture period. Biomass values are expressed in grams where actual fresh-weight measurements are available. The week-6 relative biomass value must be verified before the figure is finalized.**

### 3.2 Recorded weekly growth-rate values

The recorded weekly growth-rate percentages were 26.5, 26.8, 20.3, 26.3, 21.4 and 27.4% for weeks 1–6, respectively (Table 2). The values fluctuated during the culture period rather than showing a continuous increase. The highest recorded value was 27.4% at week 6, whereas the

lowest was 20.3% at week 3. The values recorded for weeks 1 and 2 were similar, followed by a decrease at week 3, an increase at week 4 and a further decrease at week 5.

Because the equation used to calculate these percentages and the corresponding replicate-level observations were unavailable, the values are presented as recorded growth-rate percentages. They are not interpreted as independently verified specific growth rates, and no statistical differences among weeks are inferred from these descriptive values.

### 3.3 Morphological development and vegetative regeneration

Visual observations during the six-week culture period indicated progressive morphological development of the cultured thalli (Fig. 1). The observed changes included thallus elongation, the emergence of new shoots and the development of lateral branches from the cultured explants. The formation of multiple shoots and branches provided qualitative evidence of vegetative regeneration under the modified PES culture conditions (Fig. 2).

The seawater-maintained control showed comparatively limited vegetative multiplication during the corresponding observation period (Fig. 2). In contrast, the tissue-cultured material showed visible shoot and lateral-branch development (Fig.2). However, numerical observations of shoot number, branch number and explant survival were not available, and complete replicate-level control biomass measurements were lacking. Consequently, the morphological observations are reported descriptively, without quantitative estimates of regeneration frequency or statistical comparisons between the control and tissue-culture treatments.

### 3.4 Molecular confirmation of *Gracilaria salicornia* using the *rbcL* primer

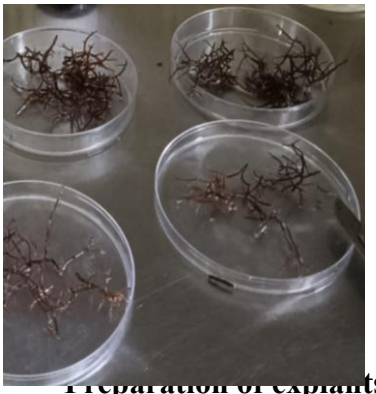
PCR amplification using the *rbcL* primer was performed to obtain molecular evidence for the identification of *Gracilaria salicornia*. The molecular conformation of the PCR product is presented in Fig. 3. The amplification profile was examined to assess the presence and apparent specificity of the target DNA fragment. The use of the chloroplast-encoded *rbcL* gene provides a molecular approach to support species identification and complement the morphological characterization of the cultured seaweed material. Further confirmation of species identity requires sequence analysis of the amplified product and comparison with reference sequences available in public nucleotide databases.



Selection of Healthy explants




Surface sterilization with disinfectants



Preparation of explants



Transfer of explants into nutrient medium



Control Seaweed



Tissue cultured grown seaweed in carboy (1 month)



Mass Culture of seaweed under invitro Lab (after 2 months)



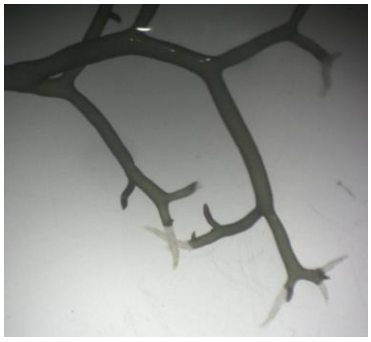
Tissue cultured seaweed thallus multiple shoots Induced after 15 days inoculated

**Figure 1. In vitro micropropagation of *Gracilaria salicornia*: Preparation of explants and morphological development during culture.**

### 3.5 Comparison of biomass in the seawater control and tissue-culture treatment

The photographic and experimental records included a comparison between *Gracilaria* material maintained in seawater and material maintained in tissue-culture medium (Fig. 2). The available observations indicated comparatively limited vegetative multiplication in the seawater-maintained control, whereas the tissue-culture treatment exhibited progressive biomass accumulation during the culture period. Nevertheless, the absence of complete replicate-level biomass measurements for the control prevents a reliable quantitative estimate of the difference between the two conditions (Fig. 4).

Accordingly, the comparison is restricted to the recorded biomass-multiplication pattern of the tissue-culture material and the qualitative observations of the seawater control. The findings support the potential of the modified PES culture system for vegetative multiplication of *G. salicornia* under the conditions investigated, while further replicated experiments with complete biomass and morphological measurements are required to quantify the treatment effect.



**Control seaweed thallus**



**Tissue cultured grown seaweed**



**Control seaweed thallus**



**Tissue cultured seaweed thallus multiple shoots Induced after 30 days inoculated**

**Figure 2. Optimization of seaweed tissue culture through growth analysis and multiple shoot induction.**

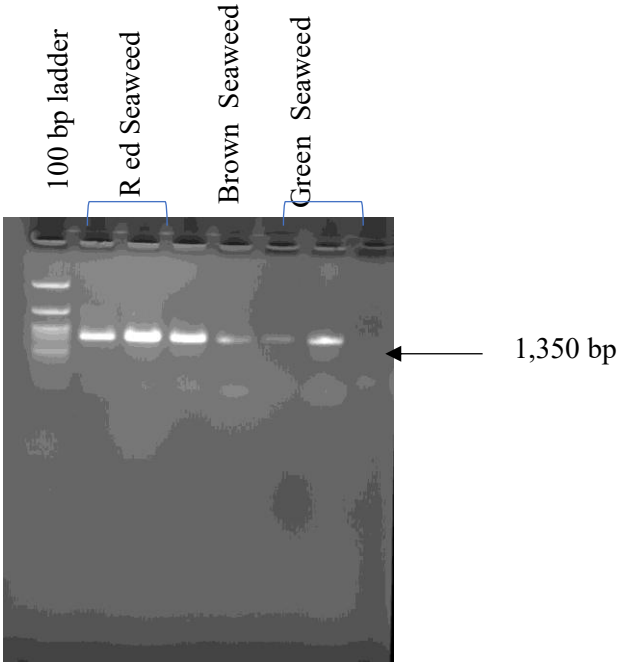



**Figure 3. Fig. 3 PCR amplification of the chloroplast *rbcL* gene from *Gracilaria salicornia* using species-identification primers.**

## 4 Discussion

### 4.1 Influence of IAA and BAP on in vitro regeneration of red seaweed

The present study demonstrated that the regeneration and growth responses of red seaweed explants varied with the combination of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP). Among the five hormone combinations tested, T3 (IAA 0.6 mg $L^{-1}$ + BAP 3.0 mg $L^{-1}$) produced the highest recorded shoot initiation percentage (80%), final shoot length (3.32 cm), shoot number (24 at week 8) and fresh biomass (10.35 g). The consistency of this response across the four measured parameters identifies T3 as the best-performing treatment among those evaluated under the experimental conditions.

The response did not increase continuously with increasing IAA and BAP concentrations. Shoot initiation increased from 20% in T1 to 80% in T3, but declined to 65% and 67% in T4 and T5, respectively. Similar patterns were evident in shoot elongation, shoot multiplication and biomass accumulation. This response suggests that the intermediate hormone combination was more favourable than the higher combinations tested. Previous studies have shown that plant growth regulators influence growth and regeneration in *Gracilaria*, with responses varying according to the species and culture conditions (Yokoya et al. 2004; Ramlov et al. 2013). However, because IAA and BAP concentrations were increased simultaneously in the present experiment, their individual effects cannot be distinguished. Therefore, T3 should be regarded as the best-performing tested combination rather than a statistically established optimum.

### 4.2 Comparison with previous studies on Gracilaria tissue culture

The treatment-dependent regeneration observed in the present study is consistent with earlier investigations of tissue culture in red seaweeds. Yokoya et al. (2004) examined the effects of plant growth regulators on callus formation, growth and regeneration in axenic cultures of *Gracilaria tenuistipitata* and *Gracilaria perplexa*. Their findings demonstrated the relevance of regulator composition to the morphogenetic response of *Gracilaria* tissues. Similarly, Ramlov et al. (2013) investigated growth, callus formation and plant regeneration in different colour morphs of *Gracilaria domingensis* under different irradiance levels and plant growth regulator treatments, demonstrating that regeneration responses may vary with culture conditions and treatment combinations.

Yeong et al. (2014) investigated the production of clonal planting material from *Gracilaria changii* and *Kappaphycus alvarezii* through tissue culture and examined the cultivation of *G. changii* explants in airlift photobioreactors. Their work illustrates the potential of tissue-culture techniques for producing planting material from commercially important seaweeds. The present study complements this research by identifying an IAA–BAP combination associated with strong shoot initiation, multiple-shoot formation and biomass accumulation in the red seaweed material examined.

Direct comparisons among these studies must be made cautiously because the species, explant types, media compositions, hormone concentrations and culture conditions differ. The findings nevertheless support the importance of optimizing tissue-culture conditions for individual seaweed species rather than assuming that a protocol developed for one species will produce equivalent responses in another.

### 4.3 Shoot initiation and the concentration-dependent response

Shoot initiation is an important indicator of the initial regenerative response of explants under in vitro conditions. In the present study, shoot initiation increased from 20% in T1 to 40% in T2 and reached 80% in T3, before declining at the two higher hormone combinations. This pattern indicates that the intermediate IAA–BAP combination supported the highest recorded shoot initiation response among the tested treatments.

Auxins and cytokinins are widely used in tissue-culture research because they can influence cellular proliferation, differentiation and shoot development. Their effects depend on the organism, explant condition, concentration and interaction with other culture variables. Previous studies of *Gracilaria* regeneration have demonstrated that responses to plant growth regulators can differ among species and culture systems (Yokoya et al. 2004; Ramlov et al. 2013). The present results are consistent with the importance of treatment-specific optimization.

Nevertheless, the experiment did not independently vary IAA and BAP concentrations. Consequently, it cannot establish whether the observed response was primarily associated with IAA, BAP or an interaction between the two regulators. A factorial experiment incorporating several independent concentrations of each regulator, together with a hormone-free control, would allow their individual and interactive effects on shoot initiation to be evaluated.

### 4.4 Shoot elongation and multiple-shoot formation

The increase in shoot length and shoot number during the eight-week culture period indicates that the tested culture treatments supported morphological development beyond initial explant establishment. T3 produced the greatest final shoot length (3.32 cm) and the highest recorded shoot number (24 shoots at week 8). In comparison, final shoot lengths in the other hormone-supplemented treatments ranged from 1.90 to 2.23 cm, while their final shoot numbers ranged from 8 to 10.

The simultaneous occurrence of longer shoots and a greater number of shoots in T3 is relevant to propagation because multiple, sufficiently developed shoots may increase the number of potential propagules produced from a given quantity of starting material. However, shoot number alone does not establish the number of viable, detachable or successfully acclimatized propagules. Further investigations should quantify the proportion of shoots that develop into independent thalli and determine their survival after transfer to nursery conditions.

Previous studies have demonstrated that plant growth regulators and irradiance influence growth and regeneration in *Gracilaria* tissue cultures (Yokoya et al. 2004; Ramlov et al. 2013). The present findings are consistent with that broader evidence, while providing a treatment-specific comparison of shoot length and shoot number. As replicate-level observations and variability estimates were unavailable, the differences among treatments should be interpreted descriptively rather than as statistically significant effects.

### 4.5 Biomass accumulation and its relevance to propagation

Fresh biomass accumulation is an important measure of culture performance because propagation requires both the development of new shoots and an increase in the quantity of algal material. In the eight-week optimization experiment, the recorded fresh biomass in T3 increased from 1.56 g initially to 3.23 g at week 4, 6.80 g at week 6 and 10.35 g at week 8. The final recorded biomass was greater than that in the other treatments, for which week-8 values ranged from 2.50 to 3.72 g.

Based on the supplied initial and final biomass values, the final biomass in T3 was approximately 6.63 times the initial biomass, equivalent to an increase of approximately 563.5%. This calculation applies only to the eight-week optimization experiment and should not be conflated with the separate six-week time-course experiment, which reported fivefold relative biomass. The two datasets indicate substantial biomass accumulation under their respective experimental conditions but should be interpreted independently unless their experimental relationship is established.

The increase in biomass in T3, together with the corresponding increases in shoot length and shoot number, suggests that this treatment supported both morphological development and biomass production. However, fresh biomass is affected by tissue water content as well as by algal growth. Future experiments should standardize the blotting procedure and, where appropriate, measure dry biomass or moisture content alongside fresh weight. Reporting biomass productivity relative to initial biomass, culture volume and culture duration would also facilitate comparisons among experiments and cultivation systems.

The importance of developing reliable propagation methods is consistent with the broader literature on seaweed production and cultivation (Buschmann et al. 2017). Nevertheless, laboratory biomass accumulation alone does not establish commercial productivity, and further validation under nursery and cultivation conditions is necessary.

### 4.6 Comparison with seawater-maintained material

The available observations indicated comparatively limited vegetative multiplication in the seawater-maintained control relative to the tissue-culture treatments. This observation is consistent with the possibility that the nutrient-enriched culture environment and associated treatment conditions supported more active shoot development and biomass accumulation.

However, the control comparison remains qualitative because complete replicate-level biomass measurements were unavailable. Furthermore, unless the control and tissue-culture treatments were maintained under equivalent conditions, the observed difference cannot be attributed solely to the composition of the culture medium or the presence of plant growth regulators.

Future experiments should include clearly defined and replicated controls with equivalent initial biomass, culture volume, aeration, temperature, salinity, irradiance, photoperiod and handling frequency. A hormone-free PES control and a seawater-maintained control would help distinguish the effects of nutrient enrichment from those associated with hormone supplementation. Replicated measurements of biomass, shoot initiation, shoot number and survival would provide a stronger basis for statistical comparison.

### 4.7 Implications for clonal planting-material production

The development of a culture treatment that supports shoot initiation, multiple-shoot formation and biomass accumulation represents a useful step towards establishing an in vitro propagation protocol for *Gracilaria*. Yeong et al. (2014) demonstrated the potential of tissue-culture approaches for producing clonal planting material from *Gracilaria changii* and *Kappaphycus alvarezii*. The present findings suggest that the tested IAA–BAP combination could provide a starting point for developing a comparable approach for the red seaweed material investigated here.

The availability of healthy planting material is relevant to seaweed cultivation because it can support the establishment and maintenance of cultivation stocks. Raja et al. (2025) examined the cultivation potential of *Gracilaria salicornia* in a brackishwater ecosystem, providing a complementary context for the species' cultivation potential. However, field cultivation and laboratory tissue culture represent different stages of production, and biomass values obtained under these systems should not be compared directly without accounting for differences in initial stocking biomass, culture duration and environmental conditions.

The present study does not establish that tissue-culture-derived material has superior field performance or economic advantages over conventionally propagated material. To determine its practical applicability, regenerated thalli should be transferred to nursery systems and evaluated for survival, attachment, growth, branching and biomass production. Subsequent field trials comparing tissue-culture-derived material with conventional vegetative fragments

under equivalent cultivation conditions would provide evidence of its potential for aquaculture use.

### 4.8 Genetic fidelity and physiological quality

Genetic fidelity is an important consideration in the development of clonal propagation systems for commercially valuable seaweeds. The present study assessed biomass accumulation and morphological development but did not investigate genetic stability. Therefore, the observed growth response should not be interpreted as evidence of genetic uniformity among the regenerated thalli.

Molecular marker-based assessments could be incorporated into future studies to evaluate genetic consistency between donor material and regenerated thalli, particularly if the protocol is intended for the multiplication and maintenance of selected strains. Physiological measurements, including pigment composition, photosynthetic performance and recovery following transfer from laboratory culture, would provide additional information on propagule quality.

Such assessments would complement the morphological and biomass measurements reported here and help determine whether the propagation system can consistently produce healthy material suitable for subsequent cultivation. Genetic and physiological stability should therefore be treated as additional validation objectives rather than as outcomes established by the present experiment.

### 4.9 Requirements for further optimization and scale-up

The present culture system provides a preliminary basis for further optimization rather than a fully validated commercial propagation protocol. The strongest recorded response in T3 identifies a promising IAA–BAP combination for additional investigation. However, the independent effects of the two regulators, their interactions with nutrient availability and their influence under different environmental conditions remain to be established.

Further studies should investigate explant size, inoculation density, nutrient composition, hormone concentration, irradiance, photoperiod, temperature, salinity, aeration and medium-renewal frequency. Replicated factorial experiments would help determine the relative contributions of these variables and identify combinations that consistently support regeneration and biomass accumulation. Longer culture trials would also establish whether multiplication continues beyond the current observation period and identify any changes associated with culture duration or increasing biomass density.

Scale-up from small vessels to larger culture systems may alter light penetration, nutrient distribution, gas exchange and biomass density. Consequently, performance should be evaluated using standardized initial biomass and culture volume, with biomass productivity reported over a defined period. Transfer experiments followed by nursery and outdoor cultivation trials would determine whether the laboratory response translates into improved survival, growth and biomass production under aquaculture conditions.

Overall, the present results identify T3 (IAA 0.6 mg $L^{-1}$ + BAP 3.0 mg $L^{-1}$) as the best-performing tested treatment based on the recorded shoot initiation, shoot elongation, shoot multiplication and biomass accumulation values. Further replicated experiments, verification of the biomass calculations, genetic and physiological assessments, and nursery validation are required before the treatment can be recommended for large-scale clonal planting-material production.

## 5 Conclusion

The present study demonstrated the feasibility of liquid modified Provasoli's Enriched Seawater (PES)-based *in vitro* culture for the vegetative propagation and biomass multiplication of *Gracilaria salicornia*. Under the tested culture conditions, relative biomass increased progressively from 1.40-fold after one week to 5.00-fold after six weeks, representing a 400% increase over the initial biomass. This increase was accompanied by thallus elongation, the development of new shoots and the formation of lateral branches, indicating sustained vegetative growth during the culture period. These findings establish a quantitative baseline for the controlled multiplication of *G. salicornia* using a liquid culture system.

The results highlight the potential of liquid *in vitro* culture as a complementary approach to conventional vegetative propagation for producing planting material from limited starting biomass. However, the findings apply to the culture conditions and plant growth regulator combination evaluated in this study; they do not establish the individual effects or optimal concentrations of the regulators. Further investigations should therefore evaluate plant growth regulator requirements, explant density, culture conditions, genetic fidelity, physiological quality, acclimatization and subsequent nursery and field performance. Validation through replicated experiments and scale-up trials will be necessary to determine the reproducibility, production efficiency and practical applicability of this approach. Overall, the study provides a foundation for further development of a controlled propagation protocol for *G. salicornia*, with potential relevance to future seaweed cultivation and seedstock production.

## Acknowledgements

The authors acknowledge the facilities and technical support provided by the Department of Aquatic Animal Health Management, Dr. M.G.R. Fisheries College and Research Institute, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Tamil Nadu, India.

## Author contributions

S. Saravanan: conceptualization, methodology, investigation, data collection, data analysis and writing—original draft. D. Kaviarasu: field sampling and seawater collection. A. Uma: technical advice and guidance. R. Jeya Shakila: project supervision, guidance on project execution and technical guidance. All authors reviewed and approved the final manuscript.

## Funding

This research was supported by the CMRG 2025–26 under Project ID: CMRG25-UE-0083, entitled "Genetic Improvement of Commercial *Kappaphycus, Gracilaria,* and *Gelidiella* Seaweed Cultivars for Sustainable Fisherfolk Livelihoods."

## Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

## Declarations

Conflict of interest. The authors declare that they have no competing interests.

Ethics approval. Not applicable.

Consent to participate. Not applicable.

Consent for publication. Not applicable.

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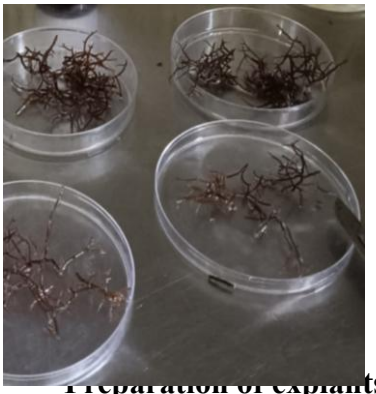
Yong WTL, Chin JYY, Thien VY, Yasir S (2017) Heavy metal accumulation in field cultured and tissue cultured Kappaphycus alvarezii and Gracilaria changii. International Food Research Journal 24:970–975.



Selection of Healthy explants




Surface sterilization with disinfectants



Preparation of explants



Transfer of explants into nutrient medium



Control Seaweed



Tissue cultured grown seaweed in carboy (1 month)

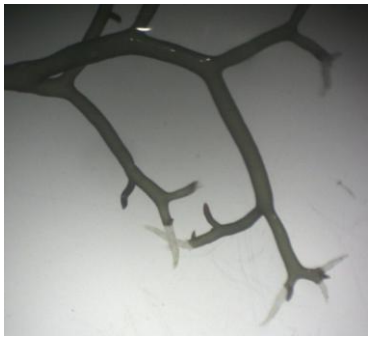


Mass Culture of seaweed under invitro Lab (after 2 months)



Tissue cultured seaweed thallus multiple shoots Induced after 15 days inoculated

**Figure 1. In vitro micropropagation of *Gracilaria salicornia*: Preparation of explants and morphological development during culture.**



**Control seaweed thallus**



**Tissue cultured grown seaweed**



**Control seaweed thallus**



**Tissue cultured seaweed thallus multiple shoots Induced after 30 days inoculated**

**Figure 2. Optimization of seaweed tissue culture through growth analysis and multiple shoot induction.**

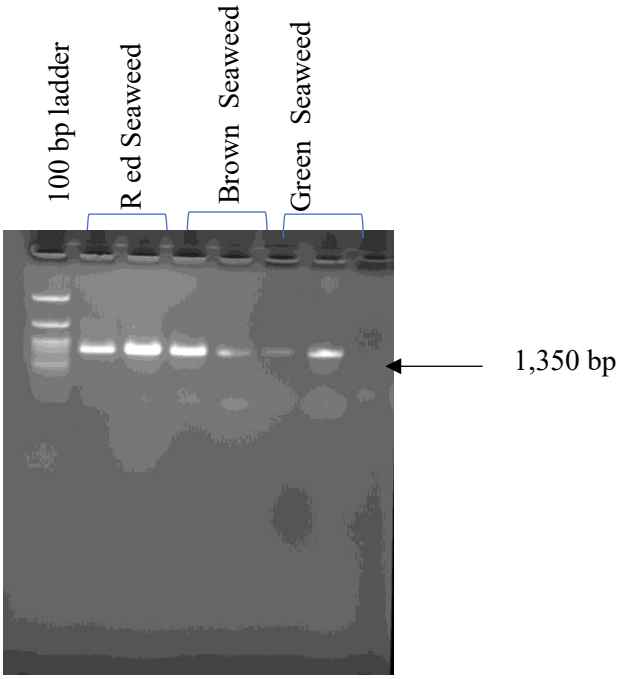
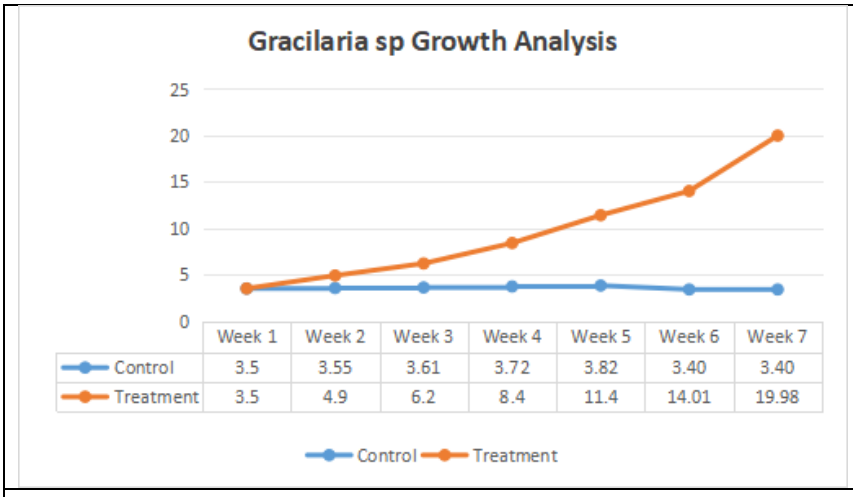



**Figure 3. Fig. 3 PCR amplification of the chloroplast *rbcL* gene from *Gracilaria salicornia* using species-identification primers.**




Control – in Seawater
Treatment – in Tissue Culture Media
Weight - in grams

**Biomass increase**
After 1st week : 1.4 times
After 2nd week : 1.8 times
After 3rd week : 2.3 times
After 4th week : 3.2 times
After 5th week : 4.0 times
After 6th week : 5.5 times

**Growth rate**
After 1st week : 26.5%
After 2nd week : 26.8%
After 3rd week : 20.3%
After 4th week : 26.3%
After 5th week : 21.4%
After 6th week : 27.4%

**Figure 4. Comparison of biomass multiplication in Gracilaria maintained in seawater (control) and modified Provasoli's enriched seawater (PES) medium (tissue-culture treatment) during the six-week culture period. Biomass values are expressed in grams where actual fresh-weight measurements are available. The week-6 relative biomass value must be verified before the figure is finalized.**