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CALLUS INDUCTION IN *Kappaphycus alvarezii* USING INDOLE-3-ACETIC ACID (IAA) and 6-BENZYLAMINOPURINE (BAP) FOR SEEDSTOCK DEVELOPMENT

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ABSTRACT

The commercially important red seaweed Kappaphycus alvarezii is extensively cultivated for carrageenan production. Despite its economic value, large-scale reproduction and genetic enhancement remain limited due to its low regeneration potential. This study aimed to optimize plant growth regulator (PGR) concentrations for efficient callus induction in K. alvarezii. A completely randomized design was employed, comprising five treatments with varying concentrations of indole-3-acetic acid (IAA) and 6-benzylaminopurine (BAP), along with a control lacking PGRs. A total of 180 explants from meristematic tissues of acclimatized thalli were cultured (30 explants per treatment). The highest callus induction rate (88%) was achieved with 1.50 mg/L IAA and 5 mg/L BAP (Treatment F), with visible callus formation beginning around day 38. A progressive color change from brown to white was observed, indicating active cellular proliferation. Other treatments exhibited lower induction rates, ranging from 0% (control) to 61% (Treatment D). These findings underscore the critical influence of auxin–cytokinin interactions on callogenesis and offer an optimized hormonal regime for improving in vitro culture efficiency. The established protocol provides a valuable platform for future large-scale propagation and genetic improvement strategies in K. alvarezii, contributing to the advancement of seaweed biotechnology.

KEYWORDS: *Kappaphycus alvarezii*, callus induction, tissue culture, indole-3-acetic acid, 6-benzylaminopurine, seaweed biotechnology

INTRODUCTION

Seaweeds, a diverse group of marine macroalgae, play essential roles in coastal ecosystems and serve as valuable resources in biotechnology and aquaculture (Segaran *et al*., 2024). They are taxonomically classified into three major groups—Chlorophyta (green algae), Phaeophyta (brown algae), and Rhodophyta (red algae)—each distinguished by unique pigmentation, morphology, and reproductive strategies (Pereira, 2021). Ecologically, seaweeds significantly contribute to marine biodiversity, primary productivity, nutrient cycling, and habitat complexity, particularly in tropical regions like Indonesia, which is among the world's richest in seaweed diversity (Basyuni *et al*., 2024). The growing scientific interest in seaweed research over recent decades reflects their multifaceted ecological roles and broad applications (Segaran *et al*., 2024). Among their valuable compounds, carrageenan—a sulfated polysaccharide extracted mainly from red algae—has attracted considerable attention for its versatile applications in food, pharmaceuticals, and biotechnology, underscoring the economic importance of seaweeds (Pradhan & Ki, 2023). Additionally, seaweed physiology, notably their responses to abiotic stresses influenced by nutrient availability such as phosphorus, highlights their ecological significance and potential for biotechnological innovation (Khan *et al*., 2023).

Indonesia is a major contributor to the global seaweed industry, ranking second after China in cultivation area, and accounting for 25% of global production as of 2024. Seaweed exports increased sharply by 26% in 2022, reaching USD 1.6 billion, with

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Indonesia becoming the leading exporter by value, largely driven by increased shipments to China (FAO, 2024). A commercially important red seaweed, *Kappaphycus alvarezii* is predominantly cultivated for carrageenan extraction, a gelling agent widely used across nutraceutical, biomedical, and beauty industries (Rupert *et al*., 2022; Fadilah *et al*., 2024). In 2020, exports of *K. alvarezii* totaled over 97 million kg (dry weight), valued at approximately USD 131 million, underscoring its economic significance for Indonesia's seaweed sector (Statistics Indonesia, 2023). The ecological and economic importance of *K. alvarezii* has stimulated efforts to optimize cultivation methods and develop genetic improvement strategies (Muyong & Tahiluddin, 2024). Given the increasing demand for carrageenan, sustainable and efficient propagation techniques have become critically important (Siddiqui *et al*., 2022). Tissue culture methods, especially optimized callus induction, present promising strategies to accelerate propagation rates and ensure a reliable supply of high-quality seaweed biomass to meet global market demands (Chandimali *et al*., 2024). However, challenges such as limited regeneration capacity and incomplete understanding of *K. alvarezii*'s physiological and developmental responses to environmental and hormonal signals are not fully understood (Kumar *et al*., 2020; Khan *et al*., 2023; Radkhah *et al*., 2024).

In vitro propagation through tissue culture has emerged as an effective tool for mass multiplication and genetic enhancement of *K. alvarezii* (Jiksing *et al*., 2022). Callus formation, a pivotal step in this process, has been shown to improve biomass production, growth performance, and stress tolerance (Sulistiani *et al*., 2012; Hlaing & Jarukamjor, 2024). Plant growth regulators (PGRs), particularly auxins and cytokinins, are essential in callus induction. Indole-3-acetic acid (IAA), a natural auxin, promotes cell elongation and differentiation, while 6-benzylaminopurine (BAP), a synthetic cytokinin, stimulates cell division and organogenesis (Lim *et al*., 2013; Yegappan *et al*., 2018). Therefore, identifying the optimal concentrations and combinations of IAA and BAP is critical for efficient callus formation in *K. alvarezii* (Sulistiani *et al*., 2012; Hlaing & Jarukamjor, 2024). Recent studies report considerable variability in callus induction outcomes depending on PGR concentrations. The interaction between auxins and cytokinins is highly context-dependent, potentially producing synergistic or antagonistic effects that influence morphogenesis and stress responses (Bielach *et al*., 2017; Yegappan *et al*., 2018). A comprehensive understanding of these hormonal mechanisms is necessary to refine tissue culture protocols and achieve consistent propagation results (Das & Prasad, 2015). Moreover, insights into the physiological effects of exogenous PGRs can inform genetic and biotechnological strategies aimed at enhancing growth, productivity, and environmental adaptability in seaweeds. Targeted hormonal manipulation may also induce somaclonal variation, promoting genetic diversity and expanding the biotechnological applications of *K. alvarezii* in sustainable aquaculture and industry (Tanaka *et al*., 2020; Khan *et al*., 2023).

Several research have also explored the use of PGRs and chemical treatments to optimize tissue culture and micropropagation of *K. alvarezii*. Notably, combining IAA and BAP with colchicine enhanced callus formation and explant regeneration, with colchicine improving regeneration potential in specific strains (Hayashi *et al*., 2008). Similarly, spindle inhibitors such as colchicine and oryzalin, used alongside phytoregulators including IAA, kinetin, and spermine, increased micropropagule growth and axis formation, facilitating improved mass cultivation under laboratory conditions (Neves *et al*., 2015). Despite these advances, a knowledge gap remains in the comprehensive evaluation of the synergistic effects of IAA and BAP alone on callus induction efficiency. In particular, the influence of varying concentrations and ratios of these key PGRs remains insufficiently studied. Hence, this study aimed to address this gap by systematically investigating the effects of different concentrations and combinations of IAA and BAP on callus induction in *K. alvarezii*. Optimizing these parameters is projected to enhance callus induction protocols, thereby supporting more efficient propagation and genetic improvement strategies for this economically important seaweed species widely used in carrageenan production.

MATERIALS AND METHODS

Research Design

This study was conducted from February to May 2023 at the Tissue Culture Laboratory, Centre for Brackish Water Aquaculture Development, Takalar, South Sulawesi. A completely randomized design (CRD) was applied to assess the effects of different PGR treatments on callus induction in *K. alvarezii*. The experiment comprised six treatments: five PGR combinations and one control (without PGRs). Each treatment was replicated three times, with 10 explants per replicate (n = 30 explants per treatment; total n = 180 explants). Explants, approximately 1 cm in length, were excised from the apical meristematic region of thalli. Randomization of treatments was performed using a generated random number sequence to allocate explants independently across all 18 treatment units. This ensured equal probability of

assignment and minimized potential bias related to environmental variation or operator influence during culture preparation (Table 1). The concentrations of IAA and BAP were selected based on prior studies reporting successful callus induction in red algae and related macroalgae (Sulistiani *et al*., 2012; Jiksing *et al*., 2022; Tirtawijaya *et al*., 2022; Hlaing & Jarukamjor, 2024; Lee *et al*., 2024), and were optimized to evaluate potential synergistic effects on callus formation.

Table 1. Formulation of PGR treatment combinations (IAA and BAP) used in this study

No.	Code	Treatment
1.	A	Control (no PGR)
2.	B	IAA 0.50 mg/L + BAP 1 mg/L
3.	C	IAA 0.75 mg/L + BAP 2 mg/L
4.	D	IAA 1.00 mg/L + BAP 3 mg/L
5.	E	IAA 1.25 mg/L + BAP 4 mg/L
6.	F	IAA 1.50 mg/L + BAP 5 mg/L

the seawater was subsequently filtered to remove any precipitates. When required, the ionic balance of the seawater was reconstituted based on the standard artificial seawater formulation described by Kester *et al*. (1967), ensuring consistency in ionic strength and suitability for marine algal tissue culture. The sterilized and filtered seawater was stored in sterile containers at a controlled temperature of 22–25°C to prevent sedimentation and maintain sterility. The callus induction medium was prepared using sterilized Provasoli's Enriched Seawater (PES) diluted to 1/20 strength, following standard preparation protocols (Provasoli, 1968). The use of diluted PES 1/20 was based on previous findings indicating that lower nutrient concentrations promote callus formation in *Kappaphycus* species by reducing microbial contamination and minimizing osmotic stress during the early stages of in vitro culture (Tirtawijaya *et al*., 2022). The medium was further supplemented with specific concentrations of IAA and BAP according to the treatment design (B–F) and solidified with 0.8% bacto agar (Difco).

Acclimatization of Seaweed

Fresh and healthy *K. alvarezii* thalli, characterized by their dense structure, bright color, and absence of disease symptoms (Azizi *et al*., 2018), were collected from the waters of Takalar, South Sulawesi. The collected seaweed was transported to the laboratory in a cool box with ice to minimize stress during transit. A multi-stage acclimatization process was implemented to reduce mortality and adapt the seaweed to laboratory conditions: (i) Initial Acclimatization: Seaweed thalli were placed in containers filled with seawater at a salinity of 30 ppt and a temperature of 28°C for 12 hours. This allowed the seaweed to recover from the initial shock of collection and transportation; (ii) Intermediate Acclimatization: The seaweed was transferred to aquariums within a greenhouse for a 2-week period. A stocking density of 300 g per aquarium was applied, based on established seaweed cultivation protocols, to minimize overcrowding and ensure optimal access to light and nutrients. This facilitated gradual adaptation to controlled conditions with natural light exposure; and (iii) Laboratory Acclimatization: The seaweed was transferred to the laboratory, washed with filtered seawater, and stored in glass jars containing sterile seawater with a salinity of 29–30 ppt. The seaweed was illuminated with fluorescent lamps (Philips) at a light intensity of 1500 lux (~20–25 µmol m^{-2} s^{-1} PAR), with a 12-hour light/12-hour dark photoperiod at a controlled room temperature of 22–23°C. The PES 1/20 medium was changed weekly for the old seaweed parent stock and daily for new parent stock over a 6-week period. This gradual reduction in the frequency of medium changes helped the seaweed adapt to the tissue culture conditions.

Preparation of Seawater and Media

Natural seawater with a salinity above 29 ppt was used as the growth medium. To eliminate microbial contaminants, the seawater was sterilized using an autoclave (Hirayama HVE-50) at 121°C and 20 psi for 15 minutes. As autoclaving may cause precipitation of salts such as calcium carbonate ($CaCO_3$), potentially altering the ionic composition of the medium,

Preparation of Explants and Callus Induction

Young tissue from the thalli of acclimatized seaweed was selected and thoroughly washed with sterile seawater. Explants, approximately 1 cm in size, were excised from the meristematic tissue ends of the thalli. To minimize contamination, the explants were surface-sterilized using a two-step process. The explants were first treated with a soap solution (1 drop of dishwashing soap containing sodium lauryl sulfate in 100 ml of seawater) for 3 minutes, followed by rinsing with sterile seawater to remove any soap residue. Subsequently, the explants were immersed in a 1% Povidone Iodine solution for 3 minutes and then rinsed thoroughly with sterile seawater until all traces of Povidone Iodine were eliminated. The ster-

ilized explants were then dried on sterile tissue paper and inoculated onto the callus induction medium in culture bottles. The cultures were maintained on culture racks in a temperature-controlled room at 22–25°C under a light intensity of approximately 1500 lux with a 12-hour light/12-hour dark photoperiod. Contamination was monitored weekly through visual inspection of explants and culture media for indicators of microbial growth. Simultaneously, callus development was also assessed weekly over the 38-day incubation period, as callus induction timing can vary among explants. Extending the observation period allowed for accurate recording of late-developing calluses and identification of unresponsive or bleaching explants (Sulistiani *et al.*, 2012; Hlaing & Jarukamjor, 2024). In addition, callus induction frequency was calculated using the formula (Noor *et al.*, 2022):

$$\text{Callus induction frequency} = \frac{\text{Number of explants with callus}}{\text{Number of explants inoculated}} x\ 100$$

Photographs of the callus were taken using a digital camera to visually document callus performance. Callus coloration was evaluated using a standardized scoring system (Table 2).

Table 2. Callus color observation scoring for *K. alvarezii*

No.	Scoring	Color
1.	0	Brown
2.	1	White
3.	2	Whitish green
4.	3	Yellowish green
5.	4	Green
6.	5	Brownish green
7.	6	Yellow

Statistical Analysis

The data obtained were tabulated and subjected to statistical analysis using analysis of variance (ANOVA) to determine the effect of PGR treatments on callus induction. Prior to ANOVA, assumptions of normality and homogeneity of variance were tested using the Shapiro–Wilk test and Levene's test, respectively. All statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA). If the ANOVA revealed a significant effect ($p < 0.05$), Duncan's Multiple Range Test (DMRT) was conducted as a post-hoc test to identify significant differences between treatment means, using a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Callus induction represents a crucial phase in plant tissue culture, providing the foundational platform for large-scale cultivation and biotechnological applications. In this study, callus formation was successfully induced in *K. alvarezii* explants, and observations were focused on the timing of initiation and the extent of callus development. Notably, the frequency of callus formation differed significantly among the treatments, stressing the pivotal role of PGRs in modulating morphogenic responses (Figure 1). The highest induction rate (88%) was observed in treatment F (1.50 mg/L IAA + 5 mg/L BAP), which presented a statistically significant enhancement ($p < 0.05$) compared to other treatments. This result was consistent with previous studies that substantiated effective callus induction in red algae using similar PGR combina-

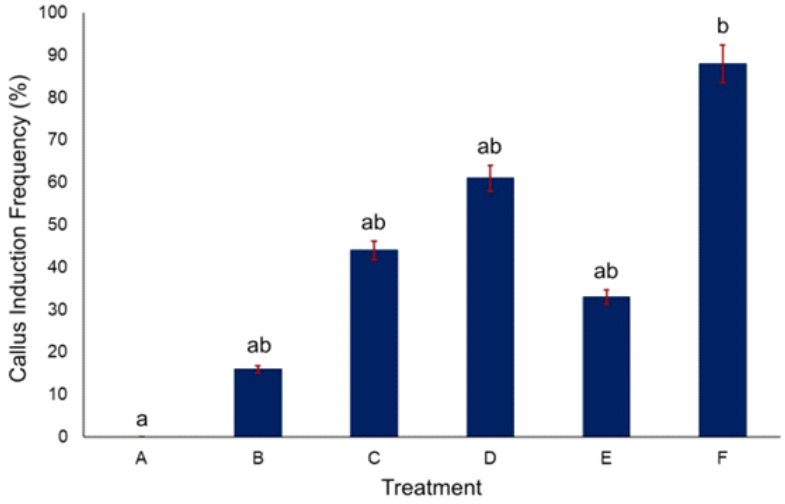

Figure 1. Frequency of callus induction in *K. alvarezii* explants under different combinations of PGRs. Different letters above the bars represent statistically significant differences ($p < 0.05$).

tions (Sulistiani *et al*., 2012; Jiksing *et al*., 2022). However, our study achieved a higher induction rate than those previously reported. The improved performance observed in this study demonstrates the potential for optimizing PGR concentrations to enhance callus formation, which is critical for advancing seaweed tissue culture techniques and supporting genetic improvement initiatives. These findings also suggest that higher concentrations of both auxin and cytokinin can synergistically promote cellular dedifferentiation and proliferation, corroborating earlier reports (Mo & Reddy, 2016). Conversely, treatment B (0.50 mg/L IAA + 1 mg/L BAP) resulted in only 16% ($p < 0.05$) callus formation, indicating that suboptimal PGR levels are insufficient to trigger effective morphogenic responses. Moreover, no callus formation was observed in the control group (treatment A), reaffirming the fundamental role of exogenous PGRs in initiating callus induction in *K. alvarezii*.

The significant influence of specific auxin and cytokinin combinations on callus induction has been extensively documented. Auxins, such as IAA, trigger the dedifferentiation of somatic cells, facilitating the formation of pluripotent callus tissue. Concurrently, cytokinins like BAP stimulate cell division and regulate callus differentiation in a concentration-dependent manner relative to auxin levels (Ma *et al*., 2022; Sosnowski *et al*., 2023). As emphasized by Fehér (2023) and Wang *et al*. (2016), achieving an optimal balance between auxin and cytokinin is critical for enhancing callus induction efficiency. The recent studies also have further underscored the importance of fine-tuning the auxin-cytokinin ratio to improve the quality of induced callus tissue, highlighting that different species respond variably to hormone combinations (Wang *et al*., 2015; Bano *et al*., 2022). Furthermore, in treatment F, explants initially turned brown (Figure 2). By day 15, a shift in pigmentation was observed, and callus initiation began on day 38, primarily at the explant tips, where white, filamentous crystalline structures appeared. By day 55, a marked increase in callus biomass was evident.

The texture and color of induced callus are major determinants of its suitability for subsequent subculture and regeneration. In treatment F, the callus appeared compact and white; however, its dense consistency hindered efficient dissociation. This suggests that although callus induction was effectively achieved, the callus had not yet attained a friable state, which is effective for the facile separation into single cells or small cell clusters necessary for downstream regeneration processes (Jiksing *et al*., 2022). The resistance to dissociation observed in compact callus may restrict the availability of viable cells required for successful organogenesis or somatic embryogenesis, thereby potentially diminishing regeneration efficiency and prolonging propagation timelines (Mo *et al*., 2020; Avila-Victor *et al*., 2023). These observations align with previous reports emphasizing that the transition from compact to friable callus is a pivotal step in enhancing subculture outcomes and regeneration success by enabling easier cell separation and stimulating vigorous cell division (Chandimali *et al*., 2024).

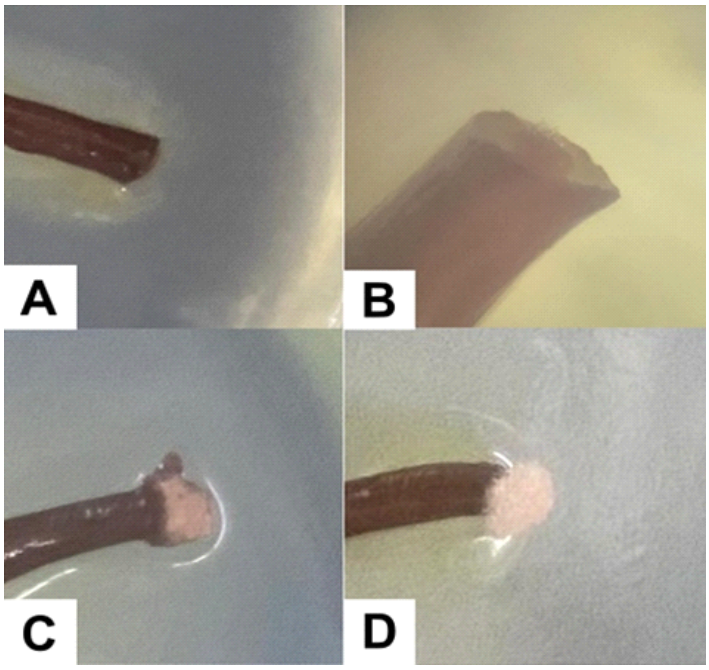

Figure 2. Callus formation in *K. alvarezii* seaweed explants under treatment F, observed at day 0 (A), day 15 (B), day 38 (C), and day 55 (D).

The observed morphological changes during callus induction provide significant understanding into the cellular responses to hormonal treatments. The shift in pigmentation from brown to white, filamentous structures serves as a key indicator of successful dedifferentiation from somatic tissue to callus (Wang *et al*., 2016; Martinez *et al*., 2021). The texture and compactness of the callus are also crucial for subsequent regeneration, with friability being an pivotal trait for efficient subculture (Kruglova, 2022). Studies by Tuskan *et al*. (2018) have shown that the physical characteristics of callus, such as color and texture, are not only indicative of successful dedifferentiation but also critical in the determination of regeneration potential. The influence of phenotypic characteristics like color and texture on differentiation competence aligns with findings in other plant species (Ge *et al*., 2016). Moreover, in treatments B, C, D, and E, the lower PGR concentrations were ineffective in promoting the formation of viable callus (Table 3; Figure 3). The resulting dark brown callus suggests inhibited growth or tissue necrosis. The lack of organized structures indicates that this callus is unsuitable for further regeneration.

This study demonstrated that treatment F (1.50 mg/L IAA + 5 mg/L BAP) yielded the most favorable callus induction in terms of both rate and quality. The other treatments did not produce optimal results, accentuating the critical importance of a balanced auxin-cytokinin ratio for effective callus formation. Sub-optimal PGR concentrations failed to stimulate adequate cell division, while supra-optimal concentrations were toxic, hindering callus development. In line with recent studies, the impact of both low and high PGR concentrations on callus induction has been clearly observed, with a fine-tuned hormone balance being important for high-quality tissue culture (Ikeuchi *et al*., 2013; Tirtawijaya *et al*., 2022).

The important contribution of a finely tuned auxin–cytokinin balance in promoting effective callus induction and plastid differentiation is well-documented in plant tissue culture studies (Stirk & Staden, 2014; Chandler & Werr, 2015; Rademacher, 2015). In this study, treatments B, C, D, and E, which utilized relatively low concentrations of PGRs, failed to initiate adequate cell division and dedifferentiation—processes essential for the initiation and proliferation of viable callus tissue. Conversely, treatments with elevated PGR concentrations exhibited reduced callogenic responses, likely due to phytotoxic effects that compromised tissue viability and cellular function (Klimek Chodacka *et al*., 2020; Mazzoni *et al*., 2021; Ma *et al*., 2022). This suppression of callus formation may be attributed to hormonal overdose, which disrupts cellular homeostasis and triggers oxidative stress, membrane damage, and abnormal metabolic responses, ultimately impairing the reprogramming of somatic cells. Elevated cytokinin levels, in

Table 3. Proportion of callus color in different PGR treatments

Treatment	Callus proportion (%)	
	Brown	White
A	100	0
B	66.67	33.33
C	66.67	33.33
D	33.33	66.67
E	66.67	33.33
F	0	100

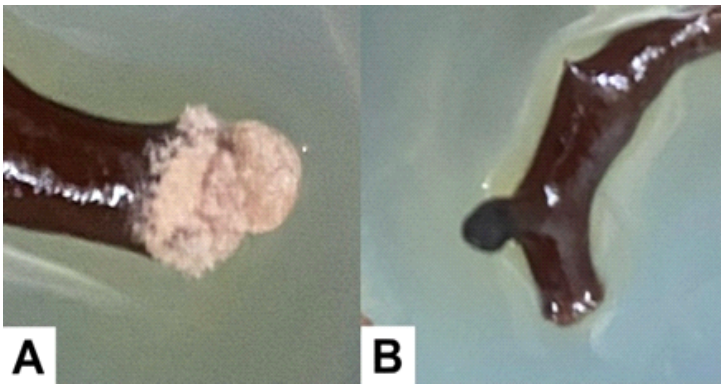

Figure 3. Callus structure of *K. alvarezii* under treatment F (A) and treatment B (B).

particular, have been associated with increased production of reactive oxygen species, which, if not adequately regulated, can lead to diminished cell viability and regenerative potential (Ozden & Karaaslan, 2011; Chen *et al*., 2019). These findings support the notion that an optimal, carefully calibrated range of PGR concentrations is essential not only for promoting dedifferentiation and cell proliferation but also for avoiding cytotoxic stress. Thus, maintaining this delicate hormonal equilibrium is fundamental to ensuring successful callus induction and the long-term viability of in vitro cultures.

The underlying mechanisms of callus induction involve complex processes, including histone modifications and transcription factor activity, in response to PGRs. Notably, studies in model plant systems have shown that high auxin levels induce significant lineage reprogramming, which is essential for callus formation and plant regeneration (Long *et al*., 2022; Ma *et al*., 2022; Hong *et al*., 2024). These findings suggest that the co-application of BAP and IAA plays a crucial role in the transcriptional regulation of genes involved in callus growth, promoting cell proliferation and differentiation (Ge *et al*., 2016; Ma *et al*., 2022). This is further corroborated by recent studies on other species, where auxin and cytokinin signaling pathways were shown to modulate gene expression related to callus formation and differentiation, providing new revelations into the molecular mechanisms of tissue culture (Perianez-Rodriguez *et al*., 2014; Long *et al*., 2022). In addition, comparative studies across species emphasize the need for species-specific PGR regimens, as validated in this *K. alvarezii* research. The differential responses of callus tissues to varying PGR concentrations highlight the importance of careful calibration for protocol optimization (Meiyana *et al*., 2023; Nie *et al*., 2023). This aligns with the work of Bennur *et al*. (2025), who emphasized that PGR regimens must be tailored to the specific biological characteristics of the species in question for optimal tissue culture outcomes. These findings also underscore the significance of adjusting PGR concentrations based on the specific requirements and responses of plant tissues during callus induction (Suryati *et al*., 2015; Maradhy *et al*., 2022).

These findings illuminate the critical role of an appropriate auxin–cytokinin balance in promoting effective callus formation. However, it is significant to note the potential risks of somaclonal variation in callus-derived plants, which could affect the genetic stability of regenerated tissues. Despite this limitation, the optimized PGR conditions provide a valuable foundation for advancing tissue culture techniques and propagation efficiency. Additionally, the insights gained from this study offer exciting prospects for the application of genetic engineering strategies, which can enhance the genetic improvement of *K. alvarezii* in seaweed biotechnology. Future research should explore the regeneration potential of induced callus and assess the genetic fidelity of plantlets, thereby enabling the development of stable and commercially viable propagation protocols.

CONCLUSION

The optimal condition for callus induction in *K. alvarezii* was achieved with treatment F, comprising 1.50 mg/L IAA and 5 mg/L BAP. This treatment resulted in the highest callus formation rate of 88%, with initiation observed on day 38. The induced callus was characterized by a white, crystalline morphology, indicating healthy and active tissue development.

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