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CALLUS INDUCTION AND FILAMENTS REGENERATION FROM CALLUS OF COTTONII SEAWEED (*Kappaphycus alvarezii* (Doty) COLLECTED FROM NATUNA ISLANDS, RIAU ISLANDS PROVINCE

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ABSTRACT

The objective of this study was to obtain the optimal medium for callus induction from thallus explants of *Kappaphycus alvarezii (*Doty*)* and to regenerate filamentous callus from induced callus. Before cultured, cottonii seaweeds collected from the Natuna Islands (Riau Islands Province) were acclimatized in greenhouse and in semi-sterile culture in the laboratory. Sterilized explants were cultured on PES and Conwy media solidified with 0.8% Bacto Agar. In each of these media two combinations of plant growth regulators i.e. BA+IAA and BA+NAA were added. The concentrations of BA used were 0, 0.5, 1 mg/l, the concentrations of IAA were 0, 2.5, 5 mg/l, whereas the concentration of NAA were 0, 0.5, 1 mg/l. The result indicated that the optimal medium for callus induction was PES solidified medium supplemented with BA 1 mg/l. Types of callus formed were (a) white compact callus, (b) white filamentous callus, (c) greenish/brownish callus. Regeneration of callus into clumps of filament had been done by subculturing the callus into PES solidified medium supplemented with BA 1 mg/l + IAA 2.5 mg/l.

Key words: tissue culture, callus induction, filamentous callus, seaweed, *Kappaphycus alvarezii*

INTRODUCTION

Cottonii seaweed or *Kappaphycus alvarezii* is one type of carrageenan-producing seaweed massively cultivated in Indonesia. The Ministry of Marine Affairs and Fisheries of Republic of Indonesia continues to attempt the development of seaweed farming and cottonii seaweed processing industry. To support the efforts to increase the production of this seaweed, continuous availability of quality seeds is required.

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Since cultivated in the 1970s, seeds of cottonii seaweed are obtained from vegetative propagation. Repeated clonal propagation caused a decrease in genetic variability resulting in decreased growth rate, carrageenan yield and gel strength. In addition, the decrease in variability also causes increased susceptibility to disease (Hurtado & Cheney 2003).

Application of tissue culture techniques in seaweed is expected to help produce a superior clone, thereby increasing the provision of seeds required for cultivation and to produce uniform seedlings in large quantities but in a short time. Generally, seaweed tissue culture stages include preparation of axenic explants, callus induction and regeneration of callus into thallus and young plantlets of seaweed. The objective of this study was to obtain the optimal medium for callus induction from thallus explants of *K. alvarezii* collected from Natuna Islands, Riau Islands Province, Indonesia. to regenerate filamentous callus from induced callus.

MATERIALS AND METHODS

Seaweed acclimatization.

Seaweeds were collected twice from the Natuna islands, first on April 20 and the second on May 20, 2010. To keep it fresh during the journey to Bogor, seaweed was inserted into cardboard boxes in a quite wet condition. Every two clumps of seaweeds were placed separately from each other on the boxes, while old newspapers were used as partition.Then the cardboard boxes were closed and inserted into a sack tied using raffia.

To reduce mortality at the time of semi-sterile culture in the laboratory, new seaweed brought from the sea need to be acclimatized and maintained in a clean and controlled environment in greenhouse before the semi-sterile culture in the laboratory. The purpose of acclimatization was to adapt the seaweed from the sea to the new environment of the aquarium placed in greenhouse. Seaweeds were maintained in the aquarium containing seawater as much as 7/8 part of the aquarium sized 30 cm wide x 35 cm high x 90 cm long. Using recirculation system, seawater in the aquarium was flown into the container filter consisting layers of fiber glass wool, activated carbon and coral. Seawater that had been filtered was reflown using a pump and PVC pipe to the aquarium. The aquaria also were equipped with an aerator, and shaded by 65% shading net.

After 1 month, the seaweed with light green color, clean from dirt and epiphyte were acclimatized in semi-sterile culture in the laboratory. Seaweed was washed using seawater which was sterilized by autoclave. The dirt was cleaned gently using a soft brush. Mucus attached to the seaweed was cleaned by rinsing 2-3 times with sterilized seawater. Subsequently, the seaweeds were cut into pieces $\pm$ 10 cm long and cultured in 10-liter glass jar, which already contains Conwy (CW) liquid medium (Liao *et al.* 1983). Before use , the culture media were sterilized by autoclave at temperature of 121ºC for 1 hour. The experiment was repeated in 3 jars, each jar contained 4 liters of media and 150 g of seaweed. After 3 weeks, the weight of still alive seaweeds was

measured again to obtain the survival rate of seaweed (%) in the semi-sterile culture system.

The cultures were placed on culture rack, illuminated with fluorescent lamps at 1500 lux of light intensity with a 12:12 light and dark cycle. The room temperature was set between 22-25ºC. In the first 2 weeks, the media was replaced 2 times a week with fresh media. After 2 weeks, the media was replaced once a week, for another 3 weeks.

Preparation of axenic explants

Preparations of axenic explants in this study were mostly obtained according to methodology described by Reddy *et al.* (2003). The apical parts of thallus were selected from semi-sterile culture to be used as explants for callus induction. Explants were taken from the cultures age 5 weeks and 1 week. Thalli were cut into pieces with a length of 4-5 cm. Subsequently, the explants were soaked in Tween solution (5 drops in 200 ml) for 10 minutes while occasionally shaken, and then the explants were rinsed twice with sterilized seawater. Explants then were soaked in 0.5% povidone iodine (Betadine) (0.5 ml in 100 ml of sterilized seawater) for 3 minutes. After that, the explants were rinsed 3-4 times with sterilized seawater. Explants were then dried with sterilized tissue papers and immersed in CW medium containing 3% antibiotics mixture (penicillin G 1 g, streptomycin sulphate 2 g, kanamycin 1 g, nystatin 25 mg, Neomycin 200 mg in 100 ml distilled water). The cultures were shaken with a shaker for 60 hours, at room temperature of 22-25ºC and illuminated with fluorescent lamps at 1500 lux of light intensity with a 12:12 light and dark cycle. Then the explants were washed with sterilized seawater, and dried with sterilized tissue papers then planted in the CW medium solidified with 0.8% Bacto agar. Observations were carried out to record the condition of explants and the percentage of contaminated explants.

Callus induction

Explants that had been observed for 2 weeks and did not contaminate were used as explants for callus induction. Explants were cut into pieces with length of 4-5 mm. Each explant then was wiped gently with sterile tissue papers to remove moisture and any mucilaginous substances exuded from the cut ends. Then explants were planted on treatment media for callus induction.

To investigate the optimal basic culture media, explants were cultured on Provasoli enriched seawater/PES (Provasoli 1968) and Conwy/CW (Liao *et al.* 1983) media solidified with 0.8% Bacto Agar. In each of these media two combinations of plant growth regulators i.e. BA+IAA and BA+NAA were added. The concentrations of BA were 0, 0.5, 1 mg/l, the concentrations of IAA were 0, 2.5, 5 mg/l, whereas the concentrations of NAA were 0, 0.5, 1 mg/l. Each treatment was done with 10 replications. The cultures were stored in a culture room, with room temperature between 22-25ºC, and 60-70% of relative humidity. The cultures were illuminated with fluorescent lamps at 1500 lux of light intensity with a 12:12 light and dark cycle.

Observations on callus growth were done by giving a rating (score). Quantity of callus growth was determined in 6 values representing 6 categories of callus growth, namely: 0 = no callus induction, the explants were bleached ; 1 = quantity of callus growth was very low; 2 = quantity of callus growth was low; 3 = quantity of callus growth was medium, 4 = quantity of callus growth was high; and 5 = quantity of callus growth was very high. Data on callus growth were analyzed with non-parametric statistical test of Kruskal-Wallis using SPSS 16.0 software. The Dun's multiple comparison test was used to distinguish treatments with significant differences.

Regeneration of filamentous callus

After 6 weeks in treatment media of callus induction, all explants were subcultured in PES solid medium containing 1 mg/l BA + 2.5 mg/l IAA for 2 months to enhance callus growth. Subsequently, all callus outgrowth were excised from the explants and subcultured separately in PES solid medium containing 1 mg/l BA + 2.5 mg/l IAA for another 2 months to regenerate filamentous callus.

RESULTS AND DISCUSSIONS

Seaweed acclimatization

Installation of seaweed maintenance equipment in the greenhouse, was good enough to maintain the life of seaweeds brought from the sea. Cottonii seaweed harvested from Natuna Islands, on 20th of May 2010 had survived up to one month, with 20% of mortality rate. The highest mortality occurred in the first two weeks, where the seaweed suffered stress during travel distance from the sea to the laboratory and the influence of packaging. After 3 weeks maintained in greenhouse, the tip of thallus began to grow. It was characterized by the formation of light green buds at the tip of thallus.

Installation of seaweed maintenance equipment in the greenhouse is useful to adapt the seaweed to the new environment (acclimatization). It also can be used to store seaweed stock for a long time as source of explants for seaweed tissue culture studies and genetic improvement to produce quality seeds of seaweed.

Observations after three weeks maintained in semi-sterile culture showed that survival rate of seaweeds that came from greenhouse was 72%. In our first experiment, seaweeds brought directly from the sea were acclimatized in the semi-sterile culture. As a result, the survival rate of seaweeds was 0-25%. This indicated that the acclimatization of seaweed *K. alvarezii* in greenhouse, succeeded to increase the survival rate of seaweed in acclimatization phase in semi-sterile culture in the laboratory.

Due to long distance transportation from Natuna Islands to Bogor, which took two days one night and humid condition of packaging, cottonii seaweed produced a lot of mucus. The mucus caused the seaweed thallus to bleach and die.. In addition, the high concentration of mucus in the static culture may also be a factor causing the death of seaweed.

Based on these results, the new seaweeds brought from the sea need to be adapted first and maintained in a cleaner and controlled environment in the greenhouse before semi-sterile culture in the laboratory. Acclimatization in a greenhouse with a recirculation system could refresh the seaweed conditions which suffered stress during delivery. In addition, the mucus contained in the seaweed could be reduced by recirculation system of water management. Seawater in the aquarium was flown into the filter container to clean the dirt and slime carrying bacteria, then the filtered seawater was reflown to the aquarium.

Acclimatization stages in laboratory was very important to be optimized, because the sterilization of explants to obtain axenic explants could be more easily obtained from explants that had been acclimatized first in laboratory than seaweed explants collected directly from the sea. In addition, rate of callus induction would be higher when thallus explants were used that had been acclimatized first in the laboratory (Reddy *et al.* 2003).

Preparation of axenic explants

The number of explants that had been sterilized was 283 explants. Observation data 2 weeks after planting indicated that explants which were acclimatized 5 weeks in semi sterile culture produced higher sterile explants (64.5%) than the explants that has been acclimatized 1 week (43.4%). All contaminations were caused by bacteria. However, the explants remained green during the entire section, where for 1 week old explants (44%) the percentage was higher than for 5-week old explants (27.4%). The rest changed color to brown (browning) or white (bleaching) as a sign that explants were dead, because 5-week-old explants had more young thallus with a diameter <4 mm. These thalli were the result of seaweed growth during acclimatization on semi-sterile culture which had smaller diameter than thallus of seaweed which grows in the sea (Fig. 1). While for 1 week old explants grown on semi-sterile culture, thalli were originally derived from the sea, and had larger diameter between 4-5 mm. These thalli had a higher resistance to the sterilization process than <4 mm diameter of thallus explants (Muñoz *et al.* 2006).

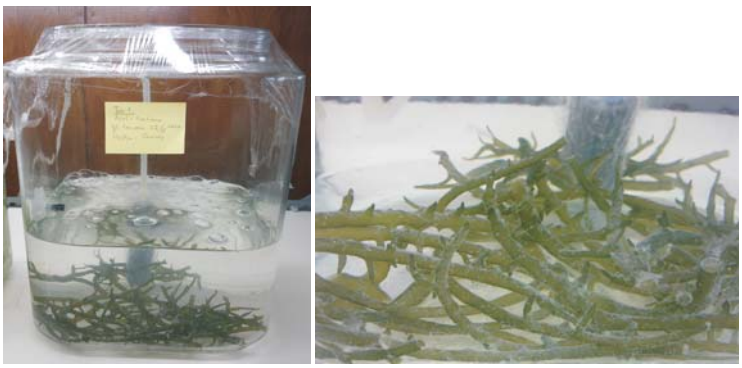

Figure 1. Cottonii seaweed on CW medium has survived and grown up to 5 weeks after planting in semi-sterile culture.

Callus induction

The most rapid callus induction occurred at 14 days after planting in the media treatment, while the slowest occurred at 28 days after planting. Explants which did not show the growth of callus over 28 days after planting become bleached. The growth of callus usually occurred in medullar region (Fig. 2A), but also on cortical regions in some explants i.e. the skin surface of the thallus and the tip of apical thallus (Fig. 2B). Most explants in PES medium formed callus faster than in CW medium. The average time required for callus induction on PES media was 18 days, while for CW medium it was 22 days (Table 1).

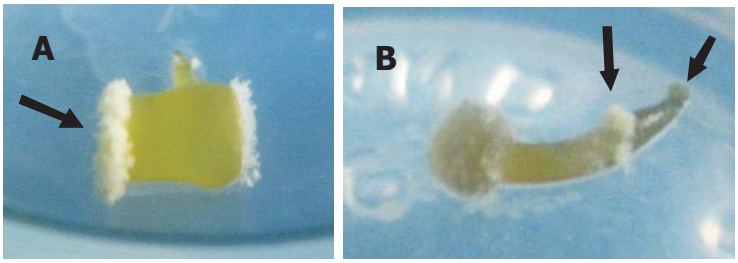

Figure 2. The location where the callus began to grow on thallus explants namely: (A) in the medullar region; (B) in the cortical region and apical thallus tip.

Tabel 1. Average time required for callus induction in PES and CW media supplemented with combination of BA + IAA and BA + NAA.

Concentration of PGR	Time required for callus induction (days) in basic medium :	
	CW	PES
Without PGR	16	15
BAP 0 mg/l + IAA 2.5 mg/l	19	14
BAP 0 mg/l + IAA 5 mg/l	NC	16
BAP 0.5 mg/l + IAA 0 mg/l	22	18
BAP 0.5 mg/l + IAA 2.5 mg/l	24	19
BAP 0.5 mg/l + IAA 5 mg/l	25	NC
BAP 1 mg/l + IAA 0 mg/l	26	22
BAP 1 mg/l + IAA 2.5 mg/l	26	15
BAP 1 mg/l + IAA 5 mg/l	25	15
BAP 0 mg/l + NAA 0.5 mg/l	25	15
BAP 0 mg/l + NAA 1 mg/l	20	25
BAP 0.5 mg/l + NAA 0 mg/l	22	18
BAP 0.5 mg/l + NAA 0.5 mg/l	15	15
BAP 0.5 mg/l + NAA 1 mg/l	21	21
BAP 1 mg/l + NAA 0 mg/l	26	22
BAP 1 mg/l + NAA 0.5 mg/l	19	17
BAP 1 mg/l + NAA 1 mg/l	16	NC
Average time (days)	**22**	**18**

Note: NC = no callus induction and explants bleached

Six weeks after planting, PES medium generated an average rate of callus induction higher (35.6%) than in CW medium (31.7%) (Table 2). This result was different from the research of Suryati and Mulyaningrum (2009) which indicated that the CW medium had a better rate of callus induction than PES medium. This difference could be due to different sources of seaweed explants used, because according to George (1993) in addition to the culture medium, there are three factors that could affect growth and morphogenesis in tissue culture, namely genotype of explants source, environment of culture and tissue-dependent factor.

PES Medium had been widely reported to successfully establish and regenerate *K. alvarezii* callus into plantlets of young seaweeds (Reddy *et al.* 2003; and Muñoz *et al.* 2006). In addition to *K. alvarezii*, PES medium had also succeeded to induce and regenerate callus on other species of seaweed such as *Grateloupi* sp, *Carpopeltis* sp, *Ptilophora* sp (Huang & Fujita 1997), *Gracilaria* sp (Collantes *et al.* 2004; Kumar *et al.* 2007) , *Hypnea musciformis* and *Turbinaria conoides* (Kumar *et al.* 2007).

In addition to PES medium, research of Hayashi *et al.* (2008) showed that the induction of *K. alvarezii* callus had succeeded either by using the F/2 50 medium (seawater enriched with Guillard & Ryther 50% solution) and VS 50 medium (seawater enriched with 50% von Stosch's solution). Hurtado and Biter (2007) had done plantlet regeneration of *K. alvarezii* var. adik-adik by using ESS (Erd Schreibers Seawater) medium. While Hurtado *et al.* (2009) and Yunque *et al.* (2010) had established procedures for production of tissue culture seedlings of *K. alvarezii* with media AMPEP (Acadian Marine Plant Extract Powder).

The highest rate of callus induction was found in PES medium + BA 1 mg/l (70%) (Table 2). Explants that did not form callus generally bleached on the third week after planting. Explants which mostly bleached were thallus explants with diameter less than 3 mm. The addition of IAA or NAA produced a reduction on the average rate of callus induction, both on CW and PES media, as shown on most media added with auxins IAA or NAA had an average rate of callus induction lower than in media without auxins.

According to Bradley (1991) and Yokoya *et al.* (2010), some types of plant hormone naturally occur in seaweed tissue both auxin and cytokinin (endogenous Auxin/cytokinin), such as IAA, ABA (Absisic acid), PAA (Phenyl acetic acid), iP (isopentenyladenin), and CZ (cis-zeatin). The decrease rate of callus induction (%) due to the addition of auxin IAA/NAA was probably due to hormones content in thallus explants was quite optimal to form callus, so that the addition of plant growth regulator caused in excessive concentrations resulting in decreased rate of callus induction.

Negative influence of IAA on callus growth was probably caused by the use of too high concentration , while Suryati and Mulyaningrum (2009) found that the optimal concentration was 0.4 mg/l. Concentrations higher than 0.4 mg/l caused a decrease rate of callus induction.

Observations on callus growth on each treatment media was done by giving a rating (score). Quantity of callus growth was determined in 6 values representing 6 categories of callus growth. Examples of callus growth in each category were shown in Figure 3.

Table 2. Callus induction rate (%) on PES and CW media supplemented with combination of BA + IAA and BA + NAA.

Concentration of PGR	Average rate of callus induction (%) in basic medium	
	CW	PES
Without PGR	40.00	20.00
BAP 0 mg/l + IAA 2.5 mg/l	20.00	50.00
BAP 0 mg/l + IAA 5 mg/l	0.00	40.00
BAP 0.5 mg/l + IAA 0 mg/l	40.00	40.00
BAP 0.5 mg/l + IAA 2.5 mg/l	30.00	20.00
BAP 0.5 mg/l + IAA 5 mg/l	40.00	0.00
BAP 1 mg/l + IAA 0 mg/l	30.00	70.00
BAP 1 mg/l + IAA 2.5 mg/l	30.00	50.00
BAP 1 mg/l + IAA 5 mg/l	30.00	30.00
BAP 0 mg/l + NAA 0.5 mg/l	20.00	30.00
BAP 0 mg/l + NAA 1 mg/l	30.00	50.00
BAP 0.5 mg/l + NAA 0 mg/l	40.00	40.00
BAP 0.5 mg/l + NAA 0.5 mg/l	10.00	50.00
BAP 0.5 mg/l + NAA 1 mg/l	40.00	50.00
BAP 1 mg/l + NAA 0 mg/l	30.00	**70.00**
BAP 1 mg/l + NAA 0.5 mg/l	60.00	10.00
BAP 1 mg/l + NAA 1 mg/l	40.00	0.00
Average rate (%)	**31.18**	**36.47**

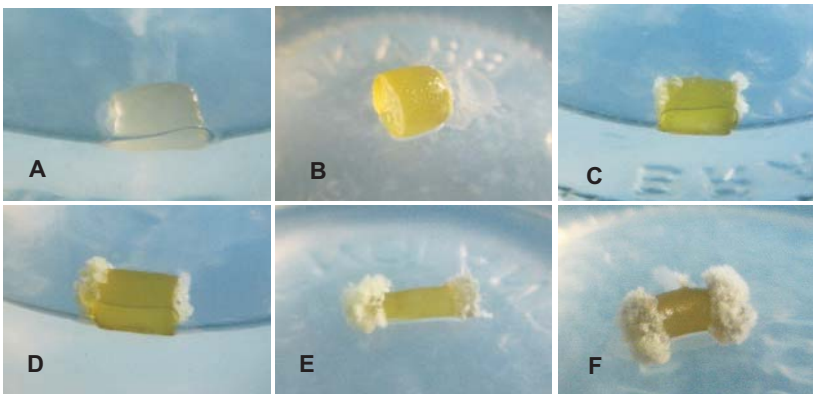

Figure 3. Callus represented by 6 values of callus growth, namely: (A) 0 = No callus induction, the explants become bleached; (B) 1 = quantity of callus growth was very low; (C) 2 = quantity of callus growth was low; (D) 3 = quantity of callus growth was medium, (E) 4 = quantity of callus growth was high; and (F) 5 = quantity of callus growth was very high (Scale bar = 3 mm).

The data of callus growth after 6 weeks in treatment media were analyzed with non-parametric statistical test of Kruskal-Wallis using SPSS 16.0 software. The results showed that the media types, the addition of plant growth regulator BA, IAA and NAA did not significantly affect quantity of callus growth of Cottonii seaweed. Similarly, the interaction between these factors had no significant effect on quantity of callus growth ($p < 0.05$).

The data on PES mediated callus growth were re-tested for statistical analysis of Krukall Wallis for two factors, namely the concentration factor of BA and IAA, and concentration factor of BA and NAA. Statistical test of data on callus growth in PES medium with BA and IAA treatment indicated that both of these factors and their interactions had no significant effect on callus growth ($p < 0.05$). Meanwhile, in PES medium with BA and NAA treatment, the results of statistical tests showed the two factors also had no significant effect, but the interaction of both factors significantly affected callus growth ($p < 0.05$). The highest mean rank of callus growth was PES medium + BA 1 mg/l without the addition of NAA. Based on the results of multiple range test of Dunn, the treatment was significantly different from other treatments ($p < 0.05$) (Table 3).

The addition of plant growth regulator was often used to increase the rate of callus induction (%) and callus growth in seaweeds (Dawes & Koch 1991; Huang & Fujita 1997; Yokoya & Handro 2004; Yokoya *et al.* 2004; Muñoz *et al* 2006; Hayashi *et al.* 2008). Plant growth regulators of auxin and cytokinin were used either individually or in combination. In this study, the addition of plant growth regulator BA + NAA and BAP + IAA in the medium did not significantly result in increased rate of callus induction and growth of seaweed *K. alvarezii*. Similar results of this study were also reported by Reddy *et al.* (2003).

Table 3. Effect of NAA and BA concentration on the growth of callus 6 weeks after planting

NAA Concentration (mg/l)	BA Concentration (mg/l)		
	0	0.5	1
0	37.85 *abc*	51.00 *abc*	58.50 *a*
0.5	42.50 *abc*	55.45 *abc*	32.60 *bc*
1	51.35 *abc*	51.25 *abc*	29.00 *c*

Note: Numbers followed by same letter are not significantly different based on Dunn's multiple range test ($p < 0.05$).

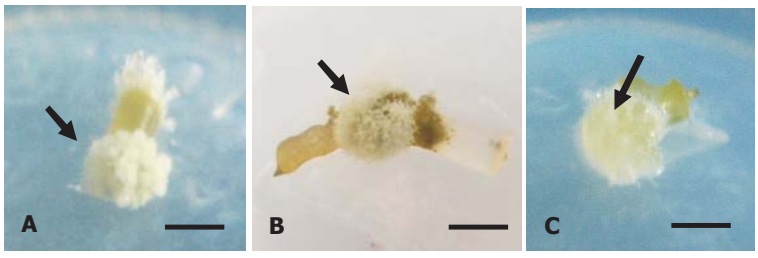

Figure 4. Types of callus formed after 6 weeks in callus induction media : (A) white compact callus; (B) white filamentous callus; (C) greenish/brownish callus (Scale bar = 4 mm).

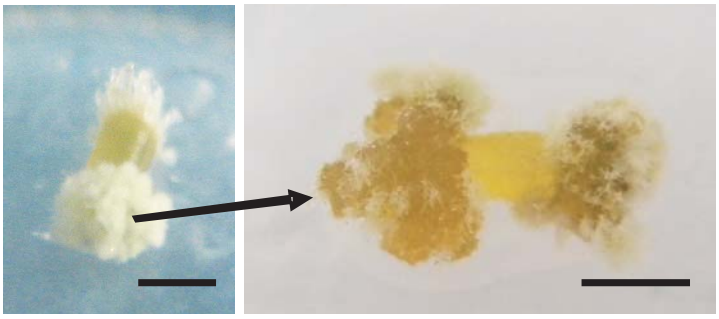

Figure 5. Changes of white compact callus to greenish/brownish callus after 2 months subcultured in PES medium added with BA 1 mg/l + IAA 2.5 mg/l (scale bar = 4 mm).

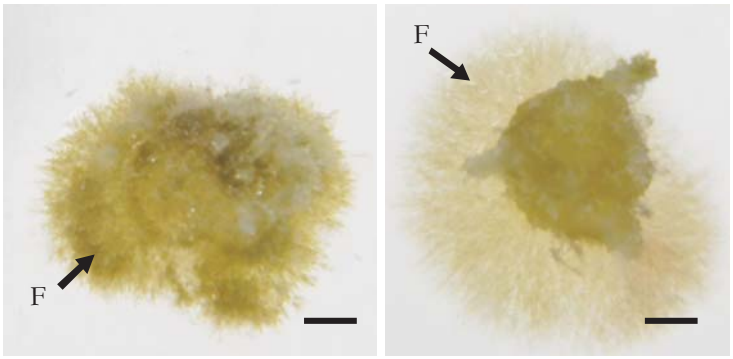

Figure 6. Growth of filaments clumps (F) on greenish/brownish callus 8 weeks after subcultured in PES medium +BA 1 mg/l + IAA 2.5 mg/ (scale bar = 2 mm).

The role of plant growth regulators (PGRs) on callus induction in multi-cellular algae such as seaweed is still debatable and showed no definite trend (Bradley 1991; Evan & Trewasvas 1991; Baweja *et al.* 2009; Yokoya *et al.* 2010). The inconsistency in responses to PGRs by seaweeds could be mostly due to lack of understanding of the physiological role of these substances in their growth and differentiation (Reddy *et al.* 2008).

Regeneration of filamentous callus

Based on the observations after 6 weeks on induction medium, there were several types of callus, namely: (a) white compact callus, (b) white filamentous callus, (c) greenish/brownish callus (Fig. 4). Filaments are rows of cells which form long chains. Generally in seaweed, the long chain of cells are not branched (unbranched filaments) except of Oscillatoriaceae species (Lobban & Harrison 1994).

After 6 weeks in the treatment media for callus induction, all of formed callus were sub-cultured into PES medium + BA 1 mg/l + IAA 2.5 mg/l in order to enhance callus growth. After 2 months in the media, the appearance of callus changed. The

white compact callus turned into greenish/ brownish friable callus with filaments that grow on it (Fig. 5).

The greenish/brownish callus was isolated from thallus explants then subcultured in PES medium + BA 1 mg/l + IAA 2.5 mg/l. After 2 months, greenish/brownish filaments in large quantities grown on the callus. Growth of these filaments aside from growing on the surface of the callus also grow at the bottom of the callus penetrating the culture medium (Fig. 6).

PES medium supplemented with BA and IAA had successfully regenerated callus into more clumps of filaments. Filament formation is the initial phase of young seaweed formation (Shao *et al.* 2004). Addition of BA and IAA also had been reported to regenerate callus into young seaweed as in the study of Huang and Fujita (1997) and Hayashi *et al.* (2008).

CONCLUSIONS

The procedures of callus induction and filaments regeneration from callus of cottonii seaweed (*Kappaphycus alvarezii)* derived from Natuna Islands (Riau Islands Province) were as follows:

1. Due to long distance transportation, seaweed brought from the sea has to be acclimatized in greenhouse for 4 weeks, then 1 week in laboratory before cultured in medium of callus induction
2. The most effective medium for callus induction of *K. alvarezii* seaweed was PES medium supplemented with BA 1 mg/l.
3. Regeneration of callus into clumps of filaments could be done in PES medium supplemented with BA 1 mg/l and IAA 2.5 mg/l.

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