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Effect of plant growth regulators on direct regeneration and callus induction from *Sargassum polycystum* C. Agardh

Siti Nor Safriena Muhamad[1] · Anna Pick-Kiong Ling[2] · Ching-Lee Wong[1]

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Abstract

Seaweed tissue culture is one of the potential technologies that can be used to increase seaweed production to cope with increasing demand. Seaweed tissue culture can be divided into two methods, namely, direct regeneration and callus induction (indirect regeneration). These methods provide several advantages, including generating seaweed cultures that are disease-free and able to mature faster to support mass production. The present study aims to develop a suitable protocol for tissue culture of *Sargassum polycystum* C. Agardh. We investigated different methods of sterilizing the stipe, stolon and leaf explant surfaces to allow for axenic tissue growth. In addition, we also investigated the effects of different plant growth regulators (PGRs) on seaweeds that are grown via direct regeneration and callus induction method. Our results showed that a combination of physical (brushing using soft brush under microscope) and chemical treatments (detergent, povidone iodine (PI) and antibiotic solution of streptomycin sulphate) was most suitable for growing axenic stipe of *S. polycystum*. For obtaining axenic leaf and stolon explants, the best treatment option was using germanium oxide (GeO_2) as a pre-treatment, followed by treatments with detergent, PI and streptomycin sulphate antibiotic solution. We tested the effects of nine different plant growth regulators on the growth of tissue explants, which include indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-napthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), picloram, forchlorfenuron (CPPU), uniconazole, 6-benzylaminopurine (BAP) and kinetin. Results showed that only bud explant was able to induce callus growth when treated with kinetin (4.65 μM and 13.94 μM) and uniconazole (3.00 μM, 7.00 μM and 10.00 μM). In addition, only stipe explant showed regeneration of adventitious bud when grown in the absence of plant growth regulators.

Keywords *Sargassum polycystum* · *Phaeophyceae* · Adventitious bud regeneration · Callus induction · Plant growth regulator · Seaweed tissue culture

Introduction

Commercial seaweed mariculture activity is widely carried out in 42 countries (Kaur and Ang 2009). Marine seaweed cultivation is an important economic activity that has contributed to the economic growth in many parts of Southeast Asia (Krishnaiah et al. 2008). There are many factors that can affect industrial seaweed farming such as seaweed species, purpose of farming, market price, and scale of operation. In 2015, 24 million tonnes were produced via seaweed farming (Radulovich et al. 2015) with 99% occurring in merely eight Asian nations (China, Indonesia, Philippines, Republic of Korea, Japan, Korea DRP, Malaysia, and Vietnam). There are massive increases of aquatic plants which contributed 29.4 million tonnes (27.7%) to the global aquaculture production in 2015 (Food and Agriculture Organization 2017) to meet the demands of phycocolloids production, food and emerging seaweed-based pharmaceutical, nutraceutical, and biofuel industry with total value of 6701 million US$. Recently, seaweeds have also been used as liquid fertilizer and a source of biofuel production.

Brown seaweeds are edible and have been reported to contain various nutritional values. Their benefits to human health have been recognized for thousands of years and some studies

✉ Ching-Lee Wong
chinglee.wong@taylors.edu.my

[1] School of Biosciences, Taylor's University, Taylor's Lakeside Campus, No.1, Jalan Taylor's, 47500 Subang Jaya, Selangor, Malaysia

[2] Division of Applied Biomedical Sciences and Biotechnology, School of Health Sciences, International Medical University, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Kuala Lumpur, Malaysia

propose that these seaweeds have potential to become a staple food in the future (Kumar et al. 2015). The brown color of this seaweed can be attributed to a xanthophyll pigment known as fucoxanthin, which masks the colors of other pigments such as chlorophyll *a*, chlorophyll *c*, and beta-carotene. The secondary metabolites extracted from *Sargassum polycystum* C. Agardh have shown a broad spectrum of biological activities.

There are many potential problems that may arise when developing large-scale seaweed farming (Titlyanov and Titlyanova 2010). For example, due to the large areas occupied by seaweed farms, shipping and fishing activities at the farming area may be disrupted. In addition, this may cause irreparable damage to natural aquatic and terrestrial ecosystems. Hence, the tissue culture method is recommended for culturing of industrially important seaweeds.

Seaweed tissue culture was first introduced by Chen and Taylor (1978), where they successfully demonstrated the totipotency of the red algae, *Chondrus crispus* Stackhouse, using the tissue culture method. Tissue culture can enable mass propagation of disease-free seaweeds and promote rapid propagation of species that are difficult to grow via conventional methods. Successful cultivation of seaweed via tissue culture will allow for production of seedling stock for cultivation and provide constant supply of selected breeds with desired characteristics (Reddy et al. 2008). Despite all the advantages, the development of seaweed tissue culture is much slower compared to that of terrestrial plants (Baweja et al. 2009). The knowledge on seaweed tissue culture is still rather limited. For example, there is a lack of study on the development of suitable surface sterilization protocols for each species of seaweed and also the suitable medium for culturing the seaweed in either obtaining callus formation or regeneration of explants. It is difficult to maintain sterility of the seaweeds to be used in tissue culture because in contrary to land plants, seaweeds do not have a waxy cuticle that makes them fragile towards some of the chemical sterilants (Baweja et al. 2009). Generally, for successful seaweed tissue culture, there are few important factors to be considered such as surface sterilization protocol, media composition and types, hormone combination and concentration, and culture condition. These factors varied between species and are also affected by the locations where the species are collected. Since 1987 until 2017, only 12 species of brown seaweed have been studied and five of them are from *Sargassum* species [*S. confusum* C. Agardh, *S. fluitians* Borgesen (Borgesen), *S. horneri* Turner (C. Agardh), *S. muticum* Yendo (Fensholt), and *S. tenerrimum* J. Agardh] (Fisher and Gibor 1987; Polne-Fuller and Gibor 1987; Kirihara et al. 1997; Kumar et al. 2007; Uji et al. 2015). Based on reviews by Bradley (1991) and Baweja et al. (2009), there are several issues related to tissue culture of brown seaweed that need to be further investigated, such as establishing suitable surface sterilization protocols to obtain axenic explants, determining the best part of the explants, that is, suitable for tissue culture and micropropagation, culture medium, culture condition, effect of plant growth regulators, and time taken for the explants to form bud or callus (Polne-Fuller and Gibor 1987; Kumar et al. 2007; Baweja et al. 2009; Uji et al. 2015).

Thus, this study aims to develop a suitable protocol for tissue culture of *S. polycystum* by determining the most suitable surface sterilization protocol for the formation of axenic culture of stipe, stolon, and leaf explants and determining the best plant growth regulators (PGR) for direct regeneration and callus induction. The results obtained from *S. polycystum* tissue culture can be used for the establishment of large-scale seaweed propagation, hence preventing overharvesting and destruction of natural seaweed population.

Materials and methods

Plant materials *Sargassum polycystum* was collected from Teluk Kemang (2° 26′ 23.03″ N, 101° 51′ 25.2″ E), Port Dickson during low tide (≤ 0.5 m above the sea level). The seaweed was placed on ice during transportation to the laboratory and kept in an aquarium for a maximum of 3 weeks. For the *S. polycystum* tissue culture, healthy seaweeds, defined as those free of any visible epiphytes, were selected.

For this study, the explants used were the stipe, leaf, and stolon. Stipes from the primary branch were selected for use and all the leaves were cut off before the washing process. The leaves from the top of the primary and secondary branches were selected, as they were juvenile and cleaner compared to the ones at the bottom of the branch.

Culture medium preparation and culture condition The medium used for this study was half-strength Provasoli's enriched seawater (PES) (Starr 1978), pH 8.4. This was prepared by adding Provasoli solution to autoclaved artificial seawater with the salinity of 35 psu. The artificial seawater was prepared by adding 35 g of artificial sea salts (Instant Ocean, Aquarium System) into 1 L of ultrapure water. One liter of half-strength PES medium was prepared by adding 10 mL of PES solution to 990 mL of sterilized seawater (Starr 1978). The media were adjusted to the required pH using 0.1 M HCl. For this study, 10.0 g L^{-1} of agar powder and 30 g L^{-1} sucrose were added to the medium. This PES medium was supplemented with plant growth regulator(s) accordingly based on the objective of the study. All components used for the preparation of medium were autoclaved except for the plant growth regulators. All PGRs were filtered sterilized using a 0.22-μm filter and added to the autoclaved media individually. PES medium was left to solidify in sterilized Petri dishes (90 mm × 15 mm) with the volume of 20 mL per Petri dish. For the liquid media, preparation was similar to the solid medium except that no agar powder was

supplemented into the medium. The medium was prepared in a 20-mL conical flask, with 5 mL liquid medium in each flask.

All explants used in this study were cultured at 22 ± 1 °C under the cool white fluorescent tube light at 25–35 μmol photons m^{-2} s^{-1}, 12:12 light and dark photoperiod and with humidity of 60–70%, unless stated otherwise. When calluses or buds have successfully formed, they were subcultured regularly at least once a month to sustain their growth.

Preparation of axenic culture Four types of chemical sterilants were used: germanium oxide (GeO_2) (Sigma, USA), povidone iodine (PI) (Sigma, USA), liquid detergent (Chemsoln, USA), and streptomycin sulphate (590.60 IU mg^{-1}; Amresco, USA). All chemical sterilant solutions were prepared using autoclaved sterilized salt water except for the streptomycin sulphate solution. Four surface sterilization protocols modified from Kumar et al. (2004) as outlined in Table 1 were tested to determine the most suitable surface sterilization protocol for each explant (stipe, stolon, and leaf). For each protocol, the explants were not rinsed between the chemical treatments of surface sterilization. The protocol that produced the highest percentage of sterilized explants was further used for the entire study. Epiphytes and other microscopic contaminants were removed by manual brushing under a stereoscopic or dissecting microscope and were washed several times with sterilized artificial seawater to remove all the dirt and mucilage prior to surface sterilization. Selected healthy vegetative parts were cut into fragments of 4–6 cm in length for the sterilization process. For each protocol, five explants were cultured on each Petri dish with a replication of ten Petri dishes for each individual experiment. Each experiment was repeated three times for every protocol. This experimental setup provided a population size (*N*) of 150.

Table 1 Surface sterilization protocols

Protocols	Treatment	Concentration	Time
A	Detergent	1.0% *v/v*	10 min
	Povidone iodine (PI)	2.0% *w/v*	5 min
	Antibiotic	3.5% *w/v*	48 h
B	Detergent	1.0% *v/v*	10 min
	Povidone iodine (PI)	5.0% *w/v*	3 min
	Povidone iodine (PI)	2.0% *w/v*	5 min
	Antibiotic	3.5% *w/v*	48 h
C	Germanium oxide (GeO_2)	10 mg L^{-1}	24 h
	Detergent	1.0% *v/v*	10 min
	Povidone iodine (PI)	2.0% *w/v*	5 min
	Antibiotic	3.5% *w/v*	48 h
D	Germanium oxide (GeO_2)	10 mg L^{-1}	72 h
	Detergent	1.0% *v/v*	10 min
	Povidone iodine (PI)	2.0% *w/v*	5 min
	Antibiotic	3.5% *w/v*	48 h

Determination effects of plant growth regulators (PGRs) on *S. polycystum* explants The PGRs used for this study were divided into three groups which were auxin [indole-3-acetic acid (IAA) (Duchefa Biochemie, Netherlands); 1-napthaleneacetic acid (NAA) (Duchefa Biochemie, Netherlands); indole-3-butyric acid (IBA) (Duchefa Biochemie, Netherlands); 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa Biochemie, Netherlands); picloram (Sigma, India)]; cytokinin [6-benzylaminopurine (BAP) (Chemsol, England); kinetin (R&M, UK), forchlorfenuron (CPPU) (Sigma, Israel)]; and growth retardant [uniconazole (Sigma, Switzerland)]. The PGR concentrations used were IAA (5.70, 17.13, 28.54 μM); NAA (5.37, 16.11, 26.85 μM); IBA (4.92, 14.76, 24.60 μM); 2,4-D (4.52, 13.57, 22.62 μM); picloram (1.00, 3.00, 5.00 7.00, 10.00 μM); BAP (4.44, 13.32, 22.20 μM); kinetin (4.65, 13.94, 23.23 μM); CPPU (1.00, 3.00, 5.00, 7.00, 10.00 μM); and uniconazole (1.00, 3.00, 5.00, 7.00, 10.00 μM). The PGRs were tested on stipe, leaf, stolon, and bud explants (adventitious bud regenerated on stipe explants).

The effect of plant growth regulator on stipe, leaf, and stolon explants were quantified based on elongation of the explant without any callus induction or adventitious bud regeneration. The data of the effect of plant growth regulators on stipe, leaf, and stolon size elongation were not presented in this paper. The adventitious buds grew on the stipe explants without any intervention of plant growth regulators in the medium. Five plates of medium without PGRs with four explants for each plate were cultured to multiply the growth of adventitious buds on stipe explants. A total of 20 explants were cultured on the medium without PGRs and 10 explants (50%) showing the growth of adventitious buds. The number of buds increased every week. Observation was conducted until 6 weeks of culture, with the result showing that the average multiplication of adventitious buds per plate was about 70% per week (Eq. 3). To study the effect of plant growth regulators, the adventitious buds that grew on the stipe were cut (notify as bud explants) (Fig. 1) and cultured on medium with plant growth regulators. For each PGR, the individual experiments were repeated three times. Each individual experiment consisted of ten replications of Petri dishes with five explants in each Petri dish. The bud explants were cultured on the solid and liquid medium.

The effect of PGRs on bud explants were determined by observing the number of callus formed and measuring the

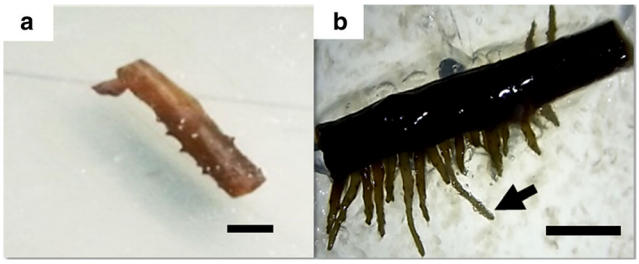

Fig. 1 Explants tested for PGRs effect. **a** Stolon explant (size 2 cm in length; bar = 1.0 mm); **b** stipe with adventitious buds formation was cut into smaller parts and cultured on solid and liquid medium and noted as bud explants (arrow: adventitious bud, bar = 5.0 mm)

multiplication of buds that branched or regenerated during the culture period (6 weeks). Three bud explants were cultured in each Petri dish or conical flask with three replications and three repeated individual experiments. This experimental set-up provided a population size (*N*) equaled to 27 bud explants for each culture medium tested.

Statistical analysis For the surface sterilization protocols, the number of explants that survived and was free from contamination was observed and recorded 2 weeks after cultured. These explants were considered as surviving explants. The percentage of these explants was calculated according to Eq. (1). The numbers of adventitious buds and number of callus produced on the explants during PGRs study were

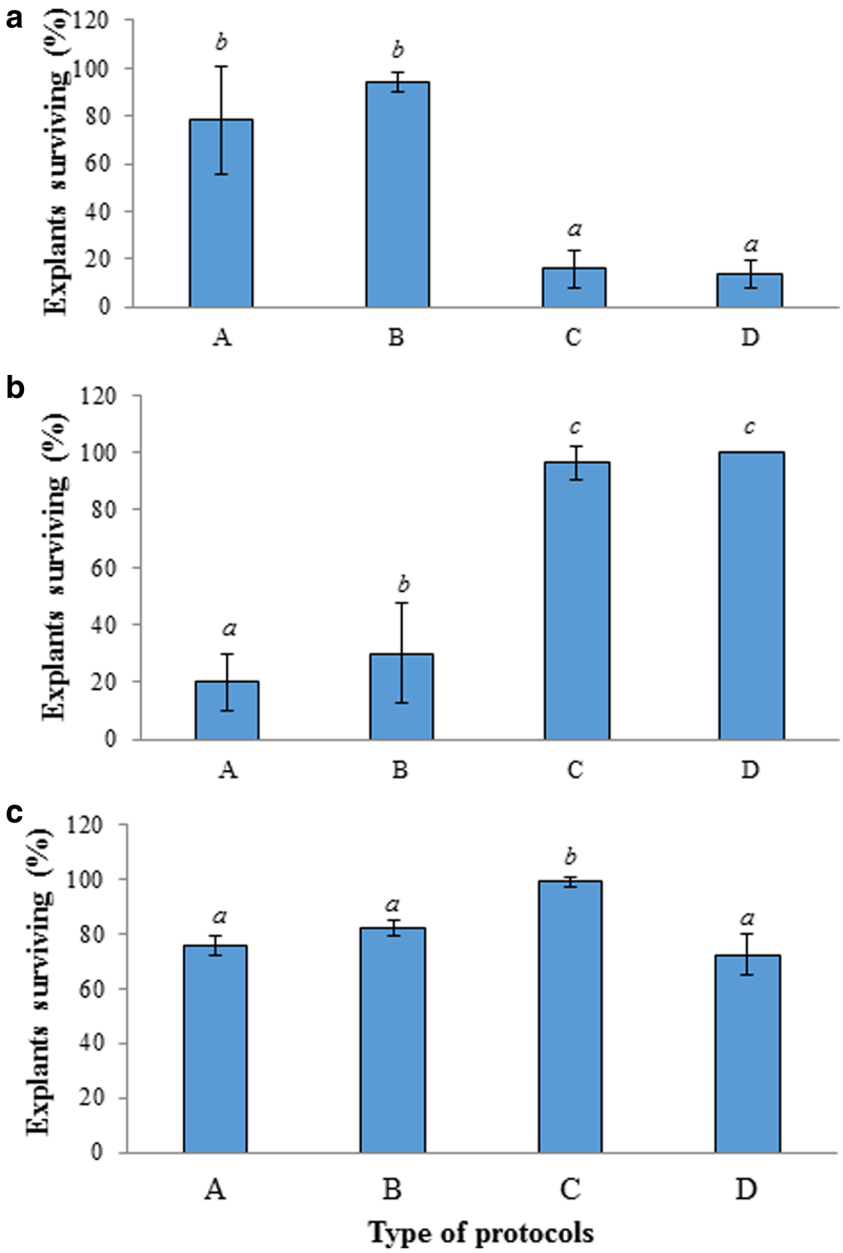

Fig. 2 Effects of different surface sterilization protocols on the percentage explants surviving of *S. polycystum*. **a** Stipe. **b** Leaf. **c** Stolon. Values represent the mean ± SD ($n = 5$, replication = 10, repeated = 3). Mean with different alphabet indicates significant different at level $p < 0.05$ according to one-way ANOVA post hoc Tukey's HSD

observed, recorded, and calculated for 6 weeks of culture as in Eqs. (2) and (3).

All data are presented as mean ± standard deviation (SD). Data was analyzed by using IBM SPSS software (Version 22.0), via one-way ANOVA and Tukey's post hoc test ($p < 0.05$).

$$\text{Sterilized explants (\%)} = \frac{\text{number of sterilized explants}}{\text{total number of explants cultured}} \text{ x } 100 \quad (1)$$

$$\text{Callus induction (\%)} = \frac{\text{number of bud explants with callus}}{\text{total number of bud explants cultured}} \text{ x } 100 \quad (2)$$

$$\text{Bud formation rate } (\%\text{week}^{-1}) = \left(\frac{\text{final number of buds grow} - \text{initial number of buds grow}}{\text{initial number of buds grow}} \text{ x } 100\right)/6 \quad (3)$$

Results

Surface sterilization Figure 2 shows the results of different surface sterilization protocols for stipe explants (Fig. 2a), leaf explants (Fig. 2b), and stolon explants (Fig. 2c) of *S. polycystum*. Each explant showed different degrees of viable axenic culture upon treatment with different protocols.

For stipe explants, protocol A (78.33 ± 22.55%) and B (94.00 ± 4.37%) generated significantly higher viable explants than protocol C (15.72 ± 8.10%) and D (13.33 ± 5.77%), but no significance was noted among protocol A and B. Protocol B (Fig. 2a) had the highest percentage of surviving axenic stipe explants at 15.67% higher than that of protocol A. The high fatality rate in protocols C and D was due to the pretreatment with germanium oxide which had led to tissue death. On the other hand, the fatality recorded from protocol A and B was likely due to both sterilization treatment and bacterial growth.

The most suitable protocol in getting axenic leaf explants for *S. polycystum* was protocol D (100 ± 0.00%) (Fig. 2b). All dead explants recorded were caused by bacterial contamination. There was no contamination by fungus recorded in the surface sterilization for leaf explant.

Figure 2c shows the results of screening treatment with different surface sterilization protocols on the stolon explants of *S. polycystum*. The highest axenic explants survival was obtained following treatment with protocol C (98.89 ± 1.93%), which was significantly higher than that recorded in other protocols. All explant fatality was due to bacterial contamination except for protocol D, where fatality was due to both tissue death and bacterial contamination. There was no fungus contamination observed in all treatments on stolon explants.

Effect of plant growth regulators on buds explants All results for the buds explants were tabulated in Table 2 for solid medium and Table 3 for liquid medium. There was no callus induced for all auxin treatments (Table 2). The highest bud formation was recorded in PES medium supplemented with 22.62 μM of 2,4-D (93.06 ± 17.35%), which was significantly higher ($F_{17,36} = 27.257$, $p = 0.0001$) than the bud formation rate recorded in other auxin treatments. The bud formation rate was increased when the concentration of 2,4-D increased. A similar trend was noted in the IBA treatment. For NAA treatment, no bud formation was observed when the NAA concentration was higher than 5.37 μM. For cytokinin treatment, callus induction was observed in PES medium supplemented with 4.65 μM and 13.94 μM of kinetin (Table 2). The highest bud formation was obtained following 4.65 μM kinetin treatment (32.22 ± 14.02%). Table 2 shows that the highest bud formation rate was when using 3.00 μM (40.97 ± 5.48%) and 7.00 μM (43.61 ± 3.94%) uniconazole and it is statistically significant ($F_{5,12} = 14.369$, $p = 0.0001$) compared to other uniconazole treatments. Callus induction was observed in the PES media supplemented with 3.00 μM, 7.00 μM, and 10.00 μM of uniconazole.

Table 3 shows the effect of different classes of PGRs on bud explants cultured in liquid medium. There was no callus observed in all auxins, cytokinins, and uniconazole treatments. The highest percentage of bud formation was recorded in media supplemented with 16.11 μM NAA (103.96 ± 31.71%). For IBA and picloram, the bud formation rate increased steadily when the concentration of the respective PGR was increased. For cytokinin treatment, the highest percentage of bud formation was recorded in media supplemented with 4.65 μM of kinetin (89.15 ± 31.66%) and it is statistically significant ($F_{11,24} = 18.960$, $p = 0.0001$) compared to other cytokinin treatments with the exception for 13.32 μM BAP (65.41 ± 0.42%) and 13.94 μM kinetin (60.21 ± 11.98%). For both CPPU and kinetin, bud formation decreased when concentrations of the respective PGRs increased. With regard to growth retardant treatment, the highest percentage of bud formation rate was recorded in the treatment supplemented with 1.00 μM (119.52 ± 21.19%) and 7.00 μM (97.86 ± 0.01%) uniconazole, which was significantly ($F_{5,12} = 27.165$, $p = 0.0001$) higher than that recorded in 3.00 μM (14.43 ± 10.18%) and 5.00 μM (56.74 ± 24.48%) uniconazole (Table 3).

Morphology of bud and callus induced from bud explants Figure 3 shows the buds that were cultured on solid PES media supplemented with plant growth regulators. The brownish color buds indicate viability while the white color buds indicate dead buds. Some buds were noted to have a

Table 2 Effect of PGRs on buds explants of *S. polycystum* cultured on solid medium

	Treatments	Concentration (μM)	Bud formation rate ± SD (% week^{-1})	No. of callus induced	Callus induction (%)
Control	PES	0.00	16.67 ± 0.00^{a}	NC	0.00
Auxins	2,4-D	4.52	3.18 ± 2.76^{a}	NC	0.00
		13.57	20.48 ± 3.43^{ab}	NC	0.00
		22.62	93.06 ± 17.35^{c}	NC	0.00
	IAA	5.71	21.76 ± 5.61^{ab}	NC	0.00
		17.13	4.94 ± 2.18^{a}	NC	0.00
		28.54	11.14 ± 1.51^{a}	NC	0.00
	IBA	4.92	23.611 ± 6.36^{a}	NC	0.00
		14.76	24.29 ± 1.65^{ab}	NC	0.00
		24.60	41.94 ± 3.37^{b}	NC	0.00
	NAA	5.37	13.70 ± 2.80^{a}	NC	0.00
		16.11	0.00 ± 0.00^{a}	NC	0.00
		26.85	0.00 ± 0.00^{a}	NC	0.00
	Picloram	1.00	41.72 ± 23.07^{b}	NC	0.00
		3.00	15.26 ± 2.82^{a}	NC	0.00
		5.00	15.24 ± 4.69^{a}	NC	0.00
		7.00	0.00 ± 0.00^{a}	NC	0.00
		10.00	4.03 ± 1.59^{a}	NC	0.00
Cytokinins	BAP	4.44	8.89 ± 0.96^{a}	NC	0.00
		13.32	5.45 ± 2.54^{a}	NC	0.00
		22.20	20.37 ± 2.23^{ab}	NC	0.00
	Kinetin	4.65	32.22 ± 14.02^{b}	4	14.81
		13.94	8.20 ± 5.37^{ab}	3	11.11
		23.23	13.25 ± 9.36^{a}	NC	0.00
	CPPU	1.00	9.16 ± 0.16^{a}	NC	0.00
		3.00	18.06 ± 6.36^{ab}	NC	0.00
		5.00	20.99 ± 2.14^{ab}	NC	0.00
		7.00	18.98 ± 2.12^{ab}	NC	0.00
		10.00	18.94 ± 3.93^{ab}	NC	0.00
Plant growth retardant	Uniconazole	1.00	16.72 ± 2.02^{a}	NC	0.00
		3.00	40.97 ± 5.48^{b}	4	14.81
		5.00	16.80 ± 11.59^{a}	NC	0.00
		7.00	43.61 ± 3.94^{b}	6	22.22
		10.00	19.14 ± 5.10^{a}	4	14.81

NC, no callus induced; ($n = 3$, replication = 3, repeated = 3)

Different alphabet indicates significance at $p < 0.05$ according to one-way ANOVA post hoc Tukey's HSD

filamentous structure growing on top of it. Further investigation confirmed that all buds grown on solid medium were of firm texture. The callus was grown on the apical and intercalary regions of the bud explants. Figure 4 shows the callus induced on bud explants on solid medium supplemented with different PGR. The calli were friable with a dark brown color and pigmented, with either a heart or globular shape. The structures were soft in texture, fragile when touched, and easily detached from the explants. Figure 5 shows the morphology of the buds that were grown in the liquid medium. The color of buds was brownish (alive) and some were white (dead). There was no sign of filament growth. The structure of the buds was fragile.

Discussion

Surface sterilization protocol and optimization study for the formation of axenic explants Protocol B (Fig. 1a) was found to be the most effective method in producing the highest

Table 3 Effect of PGRs on buds explants of *S. polycystum* cultured on liquid medium

	Treatments	Concentration (μM)	Bud formation rate ± SD (% week^{-1})	No. of callus induce	Callus induction (%)
Control	PES	0.00	19.47 ± 3.03^{ab}	NC	0.00
Auxins	2,4-D	4.52	90.51 ± 1.01^{ef}	NC	0.00
		13.57	37.10 ± 0.94^{abcd}	NC	0.00
		22.62	79.58 ± 13.11^{def}	NC	0.00
	IAA	5.71	15.15 ± 1.70^{ab}	NC	0.00
		17.13	95.79 ± 42.38^{cdef}	NC	0.00
		28.54	73.94 ± 5.10^{ef}	NC	0.00
	IBA	4.92	31.36 ± 5.49^{abc}	NC	0.00
		14.76	41.95 ± 13.982^{abcd}	NC	0.00
		24.60	43.32 ± 14.07^{abcd}	NC	0.00
	NAA	5.37	13.12 ± 11.17^{a}	NC	0.00
		16.11	103.96 ± 31.709^{f}	NC	0.00
		26.85	0.00 ± 0.00^{a}	NC	0.00
	Picloram	1.00	0.00 ± 0.00^{a}	NC	0.00
		3.00	10.96 ± 3.29^{a}	NC	0.00
		5.00	20.82 ± 4.83^{ab}	NC	0.00
		7.00	34.55 ± 2.94^{abc}	NC	0.00
		10.00	57.82 ± 15.17^{bcde}	NC	0.00
Cytokinins	BAP	4.44	36.48 ± 3.36^{bc}	NC	0.00
		13.32	65.41 ± 0.42^{cd}	NC	0.00
		22.20	22.05 ± 0.02^{ab}	NC	0.00
	Kinetin	4.65	89.15 ± 31.66^{d}	NC	0.00
		13.94	60.21 ± 11.98^{cd}	NC	0.00
		23.23	30.72 ± 0.53^{ab}	NC	0.00
	CPPU	1.00	25.27 ± 0.01^{ab}	NC	0.00
		3.00	27.50 ± 5.54^{ab}	NC	0.00
		5.00	16.19 ± 2.13^{ab}	NC	0.00
		7.00	7.44 ± 1.35^{a}	NC	0.00
		10.00	6.64 ± 0.04^{a}	NC	0.00
Plant growth retardant	Uniconazole	1.00	119.52 ± 21.19^{d}	NC	0.00
		3.00	14.43 ± 10.18^{a}	NC	0.00
		5.00	56.74 ± 26.48^{bc}	NC	0.00
		7.00	97.86 ± 0.01^{d}	NC	0.00
		10.00	93.27 ± 0.08^{cd}	NC	0.00

NC, no callus induced; ($n = 3$, replication = 3, repeated = 3)

Different alphabet indicates significance at $p < 0.05$ according to one-way ANOVA post hoc Tukey's HSD

amount of axenic culture of stipe explants. The difference between protocol B and the other protocols was the double exposure of PI introduced to the explants (5% *w/v* PI for 3 min followed by 2% *w/v* PI for 5 min). This protocol was modified from Kumar et al. (2004), which reported that 2% *w/v* PI for 3 min led to the acquisition of 90% of viable and axenic culture from *Gelidiella acerosa*. The present study showed similar findings to that of Kumar et al. (2004), suggesting that the stipe treatment of *S. polycystum* with double PI exposure was essential to obtain axenic culture. This was mainly due to the complex morphology of *S. polycystum* where the stipes are composed of spines that are prone to trapping dust, microorganisms, and epiphytes, as compared to *G. acerosa*. Thus, double exposure of PI was beneficial to stipe explant. Free iodine from PI can cause toxicity to both eukaryotic and prokaryotic cells (Sriwilaijaroen et al. 2009). Thus, the concentration of the second treatment with PI for stipe explants was lower (2% PI) and at a shorter exposure period (3 min) to prevent fatality of the stipe cell and tissue. As for leaf and stolon explants, the suitable sterilization protocol to obtain axenic culture was protocol D

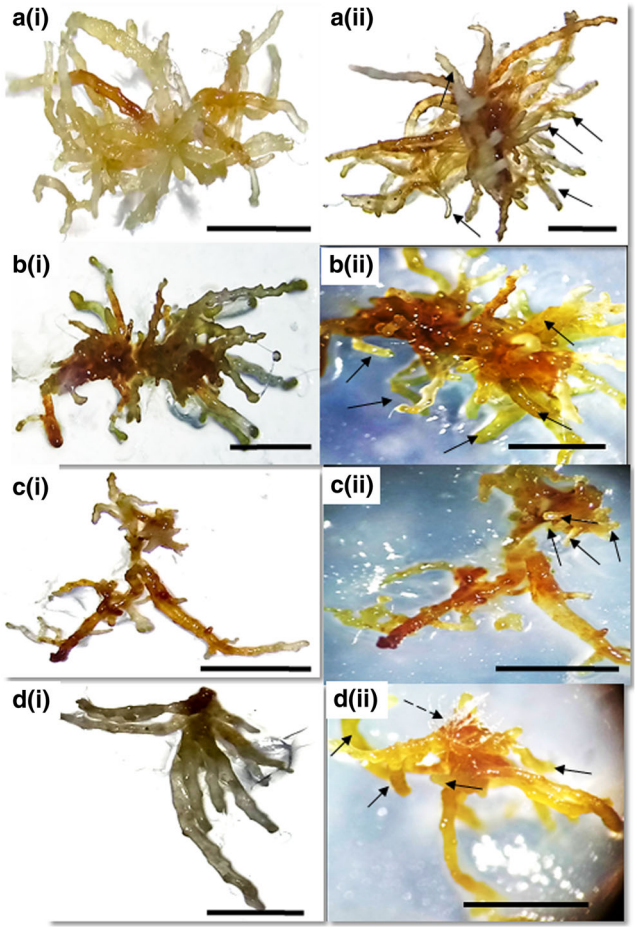

Fig. 3 Bud explants cultured on solid PES medium. The number and length of buds were increased every week. (Arrow: new buds form; dotted arrow: filament form on buds bar = 5.0 mm). **a** 22.62 μM 2, 4-D treatment (i) 1 week; (ii) 6 weeks. **b** PES (control) treatment (i) 1 week; (ii) 6 weeks. **c** 26.85 μM NAA treatment (i) 1 week; (ii) 6 weeks. **d** 3.00 μM uniconazole treatment (i) 1 week; (ii) 6 weeks.

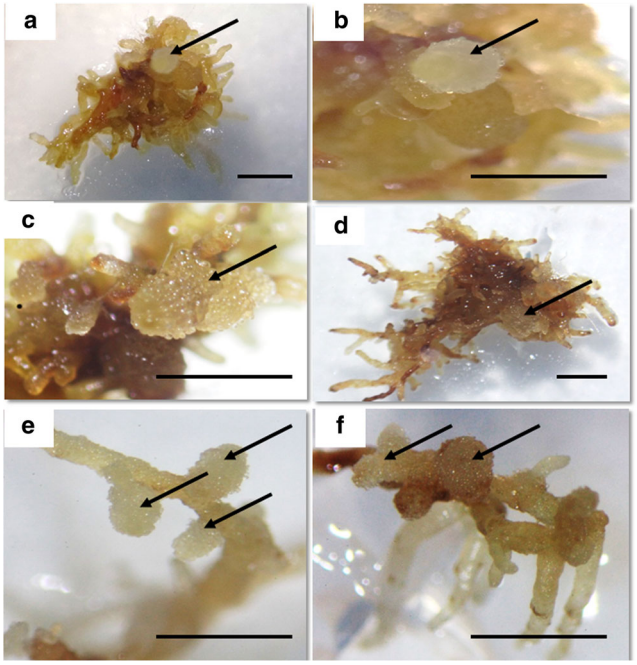

Fig. 4 Induction of callus on solid PES medium. **a** 10.00 μM uniconazole treatment medium; **b** enlarged image of callus of 10.00 μM uniconazole treatment medium; **c** 7.00 μM uniconazole treatment medium; **d** 3.00 μM uniconazole treatment medium; **e** 4.65 μM kinetin treatment; **f** 13.94 μM kinetin treatment (arrow: callus formation on buds, bar = 5.0 mm)

and C, respectively. These two protocols included the pre-treatment with 10 mg L^{-1} of GeO_2 before proceeding with other chemical sterilants. GeO_2 is an inorganic compound, that is, typically used in agriculture as an inhibitor for unwanted diatoms, whereby it penetrates into the cell of diatoms and disrupts the biochemical processes to inhibit the growth of the diatom (Lewin 1966). The pre-treatment period was longer for leaves as compared to stolon. This may be due to the morphology of the explants as the leaf has a larger surface area supported by the vein system. The higher presence of diatoms may influence the time required for the GeO_2 to take effect. The stolon of *S. polycystum* is also known as the second holdfast, which has a smoother surface with no internodes and a lower surface area compared to the leaf (Kantachumpoo 2013).

Many studies have been done on surface sterilization of seaweed tissue culture with the objective of obtaining the axenic culture, such as on *Eucheuma* species (Yong et al. 2011), *Hypnea musciformis* (Fernandes et al. 2011), *Kappaphycus alvarezii* (Yong et al. 2014), and *Stypopodium zonale* (Aguirre- Lipperheide and Evans 1993). In Asia-Pacific coastal areas, limited amount of studies was conducted using brown seaweed (especially *Sargassum* species) because of the low commercial value when compared to the red seaweeds, namely, *K. alvarezii* and *Eucheuma denticulatum* that are being actively cultured in Indonesia, Philippines, and Malaysia (Ahemad et al. 2006; Datu Razali et al. 2017).

The surface sterilization and concentrations of the chemical sterilants used are usually selected through optimization and the trial and error method (Saga et al. 1982; Bradley et al. 1988). PI is one of the common chemical sterilants used for surface sterilization of seaweed. Polyvinylpyrrolidone (in short, PVP or povidone) is a hydrophilic synthetic polymer which acts as a carrier for the iodine moiety to the cell membrane (Prince et al. 1978). PI is widely used in medical field as an antibacterial and antifungal agent against broad range of bacteria and fungus, protozoa, and virus (Zamora 1986). PI's main mode of action is the release of free iodine once the PI complex reaches the cell wall, which is cytotoxic to prokaryotic cells where they are killed within 10 s (Lacey and Catto 1993). The effectiveness of PI was proven on red seaweeds such as *K. alvarezii* at concentration of 0.3–1.0% (*w*/*v*) for 30 s (Yong et al. 2011, 2014; Yeong et al. 2014), *Gracilaria changii* at 1% (*w*/*v*) for 30 s (Yeong et al.

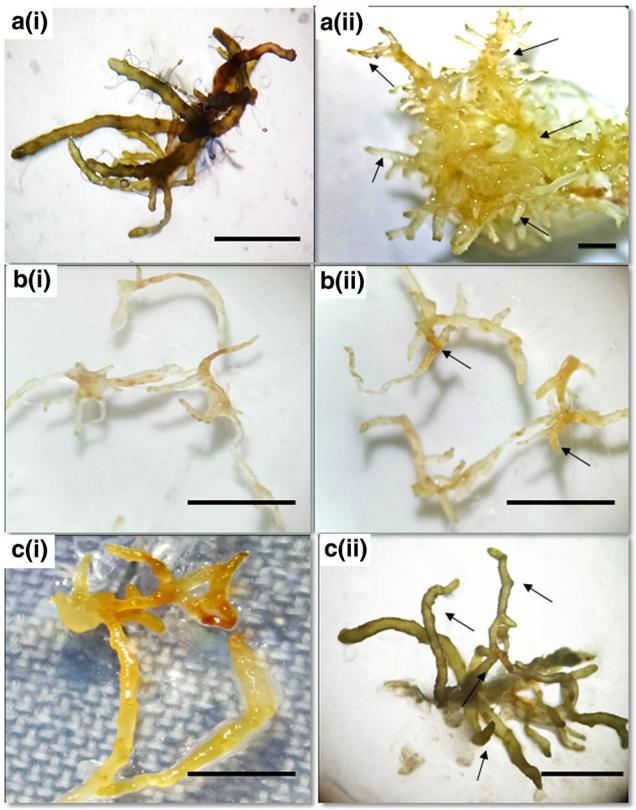

Fig. 5 Bud explants cultured on liquid medium. The number and length of buds were increased every week (arrow: new buds form, bar = 5.0 mm). **a** 1.00 μM uniconazole treatment (i) 1 week; (ii) 6 weeks. **b** 26.85 μM NAA treatment (i) 1 week; (ii) 6 weeks. **c** PES (control) treatment (i) 1 week; (ii) 6 weeks.

2014), *G. acerosa* at 2% (*v*/v) for 5 min (Kumar et al. 2004), and brown seaweed *Sargassum muticum* at 1% (*w*/*v*) for 5 min (Fisher and Gibor 1987). These studies proved that treatment with PI on seaweeds is able to provide axenic culture of explant, without affecting the viability of the explant and its ability to induce callus or adventitious buds.

Besides PI, GeO_2 is widely used as sterilant in seaweed tissue culture. Since GeO_2 is able to prevent the growth of diatoms, the chances of seaweed growth will increase with higher concentration of GeO_2 (Shea and Chopin 2007). Shea and Chopin (2007) showed that apart from inhibiting the growth of diatoms in seaweed (particularly *Laminaria saccharina*), GeO_2 also affected the growth of *L. saccharina* during the early stage of development. However, for the present study, GeO_2 showed no effect on the growth of the *S. polycystum* explants but had significant effect on the formation of axenic explants. It can be concluded that GeO_2 possesses the ability to reduce cultivation time of the seaweed culture in conventional and commercial laboratory via elimination of diatom growth.

Kumar et al. (2004) reported that the combination of detergent, PI, antibiotic and GeO_2 is able to form axenic explants with the ability to induce callus and adventitious buds when the optimal culture conditions are provided. The effectiveness of these chemical sterilants (detergent, PI, antibiotic and GeO_2) is proven to reduce the chances of microbial contamination with a relatively high production of axenic materials for tissue culture. These include cleanliness of the explant, morphology of explant, and some other environment factors (Baweja et al. 2009). For example, studies on *K. alvarezii* by Yong et al. (2011, 2014) reported that a combination of chemical sterilants such as 3.0 g L^{-1} PI and 15% ethanol for 30 s was required to obtain axenic culture. Another study by Yeong et al. (2014) using the same explant required the explants to be sonicated in autoclaved seawater intermittently, followed by PI treatment [1% (*w*/*v*)] for 30 s. Thus, there is no one universal surface sterilization protocol that can suit every single species (McCracken 1988) since each species/specimen may have its own bacterial (Bradley et al. 1988) and epiphytic flora (Ballantine 1979).

Effect of plant growth regulators on stipe, leaf, stolon, and bud explants PGRs have been used since the 1970s to study the growth of higher plants and callus induction in tissue culture (Baweja et al. 2009). Lately, the same steps have been adapted in seaweed tissue culture, utilizing the potential either of PGRs to induce callus growth or for direct regeneration of bud growth (Baweja et al. 2009).

Nine different types of PGRs were evaluated with at least three different concentrations tested for 2,4-D, IAA, IBA, NAA, BAP, and kinetin while five different concentrations were tested for uniconazole, picloram, and CPPU (forchlorfenuron). All treatments aforementioned were tested on stipe, leaf, stolon, and bud explants. Most of the PGR treatments showed effects on the growth of stipe explants, with no significant increase by size and without the formation of any callus or bud. However, adventitious bud formation was observed in about 50% of stipe explants cultured on PES only (control) medium. As for leaf explants, all PGR treatments together with the control were unable to produce any callus or bud. However, most of the treatments were able to induce increment in explant size. For the stolon explants, there was no sign of callus and bud formation. Some of the PGR treatments were reported to have no effect on elongation of stolon explant such as control, 4.52 μM 2,4-D, and all concentrations of IAA and NAA.

Both auxin and cytokinin, i.e., IAA, ABA (abscisic acid), PAA (phenyl acetic acid), iP (isopentenyladenine), and CZ (*cis*-zeatin) are referred as endogenous auxin/cytokinin which naturally occurs in seaweed tissue (Bradley 1991; Yokoya et al. 2010). The lack of ability to generate callus by all three explants may be due to the increase of PGRs level in the seaweed when the exogenous PGRs were introduced. In a

study conducted by Sulistiani et al. (2012), excessive PGR concentrations in *K. alvarezii* had decreased the rate of callus induction, and negative effect on callus growth was reported when they were treated with high concentrations of IAA.

The difficulty in obtaining callus from thallus (stipe, leaf, and stolon) explants even when supplemented with PGRs is common in seaweed tissue culture. A previous study by Huang and Fujita (1997) failed to obtain callus culture from red seaweed when IAA or BAP was supplemented and there was no significant callus induction or growth. The inability to induce callus and/or adventitious bud growth on stipe, leaf, and stolon explant in the present study may due to the differences in explant source, genotypic of explant, ecotypic/habitat differences, or environmental factors that may affect the growth and morphogenesis of the explants in tissue culture (George 1993).

In the present study, instead of using thallus (stipe, leaf, and stolon), bud explants were also used to study the effects of PGRs. The bud explants exposed to kinetin (4.65 and 13.94 μM) and uniconazole (3.00, 7.00, and 10.00 μM) on solid medium were noted to induce callus. This result was similar to that obtained in a previous study by Uji et al. (2015) where there was no formation of callus induced by other hormones (IAA, 2,4-D, BA, trans-zeatin, or GA) except uniconazole at concentration 5.00 to 10.00 μM on the leaf of *S. horneri*. Uniconazole is also known as triazole-type inhibitor for cytochrome P450 enzyme of *t*-zeatin biosynthesis (Sasaki et al. 2013), brassinosteroid biosynthesis (Iwasaki and Shibaoka 1991), abscisic acid (ABA) catabolism (Saito et al. 2006), GA biosynthesis (Izumi et al. 1985), and cellulose synthesis (Hofmannova et al. 2008). In some cases of higher plants, it was reported that due to the loss of function in cell wall synthesis (mutation impaired), callus formation was observed even in the absence of PGRs (Ikeuchi et al. 2013). For example, mutants of *Arabidopsis thaliana* which were impaired in biosynthesis of cellulose were able to form callus without the presence of auxins and/or cytokinins (Frank et al. 2002; Krupková and Schmülling 2009). Therefore, Uji et al. (2015) reported that callus formation in *S. horneri* was because of uniconazole that inhibited cellulose synthesis in *S. horneri* via same mechanism observed in higher plants (e.g., the mutant of *A. thaliana*). As *S. horneri* and *S. polycystum* are from the same genus, it may be the same mechanism that caused the formation of callus on the bud explants of *S. polycystum* when treated with uniconazole. However, this mechanism has never been reported to really affect callus induction in brown seaweed. Besides that, uniconazole can also alter levels of phytosterols, phenylpropanoids, polyamines, auxin, cytokinin, ethylene, jasmonate, and abscisic acid (Saito et al. 2006), which may contribute to callus induction observed in the present study. However, further studies are required to clarify the effect and the underlying mechanism of uniconazole on *Sargassum* species that had led to callus formation.

In this study, both solid and liquid medium were used to induce the callus of *S. polycystum.* Based on our results, only solid media supplemented with kinetin or uniconazole was able to induce callus and at the same time increase the bud formation rate (Table 2). On the other hand, liquid medium was unable to induce any callus regardless of the PGRs used. This result was similar to a study done by Baweja and Sahoo (2009) on *Grateloupia filicina* where the callus was only formed on PES solid medium with PGRs (NAA, BA, kinetin, and glycerol).

All calli formed in this study were dark pigmented brown calli, friable with globular, and heart shapes. There was no filamentous or clear callus observed in this study. The most common form of callus induction in seaweed tissue culture was filamentous callus (Reddy et al. 2003; Shao et al. 2004; Yeong et al. 2014). Brown algae calli are generally classified into two types which are clear callus and pigmented callus with filamentous callus mostly found in red seaweed species (Uji et al. 2015). Based on a previous study (Polne-Fuller and Gibor 1987; Kumar et al. 2007), about 10% of the *Sargassum* tissue sections were reported to form either clear or pigmented callus, whereas only 30% of the pigmented callus has the ability to differentiate into young plants. Callus formation in higher plants is normally caused by differentiation of tissue resulting from tissue injury (Yeoman 1987), while callus development in multicellular macroalgae is related to their thallus organization and differentiation (Aguirre-Lipperheide et al. 1995). The term "callus-like formation" was previously used to distinguish callus of macroalgae (Garcia-Reina et al. 1991; Yokoya et al. 1993) from callus of higher plants.

Calli formed on brown and red seaweeds are usually outgrowths from the medullary or cortical tissue either as clumps, uniseriate, pigmented or branched filamentous callus (Kumar et al. 2007). This is due to the regeneration of the medullary and cortical cells which outgrows the outer pigmented epidermal cells (Reddy et al. 2003). Ram et al. (2000) showed the formation of the mass of cells in the red alga *Coelarthum opuntia* from wound repairing that had involved cells in the cortical region just below the wound site. The ability of seaweed to regenerate is related to its natural wound repair potential, as seaweed is often damaged in situ by physiological stress, herbivore attack, abrasion caused by sand, and wave action injury (Bodian et al. 2013). A study conducted in Florida showed that wounds are healed by the active division of uncut cells to form a mass of undifferentiated clump cells (Fritsch 1945).

In general, comparing between stipe, leaf, stolon, and bud explants, only bud explants were able to induce callus. This may be attributed to the totipotency of the bud since there

were formations of undifferentiated and immature cells. This finding is supported by a previous study by Mooney and Staden (1985) which reported that immature explants have higher ability to form callus compared to matured explants.

In conclusion, we have developed suitable surface sterilization protocols for the stipe, leaf, and stolon of *S. polycystum* for the formation of axenic culture and determined the effect of plant growth regulators and the best explant for *S. polycystum* tissue culture. However, from the results obtained, it is difficult to get significant number of callus from *S. polycystum*.

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