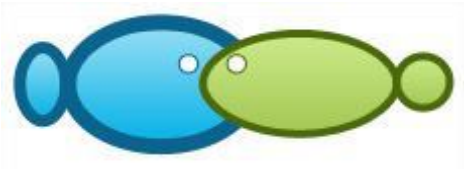

Releasing, attaching and growing of seaweed (*Gracilaria* sp.) spores in several culture media

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Abstract. The aim of this experiment was to evaluate the use of several culture media for releasing, attaching and growing of *Gracilaria* sp. spores. Media used as the treatments were Sterilized Seawater (SSW), Grund, and Provasoli's Enrich Seawater (PES). Plastic petri dish with a diameter of 5 cm were filled with 10 mL of each culture medium. Each medium had 5 replicates and a petri dish was stocked with 5 fertile thallus containing 5 spore sacs per thallus. Number of spores released into the culture medium were counted a day after culturing the thallus. Spore counting was carried out every day for 8 consecutive days. After counting the spores, the thalli were then cultured again in a new fresh medium to release the spores at the following day. The released spores were then cultured in the media and after 10 days, the number of spores attached to the substrate of plastic petridish was also counted. Plantlet survival rate was calculated after 30 days from spore releasing. Spores released in SSW, Grund and PES media ranged from 111 to 2,351, 578 to 2,249 and 404 to 2,169 spores per cystocarp per day, respectively. The range of spores number attached to the substrate under SSW, Grund and PES media were 0-3, 1-79 and 0-9 spores per cystocarp per day, respectively. Plantlet could grow for 8 consecutive days in Grund medium with plantlet survival rate ranging from 50 to 89%. Plantlet could grow on the first day until 6th day in PES medium with a survival rate ranging from 0 to 88%, while in the SSW medium, plantlet has the lowest survival rate ranging from 0 to 64% and could grow only on the 2nd and 8th day of culture. Additional nutrients were required for releasing, attaching and growing the spores. Grund medium can support spores released and plantlets grown for 8 consecutive days. These results could be applied to promote the establishment of seed production of *Gracilaria* sp. from spores that attach to polyethylene rope.

Key Words: culture medium, *Gracilaria* sp., mariculture, seed, spore, substrate.

Introduction. Indonesia is one of main producer country of *Gracilaria* (Solieriaceae, red alga) seaweed in the world (FAO 2016), and most of the *Gracilaria* seaweed production comes from aquaculture (FAO 2018). *Gracilaria*, as a source of agar has economically importance as a food for humans and marine animals (Zemke-White & Ohno 1999). It is cultivated mainly in Indonesia and Chile producing around 80% of the world's total agar (Bixler & Porse 2011). In Indonesia, *Gracilaria* is mostly cultivated by spreading it on the bottom of the earthen pond. *Gracilaria* has been cultivated in marine waters by being tied to a rope since 2013, cultivating *Gracilaria* in the sea has benefits for farmers who do not have a pond, so farmers can spend their money for buying or renting the earthen pond, and also after harvesting, seed come from spores attached to rope can culture for next crop (Lideman et al 2014). *Gracilaria* sp. cultured in marine waters has thallus which is relatively larger than *Gracilaria* cultured in earthen pond so that it is possible to be tied to polyethylene (PE) strings (Lideman et al 2016.)

This experiment was conducted based on the rapid development of *Gracilaria* sp. cultivated in the sea, so it requires a large number of seedlings. Utilization of spores for producing seed is one possible way to increase production and improvement of cultivation techniques. Carpospore are easier to use as a source of seed because their spore sacs

(cystocrap) can be observed by naked eye. The use of spores as a source of seedlings has been successfully carried out in several countries (Halling et al 2005; Yudiati et al 2004), such as in producing *Porphyra* seed (Miura 1975; Notoya et al 1993).

Gracilaria seaweed requires inorganic carbon, water, light and various minerals for photosynthesis and growth (Lobban & Harrison 1997). Spore of *Gracilaria* seaweed also requires nutrients for its development from spore to young *Gracilaria* (plantlet). Nitrogen most frequently limits the growth of seaweed (Topinka & Robbins 1976; Deboer & Ryther 1977) and is the second most limiting nutrient (Manley & North 1984; Lapointe 1986, 1987; Lapointe et al 1992). There are several culture media containing macro and micro nutrients and vitamins provided for growing of algae (Andersen 2005). In its application, the use of medium for the growth and development of *Gracilaria* seaweed often causes other problems, such as the presence of diatoms which can also develop rapidly so that it affects the release and development of spores. The presence of nutrients in the culture medium is also presumed to affect the release, attachment and growth of spores. Therefore it is necessary to evaluate the use of several media such as Grund and Provasoli's enrich sea water for the production of *Gracilaria* seaweed seeds developed from spores. The aim of this study was to evaluate the use of three kinds of culture media which were sterilized sea water (SSW), Grund medium and Provasoli's enrich seawater (PES) for releasing, attaching and growing of *Gracilaria* sp. spores.

Material and Method. This experiment was conducted in October 2017 at laboratory of seaweed, Brackishwater Aquaculture Development Center, South Sulawesi, Indonesia. Seaweed used for this experiment was *Gracilaria* sp., Family Solieriaceae (Rhodophyta) which already contained mature carpospore type of spores cultivated in Ujung Baji Village ($5^{o}27'22.6"S$, $119^{o}23'29.9"E$), Takalar Regency, South Sulawesi, Indonesia. Fertile *Gracilaria* was selectively obtained from farming area. The collected bacth was kept in a stereofoam containing seawater and then brought to the wet laboratory of the Brackishwater Aquaculture Development Center (BPBAP) in Takalar. During acclimatization, temperature of media was maintained at approximately 25°C. The collected *Gracilaria* sp. was acclimatized for 5 days using aquarium with size of 60 × 40 × 40 cm^3. Salinity and pH of seawater used during the acclimatization were in range of 30-33 ppt and pH 7.8-8.2, respectively.

Carposporophyte of *Gracilaria* sp. selected for this experiment has the following characteristics: its thallus was clean of dirt, color was yellowish to brown and the spore sacs (cystocarp) were bright brown with a relatively larger in diameter. All thalli were cultured until the spores released from the cystocarp. Culturing the thallus to release the spores in the three medium was repeated for 5 times.

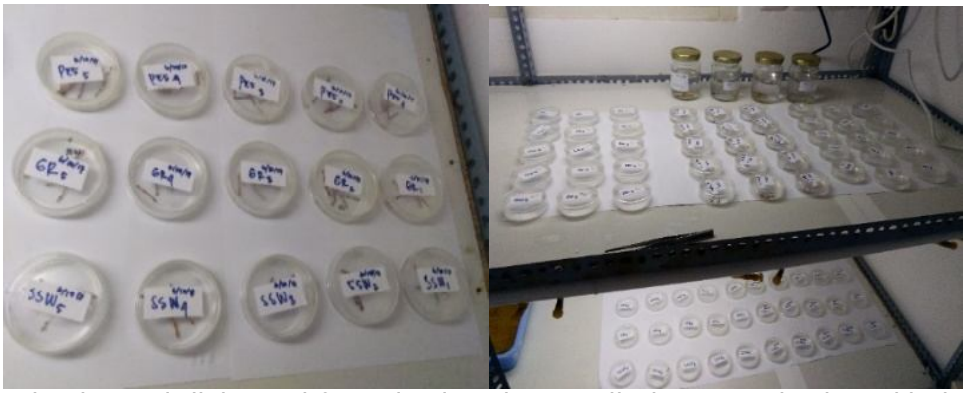


Figure 1. Plastic petri dish used for culturing the *Gracilaria* spores in three kinds of media of Grund, PES and SSW.

Culture media. There were three kinds of media used for this experiment namely 1) sterile seawater (SSW), Grund medium and Provasoli's Enrich Seawater (PES). The SSW was seawater filtered by using Whatman paper with size of 0.45 µm. This filtered sea water was then sterilized by using autoclave at $121^{o}C$ for 15 minutes with a pressure of 1

atm. Salinity and temperature of the culture media were kept at 30 ppt and 24°C, respectively. Composition of Grund and PES media used for this experiment were described by Andersen (2005). In short, Grund medium comprise of $NaNO_3$, $Na_2\beta$-glycerophosphate (modified to NaH_2PO_4), $FeSO_4$, $MnCl_2$, Na_2-EDTA, vitamin stock solution (thiamin, biotin and cyanocobalamin). And, PES medium contains Tris base, $NaNO_3$, $Na_2\beta$-glycerophosphate, iron-EDTA solution (Fe $(NH_4)_2(SO_4)_2$ and Na_2-EDTA), trace minerals solution (Na_2EDTA, $FeCl_3$, H_3BO_3, $MnSO_4$, $ZnSO_4$, $CoSO_4$), thiamin, biotin and cyanocobalamin. To prepare the enrichment stock solution of Grund and PES media, begin with 700 mL of dH_2O, add the components (vitamins should be added last, after mixing other ingredients), bring the final volume to 1 liter with dH_2O, and pasteurize (do not autoclave). To prepare Grund medium, add 1 mL of the enrichment stock solution of Grund to 980 mL of filtered natural seawater. And, and to prepare PES medium, add 20 mL of the enrichment stock solution of PES medium to 980 mL of filtered natural seawater.

Spores releasing. The selected carposporophyte was cut into a 1-1.5 cm of length containing 5 cystocarps. After being cut, the fertile thallus was then sterilized by soaking it in a solution of 1% betadine or 10% iodine for 2-3 minutes. After that, the thallus was put into a 20 mL Petri dish (Figure 1) containing 10 mL of each tested medium. The thalli were cultured until the spores released from the cystocarp. This protocol of releasing spores in each medium was repeated 5 times. The method of culturing the explant applied in this experiment was based on Lideman et al (2011).

Released spores in each tested medium were counted daily in the morning since stocking the fertile thallus in the media. Counting of spores was carried out for 8 consecutive days using Sedgewick rafter. Spores in the cultured medium were stirred evenly and taken approximately 1 ml to be put into Sedgewick rafter and counted under a stereo microscope. Number of Sedgewick rafter observed was 6 boxes per observation and repeated for 10 times. Formula of calculating the released spores was as follows:

$$Sc = (St/C) \times V$$

where: Sc = releasing spores per cystocarp (spore/cystocarp);
St = number of spores in sedgwick (x 1000 boxes per mL);
C = number of cystocarps cultured in the medium (25 cystocarp);
V = volume of medium (10 mL).

Attaching of spora and plantlet. Following the spores releasing, the spores were cultured in the same medium to observe their attachment onto the substrate. Attached spores were then observed under a stereo microscope and counted at day 10 after culture in the medium. Calculation of spores attached to the substrate was carried-out every day for 8 consecutive days. The parameters observed were the number of spores attaching to the substrate per spore sac.

Counting of spores attached on the substrate was carried out under a stereo microscope with 10x5 magnification and the area of view was 0.1256 cm^2. The calculation of the number of spores attached on the plastic petri dish substrate per cystocarp (Sm) was done 5 times, using below formula:

$$Sm = Sa \times A$$

where: Sm = number of spores attached to the substrate (spore/cystocarp);
Sa = the average number of spores observed to the field of view of the microscope;
A = substrate area at the bottom of the container with a diameter of 5 cm.

Counting of the number of spores that develop into plantlets (young *Gracilaria*) was carried-out after 30 days of spore released from spore sacs and then the percentage of the number of survived plantlets (survival rate) was calculated as follow:

$$SR = (PC/Sm) \times 100$$

where: SR = survival rate (%);
PC = number of plantlets attached on the substrate (plantlet per cystocarp);
Sm = number of spores attached on the substrate (spores per cystocarp).

Data collected was statistically analyzed using one-way ANOVA, and if there was a significant difference, data were continued to be analyzed using Duncan Post Hoc Test in order to know significantly differences among treatments. Analyses were performed for highest number of plantlets survived at day eight of 30 days culturing of spore. $P \leq 0.05$ was defined as the level of significance.

Results and Discussion. Carposporophyte of *Gracilaria* sp. collected and selected from cultivation area were shown in Figure 2. The carposporophytes used in this experiment had light brown color thalli and the spore sacs (cystocarp) were clean with dark brown color.

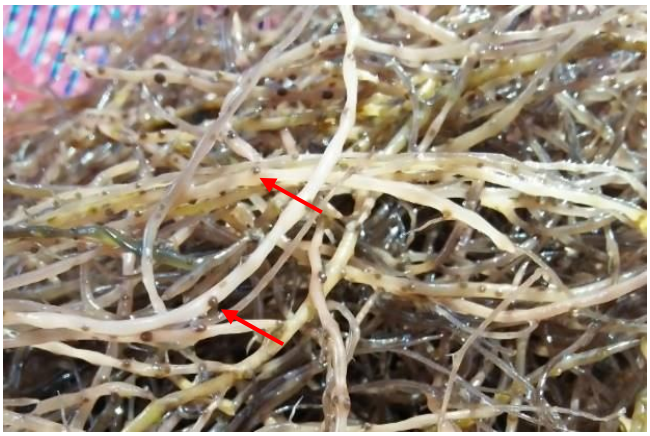


Figure 2. Thallus of *Gracilaria* sp. containing carpospore sacs (red arrows).

Releasing spora. Spores released from spore sacs in culture media are shown in Figure 3. The number of spores released from cyctocarp in SSW, Grund and PES medium observed for 8 consecutive days are presented in Figure 4. The variation of spores number released to the media were relatively high. Number of spores produced was the highest in medium of SSW, followed by Grund and PES since day 1st to day 4th. While from day 5th to 8th released spores were highest in Grund medium followed by PES and SSW as illustrated in Figure 4.

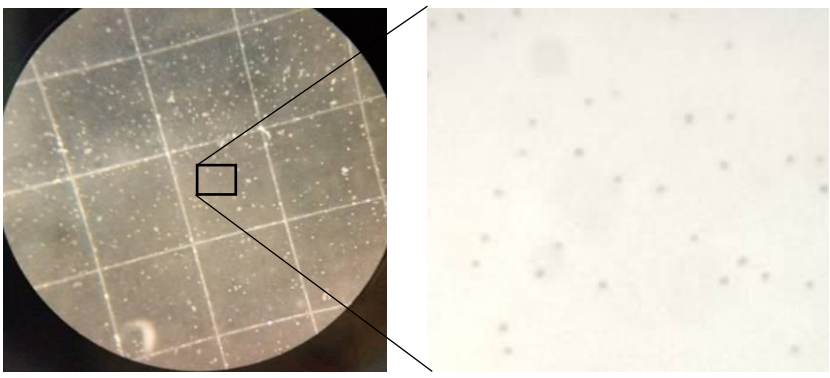
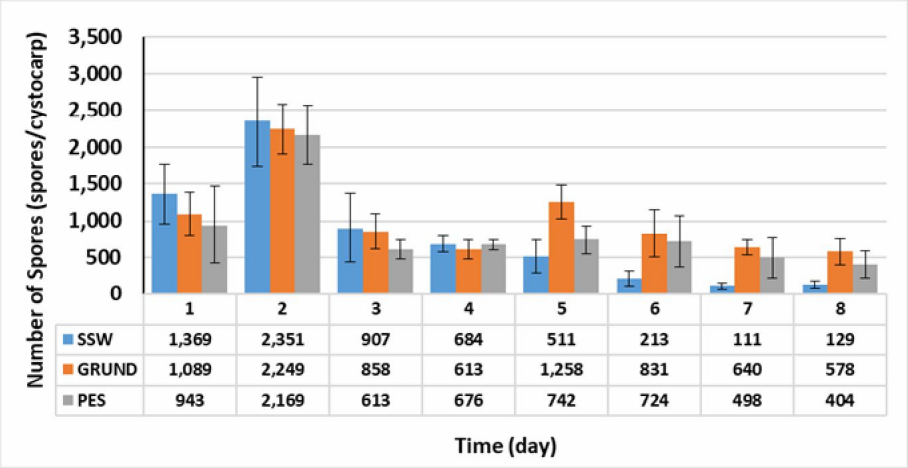


Figure 3. Spores (carpospore) that released and spread in the media (spore Ø = 20-35 µ).



	1	2	3	4	5	6	7	8
SSW	1,369	2,351	907	684	511	213	111	129
GRUND	1,089	2,249	858	613	1,258	831	640	578
PES	943	2,169	613	676	742	724	498	404

Figure 4. Number of spores released from cystocarp to culture media 8 consecutive days. Bar is standard deviation.

Number of spores released to SSW, Grund and PES medium for 8 consecutive days were 111-2,351, 578-2,249 and 404-2,169 spora per cystocarp per day respectively. The spores in SSW medium gradually decreased until day 8th after increasing at day 2nd, in Grund and PES medium the spore number increased at day 2nd and day 5th.

Attaching spores. Figure 5 shows the number of spores attached to substrate at the bottom of plastic petri dish after 10 days culturing in the media. Spores attached to the substrate in Grund medium were more than spores attached to substrate in PES and SSW media. Number of spores attached to medium SSW, Grund and PES for 8 consecutive days were 0.0-0.4, 0.2-78.9 and 0.0-1.1 spores per cystocarp per day respectively, as presented in Figure 6.

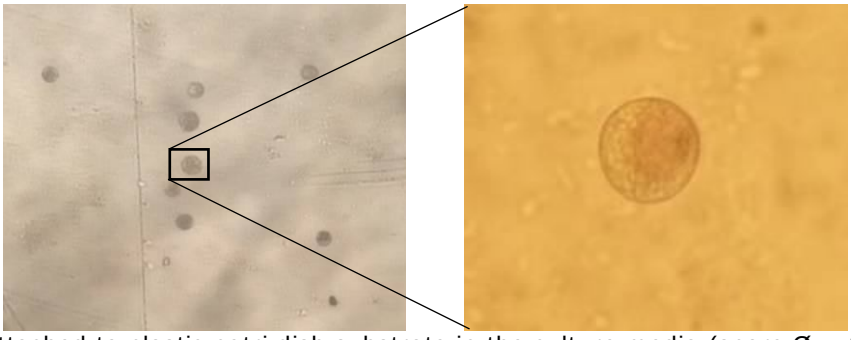
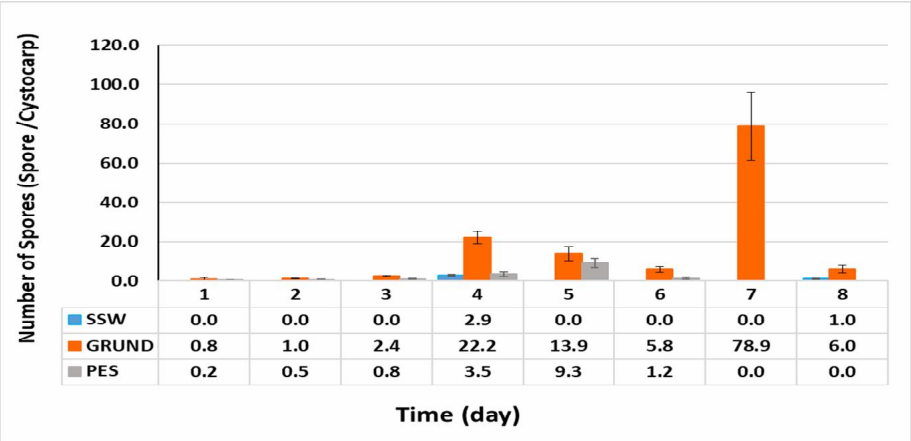


Figure 5. Spores attached to plastic petri dish substrate in the culture media (spore Ø = 80-100 µ).

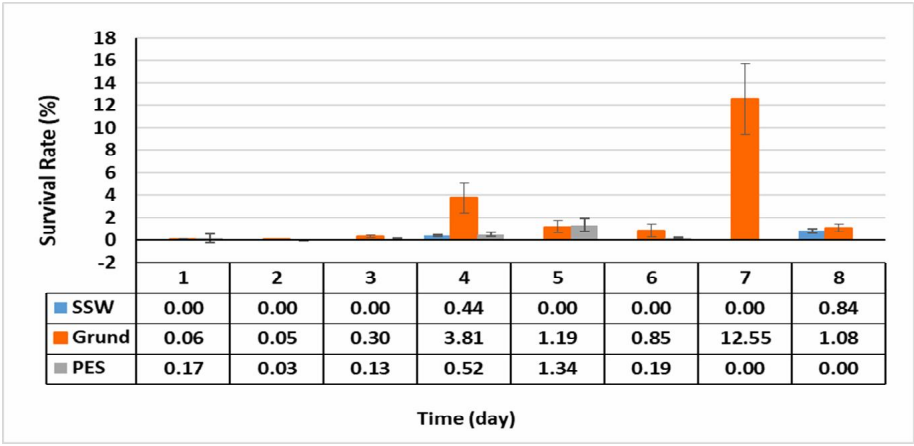


	1	2	3	4	5	6	7	8
SSW	0.0	0.0	0.0	2.9	0.0	0.0	0.0	1.0
GRUND	0.8	1.0	2.4	22.2	13.9	5.8	78.9	6.0
PES	0.2	0.5	0.8	3.5	9.3	1.2	0.0	0.0

Figure 6. Number of spores attached to substrate of plastic petri dish for 8 consecutive days culture after 10 days spore released. Bar is standard deviation.

Spores can attach to substrate for 8 consecutive days in Grund medium, day 1st to day 5th in PES medium and in medium SSW attached spores was occurred at day 4th and day 8th . Survival rate of spores attached to substrate in comparison to spores released to medium are shown at Figure 7.

SR of the attached spores was the highest at substrate in Grund medium followed by PES and SSW medium. The highest SR of the attached spores in grund was 12.5% occurred at day 7th, while in PES medium was 1.3% observed at day 5th and in SSW was 0.8% occurred at day 8th. The spore which failed to attach to the substrate shown transparent in color and its cell could not develop to next stage.



	1	2	3	4	5	6	7	8
SSW	0.00	0.00	0.00	0.44	0.00	0.00	0.00	0.84
Grund	0.06	0.05	0.30	3.81	1.19	0.85	12.55	1.08
PES	0.17	0.03	0.13	0.52	1.34	0.19	0.00	0.00

Figure 7. Survival rate of spores attached on substrate of plastic petri dish after 10 days culturing that compared to released spores. Bar was standard deviation.

***Plantlet (young Gracilaria* sp.)**. Figure 8 shows the plantlets growing on substrate in the media after 30 days culturing. The plantlet had a completed structure consisting of holdfast and thallus. The number of plantlet attached to the substrate in these media was shown by Figure 9. Number of plantlet growing on substrate in Grund medium ranged from 0.8 to 63.0 plantlet per cystocarp and plantlet could grow for 8 consecutive days. Furthermore, plantlet in PES medium could grow on the first day until day 6th with number of spores ranged from 0 to 8.0 plantlet per cystocarp. However, in the SSW medium, plantlet has a very low survival rate ranging from 0 to 1.9 plantlet per cystocarp and only spores released at the 2nd and 8th day could grow after 30 days culture (Figure 9).

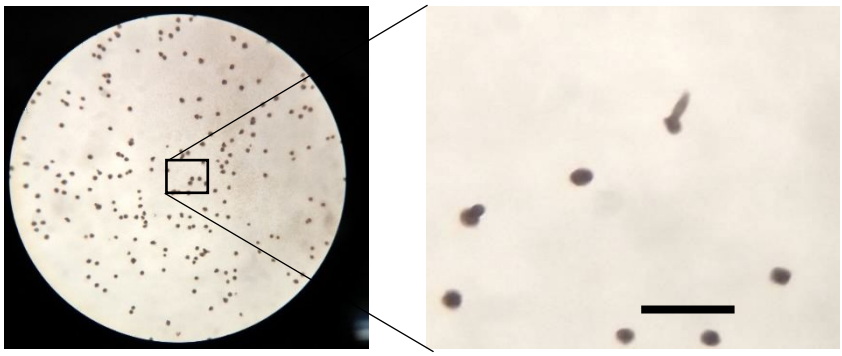
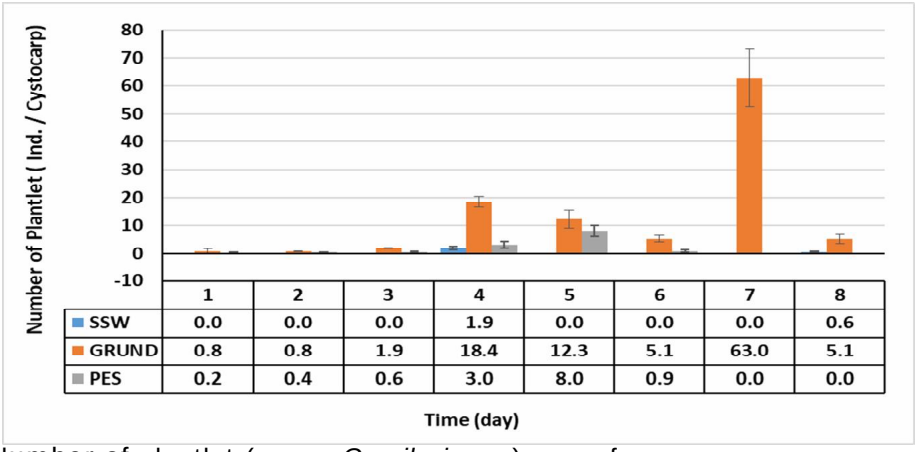


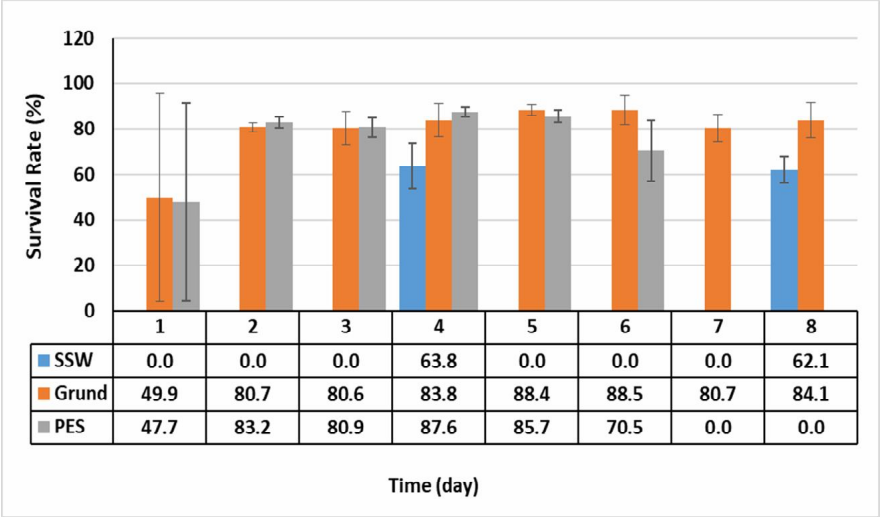
Figure 8. Plantlet growing on substrate after 30 days of releasing the spores. Bar was 1 mm.



	1	2	3	4	5	6	7	8
SSW	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.6
GRUND	0.8	0.8	1.9	18.4	12.3	5.1	63.0	5.1
PES	0.2	0.4	0.6	3.0	8.0	0.9	0.0	0.0

Figure 9. Number of plantlet (young *Gracilaria* sp.) come from spores grown on substrates in three different kinds of medium after 30 days spore released. Bar was standard deviation.

Plantlet could grow for 8 consecutive days in Grund medium with survival rate ranging from 50 to 89%. Plantlet in PES medium could grow on the first day until day 6th with a survival rate ranging from 0 to 88%, while in the SSW medium, plantlet however, has the lowest survival rate ranging from 0 to 64%. Figure 10 presents the survival rate of plantlet grown from attached spores. Number of plantlets growing on substrate in Grund medium was higher than in the medium PES and SSW ($p < 0.05$). Moreover the survival rate of plantlet after 30-d culturing of releasing spore was higher in that medium than in SSW medium ($p < 0.05$), but SR of plantlet in Grund medium was not significantly different from PES medium ($p < 0.05$).



	1	2	3	4	5	6	7	8
SSW	0.0	0.0	0.0	63.8	0.0	0.0	0.0	62.1
Grund	49.9	80.7	80.6	83.8	88.4	88.5	80.7	84.1
PES	47.7	83.2	80.9	87.6	85.7	70.5	0.0	0.0

Figure 10. Survival rate of plantlet (young *Gracilaria* sp.) grown from spores attaching on substrate in several media after 30 culture from spores releasing.

The spores were able to be released in all media for 8 consecutive days. High number of spores released into SWW medium since day 1st to day 4th indicated that nutrient contained in this medium was sufficient to support the spores to be released, thallus had enough energy for releasing the spores. However, number of spores released in SSW medium was lower than other two media after day 4th, and also released spore in SSW medium decreased sharply from day 4th to day 8th (Figure 4). Lower number of spores released to the SSW medium compared to Grund and PES media after day 4th revealed that the thalli required more nutrient from the media. According to Guzmán-Urióstegui & Robledo (1999), spora releasing in *Gracilaria cornea* in sterilized seawater was not influenced by drying process, osmotic shock and spontaneous discharge, but more affected by temperature and day length. Furthermore they found that spores discharged

of *G. cornea* was high at 26^{o}C and short days of 8:16 (L:D). Numbers of carpospores per cystocarp obtained in *G. cornea* found in the present study were lower compared with those reported in *G. corticata* (4.911 carpospores per cystocarp per day) (Umamaheswara 1976) and *G. verrucosa* (19.700 carpospores shed per cystocarpic plant) (Oza & Krishnamurthy 1968). In this study, the highest spores number of *Gracilaria* sp. released to medium was 2,351 spores per cystocarp. Released spores resulted in this study was lower than *G. corticta* and *G. verucosa* described by Umamaheswara (1976), Oza & Krishnamurthy (1968) but they were higher than *G. verucosa* that released spores of 1,858 spores per cystocarp (Yasin et al 2013)

Number of spores attaching to the substrate in Grund medium was higher than PES and SSW medium. Addition of nutrient in Grund medium showed a better effect for the spores attachment process on the substrate. The lower number of spores attached to the substrate in the medium of SSW indicated that *Gracilaria* sp. required additional nutrients for photosynthesis and growth (Lobban & Harrison 1997). In order to develop seedling of *Gracilaria* sp. from spores, several factors should be considered for a successful attachment of spores to become plantlet (Guzmán-Urióstegui & Robledo 1999). These factors include pH, salinity, temperature, light intensity, spores status, viscosity of the waters, existing microfilm layers in substrate, substrate roughness, and the spores are adhesive to the substrate (Lobban & Harrison 1997). Although the spores in SSW medium could release for 8 consecutive days, attached spore on substrate was only occurred at day 2nd and day 8th. Sterilized seawater contained low nitrogen compared to Grund and PES media. This difference was suspected to affect the spore ability to attach in medium and germination process of spore after releasing.

Further observations following a month from spore releasing showed that the spores could attach to the substrate and develop into plantlets completed with holdfast (root-like has function for attaching to substrate) and thallus. Spores attaching to the substrate and develop into plantlets in Grund medium, PES and SSW were 78.9, 1.1 and 0.4 spores per cystocarp per day, respectively. Although the number of spores attached to the substrate on Grund medium was significantly higher than in PES medium ($p < 0.05$) but the survival rate of plant in Grund medium did not significantly differ ($p > 0.05$). These result demonstrated that seed production of *Gracilaria* sp. from spores, PES medium can be used for culturing plantlet but it was not suitable for spore attachment. Moreover, Grund medium could be used for releasing and attaching spores on the substrate. The finding also showed that the spore released and attached on substrate in Grund medium was occurred for 8 consecutive days and could develop into a plantlet. In term of SSW medium, although the spore released for 8 consecutive days, plantlet could only survive on the day 4th and 8th, showing that the addition of nutrient in Grund medium can extend the period of spore releasing.

Survived plantlets derived from the first day of spore release on both Grund and PES media resulted in a large variation, even on SSW medium there was no plantlet survived. Condition of the new environment of culture medium on the first day was suspected to stimulate stress effect on explants, so that they produced poor quality of spores. Fertile *Gracilaria* were cultured in natural sea water for 3 days before culture in the SSW, PES and Grund. Therefore, based on this observation, it was not suggested to use spores from the first day of release in producing of seeds. Information obtained from the present study provides a basic information for seed production from spores including culture period of fertile thallus for releasing the spore, the use of different medium for different stage of spore/plantlet and number of fertile thalli to produce desired density of plantlets on the desired substrate.

Different chemical compositions of the media used in this experiment were caused different number of spores grown on substrate. Nitrogen and phosphor are main elements as limiting factors of algae growth (Lobban & Harrison 1997). Nitrogen is a protein component of plant cell wall, nitrogen has a function as a factor to induce cell growth (Rukmi et al 2012), while phosphate has a function as metabolic energy transformation (Kuhl 1974), and in general, seaweed need prominent element of nitrogen, phosphate and potassium (Kuhl 1974).

Conclusions. Media used in this study could resulted in different number of spore grown on the substrates. Spore grown on substrate was highest in Grund medium followed by PES and SSW medium. Additional nutrients were required for releasing, attaching and growing the spores. Grund medium can support spores released and plantlets grown for 8 consecutive days. Grund medium has less composition of compound than PES medium, utilization of the Grund medium can reduce cost and is also easier in its preparation compare to PES medium.

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