The effect of initial weight of seedlings grafted from tissue-cultured and local strain seedlings on growth and carrageenan content of the Red Seaweed (*Kappaphycus alvarezii*) using a grafting method

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Abstract. A grafting experiment using tissue-cultured and local strain *Kappaphycus alvarezii* seedlings was conducted from January to April 2019 and consisted of two steps. Firstly, grafting and propagating the seedlings using three different initial weights (IW) of 10, 15 and 20 g. Secondly, a culture experiment using the new seedlings produced from the first step. Seedlings were completely attached to each other after 18 days following the first step with a mean 75.0% grafting rate. The new seedlings were then cultured for 45 days using a long line method. The highest daily growth rate (DGR) was 4.36% day⁻¹ obtained from 20-g IW while the lowest DGR was 4.01% day⁻¹ from 15-g IW. It was found that rapid changes in water salinity and turbidity may affect the success in terms of grafting rate and DGR. The ratio of fresh weight (FW) to dry weight (FW:DW) showed no significant differences among treatments although the 20-g IW showed the highest FW:DW ratio (8.21:1) followed by 15-g IW (7.87:1) and 10-g IW (6.63:1). In contrast, 10-g IW and 15-g IW showed a significant difference in term of carrageenan content from 20-g IW. The 10-g IW carrageenan content was highest (40.38%) followed by 15-g IW (40.25%) and 20-g IW (38.34%).

1. Introduction
Indonesia is currently the largest producer of seaweed in the world [1]. Total seaweed production of Indonesia in 2017 was 10.81 MT [2]. To maintain a high level of Indonesian seaweed production, a continuous supply of high quality seedlings is urgently needed [3,4]. One of the prominent methods to improve both quality and quantity of seedling production is the tissue-culture method. Recently, this method has been used in many countries and resulted in higher production compared to the use of vegetatively produced seedlings [5,6]. However, the use of tissue culture methods still has constraints on its application by the seaweed farmers, because the seedling process through tissue-culture takes at least 2.5 years and is costly. Therefore, [7] suggested the use of grafting methods.
Grafting methods have been used in India since 2010 [8,9]. At that time, all seeds used came from local strains. The results of the study showed an increase in growth and carrageenan yield. This grafting method was then developed in Indonesia [7] by combining seeds from tissue culture and local strains. Using side-slipped and straight-slipped grafting, it was found that grafting can be a useful method to support seaweed production. However, recent studies have not provided information regarding the effect of this grafting method on carrageenan yield and grafting rate.

Since tissue-cultured seedlings have different morphological characters from local strains, some modifications need to be applied to adapt the grafting method used previously by [8,9]. One modification used in this study was trying several initial weight of seedlings, which may increase both efficiency of seaweed seedlings use and biomass production. In other words, the fewer seeds used per clump, the more seedlings planted. In many studies, initial weight (IW) of vegetatively-produced seedlings have been relatively small (around 50 g or less) or larger (around 100 g or more [10–14]. Moreover, a recent study by [15] showed higher growth rate for 5 g IW than for 10g or 15g IW. There was no grafting research conducted to examine the growth and carrageenan content using different initial weight of the seedlings from tissue-cultured seedling and local strain. Therefore, the aim of this study was to observe the effect of different initial weight of grafted *Kappaphycus alvarezii* seedlings (tissue-culture x local strain seedlings) on growth and carrageenan content.

2. Materials and Methods

This research was carried out from January to April 2019 in Sasara Coastal Waters (SCW), Kulususu Bay, North Buton Regency, South East Sulawesi (4°49’9.04”S and 123°1’49.89”E). Carrageenan content analysis was conducted in the laboratory of the Faculty of Fisheries and Marine Science (FFMS), Halu Oleo University, Kendari, Indonesia. Healthy seedlings were collected from a local cultivation farm in SCW.

2.1. Grafting of the seedlings

The grafting method was applied to selected seedlings, to join tissue-culture seedlings (TC) with local strain seedlings (LOC). The seaweed graftings process follows previous study of [8,9] with several modifications. First, both seedlings were cut in a side-slipped manner (obliquely, at an angle to the thallus orientation) from the base towards the tip of the thallus, and the off-cuts were removed. Secondly, the seedlings were tied together with the cut faces in direct contact with one another using plastic straps. (Figure 1).
Figure 1. Grafting process using tissue-cultured (TC) seedlings (D2) and local strain (LOC) seedlings (D1) of equal weight: A. 10 g IW; B. 15 g IW; C. 20 g IW; D. The seedlings were tied. Locals strain (1) and tissue-cultured seedling (2); E. The seedlings were completely tied with a plastic strap.

Grafting and propagating the seedlings was categorized as the first stage of this study. The processes were: 1) after selection, the plants were washed and cleaned completely with filtered and clean seawater to remove dirt, mud, silt, and epiphytes; 2) the selected seedlings were then cut
obliquely by a cutter into small pieces and weighed to provide material for the ±10g, 15g and 20g initial weight (IW) treatments. IW used in this study was the combination of seed weight using the same weight of seedlings derived from tissue culture and local strain. For example, the 10g IW was a combination of 5-g IW from a tissue culture seedling and 5-g IW from a local strain seedling; 3) the TC and LOC seedling cuttings were separated from the base of seaweed thalli; 4) the thalli were grafted by placing the two cuttings together using a side-slipped position; 5) finally, the two cuttings were combined and tied tightly using plastic strap. All seedlings were grown on for 18 days. Seedling condition was observed every 2 days, and at the same time grafting rate was measured and the seedlings were cleaned of mud, bryophytes and epiphytes. All the seedlings which attached successfully were then cultivated for 24 days.

2.2. Growing of the Seedlings
All successfully attached seedlings (TC + LOC) produced from the first stage were then tied to a 1.5 mm PE rope with a 10 cm planting distance. These seedlings were then cultivated for 45 days using a long line method. During the growing period, the seedlings were regularly monitored and cleaned from attached mud, silt, dirt and epiphytes. Water temperature, salinity, nitrate, phosphate, and turbidity were measured every 9 days.

2.3. Parameters Observed
- Grafting rate (GR). The rate (%) was measured by calculating the number of seedlings that failed to attach divided by the number of succeeded attach seedlings multiplied by 100. All planted seedlings were then grown for 24 days.
- Daily growth rate (DGR) of seaweed was calculated for each 9 days using the formula of 
  \[ \text{DGR}(\%) = \left(\left(\frac{W_i}{W_0}\right)^{\frac{1}{t}} - 1\right) \times 100 \% \] where \( W_0 \) is the initial weight (IW), and \( W_i \) is the final weight (FW) of the seedlings after 9 days of culture.
- Ratio of DW: FW were calculated following these processes, firstly by removing all dirt, sand and other organisms from harvested seaweed. After that, all harvested samples of each treatment were weighed as fresh weight (g) and after dried for 2-3 days using hanging method, final dried weight (g) was recorded for each sample. The Ratio of DW: FW was then calculated for all the samples.
- Carrageenan yield. Small pieces (±5 g) of dried seaweed were cut by a small scissor and washed with fresh water. After that, the samples were dried fortnight. The dried samples were soaked for 12 hours with distilled water. The samples were then sterilized using an autoclave at 121°C for 30 minutes, then the samples were smoothed using a blender. The samples were then filtered using filters, followed by precipitated the samples with 100 ml of isopropanol. The samples were then dried in the oven for 24 hours. Finally, semi refined carrageenan (SRC) was obtained and dried in sunlight. The weight of dried SRC was recorded and expressed as a percentage (%). The carrageenan content was determined using the formula: 
  \[ \text{Content} (\%) = \frac{W_c}{W_m} \times 100 \] where \( W_c \) is weight of carrageenan extract (g) and \( W_m \) is the dry seaweed weight (g) used for extraction [16]. All data of DGR, ratio DW:FW and carrageenan content from all treatments were expressed as mean ± SD.
- Water quality parameters consisted of salinity and water temperatures were measured in-situ at 6 to 8 a.m. Salinity was measured using a hand refractometer and a thermometer was used to measure the temperature. Concentration of nitrate, phosphate and turbidity were measured with the HANNA™ instrument HI9870 311 (HANNA™, Woonsocket, RI, USA) immediately after samples were collected.

2.4. Statistical Analysis
Data obtained from all treatments were analyzed using analysis of variance (ANOVA), with the Tukey post-hoc test if appropriate, at the 95% confidence level. All statistical analyses were implemented using the statistical software SPSS version 16.
3. Results and Discussion

3.1. Grafting rate (GR)
Mean grafting rate (GR) was 75.0%. The 20-g IW treatment showed the highest GR (85.0%) (Table 1)

| Days | No  | IW (g) | No of | No of | Final | Mean grafting rate GR (%) |
|------|-----|--------|-------|-------|-------|---------------------------|
|      |     |        | seedlings | failed to attach | Number of seedlings |                   |
| 6    | 1   | 10 g   | 20     | 1      | 19    | 95                        | 95.00         |
|      | 2   | 15 g   | 20     | 2      | 10    | 90                        |               |
|      | 3   | 20 g   | 20     | 0      | 0     | 100                       |               |
| 8    | 1   | 10 g   | 20     | 2      | 10    | 90                        | 90.00         |
|      | 2   | 15 g   | 20     | 3      | 15    | 85                        |               |
|      | 3   | 20 g   | 20     | 1      | 5     | 95                        |               |
| 12   | 1   | 10 g   | 20     | 3      | 15    | 85                        | 86.67         |
|      | 2   | 15 g   | 20     | 3      | 15    | 85                        |               |
|      | 3   | 20 g   | 20     | 2      | 10    | 90                        |               |
| 14   | 1   | 10 g   | 20     | 4      | 5     | 16                        | 80            |
|      | 2   | 15 g   | 20     | 3      | 15    | 85                        | 85.00         |
|      | 3   | 20 g   | 20     | 2      | 10    | 90                        |               |
| 16   | 1   | 10 g   | 20     | 4      | 20    | 16                        | 80            |
|      | 2   | 15 g   | 20     | 4      | 20    | 16                        | 80.67         |
|      | 3   | 20 g   | 20     | 3      | 15    | 85                        |               |
| 18   | 1   | 10 g   | 20     | 7      | 35    | 13                        | 65            |
|      | 2   | 15 g   | 20     | 5      | 25    | 15                        | 75.00         |
|      | 3   | 20 g   | 20     | 3      | 15    | 17                        |               |

3.2. Daily Growth Rate (DGR)
The DGR of all treatments tended insignificantly different (Figure 2 and Table 2).

Figure 2. DGR of seaweed (K. alvarezii) of the combination of local strain and tissue-cultured seedlings with different initial weight (IW).
Mean DGRs of grafted seaweed cultivated 45 days showed that 20-g IW showed the highest DGR (4.36±0.81%/day) followed by 10-g IW (4.09±0.86%/day) and 15-g IW (4.01±0.82%/day). The DGRs obtained in this study was higher than those of internationally recommended for commercial DGR (3.50%) [17]. However, the DGRs found in this study were lower than those from the grafting study conducted previously in India [9]. Obtained DGRs was 6.4%-8.19%/day. Lower DGRs in this study were attributed to salinity and turbidity level. Lower salinity (25.0°C) and higher turbidity (7.336 NTU) caused by frequently heavy flooding during this study (see Figure 4) were not suitable for seaweed growth. Heavy flooding mainly causing biomass loss of seedlings due to the breakage of thalli. Furthermore, the research period carried out from January-April is usually period of lower growth rate for local seaweed farming in Sasara Coastal Waters, Kulisusu Bay, North Buton.

Table 2. DGR and Tukey Test analysis of grafted seaweed (K. alvarezii) of the combination of local strain and tissue-cultured seedlings using different initial weight (IW).

| IW (g) | Days | DGR(%/day ± SD) | Tukey Test | p-value |
|--------|------|-----------------|------------|---------|
|        | 10   | 5.18±0.64       | 5.21a      | 0.000   |
| 9      | 15   | 5.08±1.89       | 5.11a      | 0.965   |
| 20     |      | 5.38±0.64       | 5.38a      | 0.876   |
| 18     | 10   | 4.47±0.48       | 4.48a      | 0.001   |
| 15     |      | 4.45±0.50       | 4.46a      | 1.000   |
| 20     |      | 4.71±0.47       | 4.70a      | 0.671   |
| 27     | 10   | 4.34±0.61       | 4.27a      | 0.001   |
| 15     |      | 4.10±0.47       | 4.05b      | 0.519   |
| 20     |      | 4.64±0.35       | 4.68a      | 0.046   |
| 36     | 10   | 3.09±0.41       | 3.07b      | 0.000   |
| 15     |      | 3.06±0.45       | 3.06b      | 1.000   |
| 20     |      | 3.43±0.42       | 3.48a      | 0.013   |
| 45     | 10   | 3.36±0.57       | 3.36a      | 0.000   |
| 15     |      | 3.36±0.54       | 3.36a      | 1.000   |
| 20     |      | 3.64±0.49       | 3.63a      | 0.137   |

* values followed by different letters are significantly different at p < 0.05

3.3. The ratio of Fresh Weight (FW) and Dry weight (DW)
There was no significant differences of fresh weight and dry weight (FW: DW) ratio among the treatments (Table 3).

Table 3. Ratio DW:FW of grafted seaweed (K. alvarezii) from the combination of local strain and tissue-cultured seedlings using different initial weight (IW).

| IW (g) | Wt Fresh weight (g) | Wt Dry weight (g) | DW:FW ratio | Tukey Test | p-value |
|--------|---------------------|-------------------|-------------|------------|---------|
|        | 2                   | 3                 | 4           | 5          | 6       |
| 10     | 46.50±1.14          | 7.01±0.85         | 6.63:1      | 7.01a      | 0.010   |
| 15     | 45.80±1.42          | 5.82±0.15         | 7.87:1      | 5.82a      | 0.062   |
| 20     | 50.90±1.80          | 6.20±0.30         | 8.21:1      | 6.20a      | 0.237   |
| Mean   | 43.75±8.28          | 6.94±0.40         | 6.59:1      |            |         |
The ratio (FW: DW) of 10-, 15-, and 20-g IW were 6.63:1, 7.87:1, and 8.21:1, respectively. The ratio of FW: DW obtained during this study were comparatively better than those of the un-grafted seedlings in previous studies using local strain conducted by [17] in Indian waters (9.89:1) and using tissue-cultured seedling conducted in Marobo, Indonesia (8.74:1) [18]. Based on this ratio of DW: FW, the grafting using different initial weight of a combination of local strain and tissue-cultured seedlings showed heavier and better ratio than those of un-grafted local strain.

3.4. Carrageenan Content

Carrageenan content of grafted seaweed seedlings showed significant differences among treatments (Table 4). Carrageenan content of 10-g IW was 40.38 ± 1.05% and 15-g IW (40.25 ± 1.65%) had significantly higher carrageenan content than 20-g IW (38.34 ± 1.10%).

Table 4. Carrageenan content of grafted seaweed (K. alvarezii) from the combination of local strain and tissue-cultured seedlings using different initial weight (IW).

| IW (g) | Tukey test and STDEV | p-value |
|--------|-----------------------|---------|
|        | 1                     | 2       |         |
| 10     | 40.38 ± 1.05<sup>a</sup> | 0.027   |         |
| 15     | 40.25 ± 1.65<sup>a</sup> | 0.999   |         |
| 20     | 38.34 ± 1.10<sup>b</sup> | 0.298   |         |

* values followed by different letters are significantly different at p < 0.05

The carrageenan content found for this were higher than those reported at previous grafting study done in India (31.0-39.0%) [9]. Carrageenan content found in this study fulfills international standard (minimum 27%) [17]. In addition, the carrageenan content in this study were comparatively higher with those reported using ungrafted local strain from other countries. The content in Brazil were 31-43% [19], while in Mexico were 30.3-40.7% [20]. Therefore, grafting method using a combination tissue-cultured seedling and local strain could significantly increase carrageenan content.

3.5. Water Quality Parameters

Water quality parameters in this study mostly fall within the range of optimum requirements for grafted K. alvarezii farming except for the salinity and turbidity (Figure 3). The temperature obtained was 29.0-31.0°C. salinity 25.0-32.0 ppt, turbidity 1.038-7.336 NTU, nitrates 0.324-0.420 mg/l and phosphate 0.180-0.538 mg/l. The optimum mariculture condition for cultivating K. alvarezii in term of optimum temperature is about 27-30°C and salinity is 30-33 ppt [21]. Phosphate level for seaweed growth is range from 0.02 to 1.0 ppm [22], while for nitrate level, it should be below 1.0 mg L<sup>-1</sup> [23]. Salinity decreases at the 18<sup>th</sup> days of the maintenance period and coexist with high turbidity (Figure 3). This was caused by heavy rain. The optimum temperature range is 26-33°C, salinity 30-38 ppt and turbidity ≤ 2 mg / 1 [12].
Figure 3. Water quality parameters measured during this study. A Temperature; B, Salinity; C, Nitrate; D, Phosphate; and E, Turbidity.

4. Conclusion
Side-slipped grafting using the combination of tissue-cultured and local strains of the red seaweed *Kappaphycus alvarezii* is a technique suitable to be adopted by seaweed farmers. We recommend grafting techniques using 20-g initial seedling weight.

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