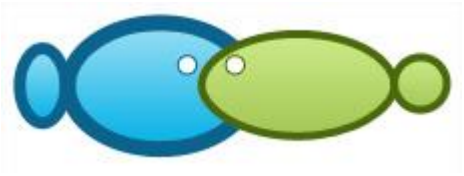

In vitro shoot micropropagation of *Gracilaria verrucosa* using plant growth dual regulators

[1]Muhammad E. Nurrahmawan, [2]Dwi Oktafitria, [3]Hery Purnobasuki, [1]Dini Ermavitalini, [1]Nurul Jadid

[1] Department of Biology, Sepuluh Nopember Institute of Technology, Surabaya, Indonesia; [2] Department of Biology, PGRI Ronggolawe Tuban University, Indonesia; [3] Department of Biology, Airlangga University, Surabaya, Indonesia. Corresponding author: N. Jadid, nuruljadid@bio.its.ac.id

Abstract. *Gracilaria verrucosa*, an agar producing seaweed, is the highest and most cultivated seaweed in Indonesia. However, its productivity is still low and has not met the global demand for agar. In addition, the lack of good quality seed stocks of this species becomes serious problem. Therefore, an efficient *G. verrucosa* in vitro culture could be an alternative to solve this obstacle. However, little is known about this method of cultivation. The present study aimed to determine the effect of dual plant growth regulators (IAA and BAP) applications on the shoot micropropagation of *G. verrucosa*. Intercalar explants of *G. verrucosa* were grown into PES medium supplemented by both IAA and BAP in various concentrations (0; 0.1; 0.3; 0.5 mg L^{-1}) for 30 days. Single BAP at 0.5 mg L^{-1} treatment showed the best result in all parameters measured, including growth rate (0.42% day^{-1}), percentage of explant producing shoot (56%) and the number of shoot per explant (2.64 shoots $explant^{-1}$).
Key Words: seaweed, plant growth regulator, agar, IAA, BAP.

Introduction. *Gracilaria verrucosa* is one of the promising red algae (Rhodophyta), which is commonly cultivated in the tropical regions, including Indonesia. The species offers important economic interest due to its agar content (Carneiro et al 2011). Therefore, *G. verrucosa* is also an agarophyte, together with other agar-producing red algae such as *Gelidium* and *Gelidiella* (Rocha et al 2019). Agar, the main product of red algae, is a mixture of agarose and agaropectin. It has been used as thickeners and emulsifiers in food industry, medicine, cosmetics, paper, textiles, industrial oils and other biotechnological industries (FAO 2003; Olatunji 2020). Due to an increased demand of agar worldwide, the cultivation of *G. verrucosa* has been increasing significantly in the last decade. According to FAO (2010), Indonesia is considered as the second largest place for *G. verrucosa* cultivation, which reaches 253 thousand tons of dried seaweed or contribute to about 30.02% of the total global *Gracilaria* production (Rejeki et al 2018). One of the centers of seaweed cultivation in the eastern part of Indonesia is located in the Jabon Subdistrict, Sidoarjo Regency with a total production value reaching 1,344 tons of dried seaweed in 2016 (BPS 2016).

According to KKP (2016), the global demand for agar in 2016 reached 550,000 tons and continuously increases every year. Various efforts have been made to cover the high demand of agar, such as the provision of sustainable seeds. To date, the provision of sustainable seeds is still facing many obstacles, such as the low growth rate of seaweed and adverse environmental conditions due to epiphytes attacks and infectious diseases (Sahu et al 2020). The provision of sustainable seeds has been done through conventional techniques by vegetative propagation using the cutting techniques. This technique includes the selection of high growth rates seaweed and the plants re-cultivation into new areas (Masak et al 2011). Nevertheless, frequent vegetative propagation could decrease the seaweed seeds quality, growth rate, agar content and gel strength, and increase the seaweed susceptibility to diseases (Hurtado & Cheney 2003). Therefore, the development of an alternative method of propagation is a priority.

In vitro micropropagation techniques provide promising prospects for the development of commercial high yield seaweed. This technique provides good quality seaweed, since the preliminary selection of high-quality of seeds is done before. Consequently, the resulting clones should have similar genetic characteristics to the mother plant. In addition, the in vitro culture method is not affected by the weather and uncertain climate factors. Furthermore, the method is not time-consuming and could produce large amounts of clones (Yong et al 2014).

The efficiency and effectiveness of in vitro shoot micropropagation is mainly determined by endogenous and exogenous factors. The endogenous factors include explant characteristics such as age, explant sources, developmental stage and physiology, whereas exogenous factors include plant growth regulators (PGRs) (Jadid et al 2015), salinity, light irradiation, photoperiodism, temperature, pH and media composition (Yokoya et al 2011). According to Yeong et al (2014), the use of PGRs plays a role in the success of in vitro shoot micropropagation. It has been demonstrated that the use of 0.1 mg L^{-1} 2,4-D and 0.1 mg L^{-1} kinetin in Provasoli's Enriched Seawater (PES) medium stimulates micropropagation of *Gracilaria changii* shoots explosively. The in vitro culture of some *Gracilaria* genus has been reported, for instant *G. textorii* (Huang & Fujita 1997), *G. vermiculophylla* (Yokoya et al 1999), *G. chilensis* (Collantes et al 2004), *G. tenuifrons* (Yokoya 2000), *G. tenuistipatata*, *G. perplexa* (Yokoya et al 2004), *G. corticata* (Kumar et al 2007). However, studies on the induction and multiplication of shoots on *G. verrucosa* are still limited to the use of F/2 medium supplemented with 5 mg L^{-1} DPU (Diphenyl urea) (Kaczyna & Megnet 1993) and PES medium with 5 mg L^{-1} kinetin (Gusev et al 1987). Therefore, the present study was conducted to determine the effect of plant growth dual regulators applications with indole 3-acetic acid (IAA) and benzylaminopurine (BAP) on the shoot micropropagation of *G. verrucosa*.

Material and Method

Preparation of explants. Seaweeds were taken using the semi-wet method, according to Sulistiani et al (2012), with modifications. The samples were collected from aquaculture ponds in Kupang Village, Jabon Sub-district, Sidoarjo Regency, East Java (7° 31'21.3 "S 112° 49'34.0" E). First, samples were cleaned from dirt and attached organisms (epiphytes). Thereafter, the samples were placed into a coolbox in wet conditions before being used in further steps.

Sterilization of explants. Sterilization was performed based on Reddy et al (2003), with modification. First, samples were cut off on the apical thallus (5 cm length) in the laminar air flow and rinsed twice with sterile seawater. Thereafter, explants were immersed in 0.1% (w/v) of detergent solution for 10 min. After that, explants were placed into 1% (v/v) povidone-iodine solution for 1 min. In each treatment, explants were rinsed with sterile seawater twice. Furthermore, explants were immersed in 3% (v/v) PES antibiotic medium solution for 48 hours. The cultures were stored on a rotary shaker, at a room temperature of 22-25^{o}C, and irradiated at a light intensity of 1,500 lux, for 12:12 hours. Finally, explants were rinsed with sterile seawater twice and dried on sterile tissue. Sterile explants were ready to use for next treatments.

Inoculation of explants. Inoculation of explants was done based on Sulistiani et al (2012), with modifications. Sterile explants were cut to a length of 1±0.5 cm using a sterile scalpel in LAF. After that, the pieces of explants were inserted into a culture glass containing 2% (v/v) solid PES medium. Each bottle contained 5 explants arranged circularly. Furthermore, the glass cultures were closed with HDPE plastic and kept in a culture rack for 4 weeks, at a room temperature of 22-25^{o}C and irradiated at a light intensity of 1,500 lux, for 12:12 hours.

Observation of explants. The observation of the explant growth rate was done by using the formula GR=[LN(Wt/W0)/t]×100 according to Reddy et al (2003), where W0 is the initial fresh weight and Wt is the final fresh weight of the explants after t days of

culture. The percentage of shoots per explants was calculated using the formula C=(Et/E0)x100, according to Yong et al (2014). Furthermore, the total number of shoots per explant, after 30 days of culture, was also counted.

Data analysis. This study used completely randomized designs with two factors, IAA and BAP. Both PGRs have 4 levels of concentration (0; 0.1; 0.3; 0.5 mg L^{-1}). Therefore, 16 treatments and 5 replications were used in this study. The growth rate of the explant, the percentage of explant forming shoots and the number of shoots per explant were observed. The study was conducted using a completely randomized design and analyzed using a two-way analysis of variance (ANOVA), followed by a Tukey post-hoc analysis.

Results and discussion

The effect of IAA and BAP combination on the growth rate of G. verrucosa explants. The combination of IAA and BAP significantly influenced the growth rate of *G. verrucosa* explants ($p<0.05$). Meanwhile, BAP alone increased the growth of the explants. Different results of growth rate suggested that the effect of the IAA and BAP combination is concentration dependent. In contrast, the use of IAA alone did not depend on the level of concentrations: the growth rate of the explants varied independently in this treatment. In addition, our observation also demonstrated that an increase of both PGRs concentrations resulted in a decrease of *G. verrucosa* growth rate. The highest growth rate performance (0.42% day^{-1}) was obtained when the explants were placed in the PES medium supplemented with 0 mg L^{-1} IAA and 0.5 mg L^{-1} BAP (Table 1).

Table 1

The effect of IAA and BAP combination on growth rate of *Gracilaria verrucosa* explants

Plant growth regulators (mg L^{-1})		*Growth rate of G. verrucosa explants (% day^{-1})*
IAA	*BAP*	
0	0	0.17^{b}
	0.1	0.23^{ab}
	0.3	0.32^{ab}
	0.5	0.42^{a}
0.1	0	0.21^{ab}
	0.1	0.30^{ab}
	0.3	0.23^{ab}
	0.5	0.30^{b}
0.3	0	0.15^{b}
	0.1	0.22^{ab}
	0.3	0.24^{ab}
	0.5	0.16^{ab}
0.5	0	0.23^{ab}
	0.1	0.13^{b}
	0.3	0.16^{b}
	0.5	0.10^{b}

The numbers followed by the same letter do not significantly differ according to the Tukey test with a significance level of 5%.

Meanwhile, the treatment with 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} BAP resulted in the lowest growth rate response (0.1% day^{-1}). Interestingly, PES medium with no PGRs also showed a non-negligible growth rate response (0.17% day^{-1}). This might be due to endogenous hormones that play a role in promoting the growth. According to Bradley & Cheney (1991) and to Yokoya et al (2010), plant hormones such as auxin and cytokinin are naturally produced within the plants. Yokoya (2000) reported that the effect of auxin and cytokinin on the growth rate of *G. tenuifrons* was associated with the function of PGRs in the process of cell division, elongation and differentiation. Auxins play a central role in regulating cell growth and elongation, while cytokinin regulates plant cell division and

morphogenesis (Ooi et al 2013). Other studies reported that the combination of 1 mg L^{-1} IAA and 2.5 mg L^{-1} BAP optimally increased the growth rate of *Kappaphycus alvarezii* seaweed explants (4% day^{-1}) (Yong et al 2014).

The growth process at the cellular level is defined as an increase of organic materials (biomass) resulting in an increase of cell size and subsequently affecting the cell division (Sablowski 2016). The relationship between growth and the production of new cells (cell cycle) is essential in the development of multicellular organisms (Dewitte & Murray 2003; Jorgensen & Tyers 2004; Jones et al 2017). Growth and cell cycle are controlled by changes in the expression of cyclin-dependent kinase (CDK) and cyclin genes, due to the effects of auxin and cytokinin. Auxins play an important role in the process of gene expression dynamics including RNA polymerase activity, ribosomal RNA level, and polyribosome protein mRNA levels enhancement (Sablowski & Dornelas 2014). Meanwhile, cytokinins play a role in the process of protein-specific synthesis during the cell cycle stages (Kieber & Schaller 2018).

In cell cycle, auxins induce CDK expression, inhibiting CDK antagonists (KRP1/KRP2) (Himanen et al 2002), improving E2FA/B protein stability (Magyar et al 2005), stimulating SKP2A degradation and inducing Telomerase expression in phase S through increased telomerase activity during replication (Tamura et al 1999). Cytokinins are also reported to induce cdc*2* gene expression in the G2/M transition phase, and induce CycD3 expression in the G1/S phase (Sablowski & Dornelas 2014).

The effect of IAA and BAP combination on shoot induction and multiplication of G. verrucosa explants. Use of PES medium supplemented with combination of IAA and BAP for 30 days showed shoot induction and multiplication on the apical, intercalary and basal part of the explants (Figure 1). These responses are promoted by the PGRs used in the present study. Meanwhile, we observed that the explants which did not undergo shoot induction showed bleaching symptoms (Figure 1c), death (Figure 1c) and contamination (Figure 1d).

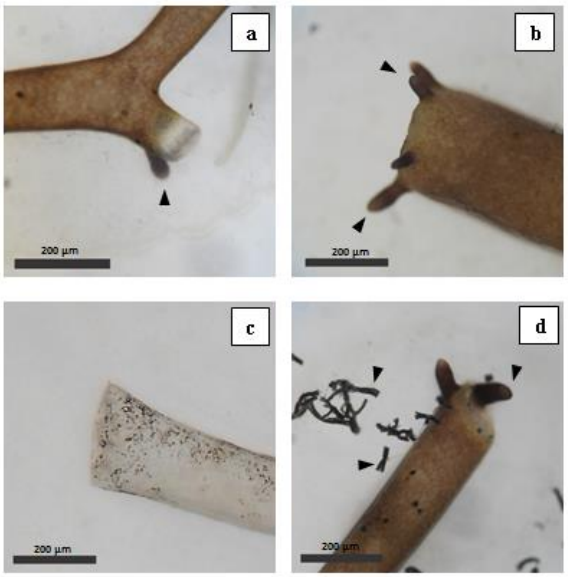

Figure 1. Condition of explant after 30 days of culture: a) shoots on apical part in 0 mg L^{-1} IAA and 0 mg L^{-1} BAP treatment; b) shoots on intercalar part in 0 mg L^{-1} IAA and 0.5 mg L^{-1} BAP treatment; c) explants experiencing bleaching and death in 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} BAP treatment; d) contaminated explant in 0.5 mg L^{-1} and 0.3 mg L^{-1} treatment (black arrow). (Bar scale=200 µm; magnification 67.5x).

The combination of IAA and BAP did not significantly induce shoot formation ($p<0.05$), but it did not promote shoot multiplication (Table 2 and 3). The highest percentage of shoot per explants and number of shoots were obtained in treatment with 0.5 mg L^{-1} BAP alone (56% and 2.64 shoots $explant^{-1}$, respectively). It seems that the use of BAP alone

was more effective compared to the treatment with IAA alone. Cytokinin has been reported to induce bud formation in *Sargassum polycystum* (Muhamad et al 2018). In addition, Uji et al (2016) has also demonstrated that cytokinin enhanced the formation of adventitious bud in *Sargassum horneri*. In contrast, an increase of both PGRs concentrations results in a decrease of *G. verrucosa* explants shoot percentage and multiplication. Previous study has reported that excessive PGRs concentration level had negatively affected the seaweed growth (Muhamad et al 2018; Sulistiani et al 2012). In the present study, the treatment of 0.5 mg L^{-1} IAA and 0.1 mg L^{-1} BAP resulted in the lowest shoot percentage (4%). In terms of number of shoot per explant, the lowest result was obtained in the treatment with 0.5 mg L^{-1} BAP and 0.5 mg L^{-1} IAA. Interestingly, zero PGRs treatments, somehow, induce shoot formation and multiplication. The later might be explained by the effect of endogenous hormones in shoot formation and multiplication (Hu et al 2017). The existence of endogenous hormones including auxin and cytokinin has been recorded in 11 species of red algae (Yokoya et al 2010). However, investigation of the endogenous hormone effects in seaweed in vitro culture are very limited (Neumann et al 2009).

Table 2

The effect of IAA and BAP combination on shoot formation

Plant growth regulators (mg L^{-1})		*Percentage of shoot induction (%)*	*Number of shoots explants^{-1}*
IAA	*BAP*		
0	0	24	1.4^{de}
	0.1	48	1.96^{bc}
	0.3	28	1.76^{cd}
	0.5	56	2.64^{a}
0.1	0	40	2.04^{bc}
	0.1	20	1.2^{ef}
	0.3	36	2.32^{ab}
	0.5	16	1.56^{cde}
0.3	0	8	0.72^{fg}
	0.1	8	0.76^{fg}
	0.3	8	0.72^{fg}
	0.5	16	1.16^{ef}
0.5	0	12	1.08^{ef}
	0.1	4	0.48^{g}
	0.3	16	0.8^{fg}
	0.5	8	0.4^{g}

The numbers followed by the same letter do not significantly different according to the Tukey test with a significant level of 5%.

In vitro shoot induction and multiplication are influenced by a variety of factors, including PGR interactions, incubation periods, agar-solidifying agents, type of explant and plant genotypes (Fatima & Anis 2012). The interaction between auxin and cytokinin is very important in controlling the process of plant development such as shoot (Boo et al 2015), root (Jing & Strader 2019) and callus formation (Schaller et al 2015). High level of auxin compared to cytokinin could trigger callus formation. Moreover, auxin alone induces the formation of roots. Finaly, a high ratio of cytokinin to auxin concentrations induces shoot regeneration (Boo et al 2015).

In the process of de novo organogenesis, explants undergoing cavity or cutting process were cultured in PES medium supplemented with exogenous hormones to produce adventitious roots or shoots (Liu et al 2014). In this study, the induction of *G. verrucosa* seaweed shoots appeared on the injury part of explant. The ability of a cell to regenerate depends on its ability to respond the hormonal signals, both at the beginning of the cell cycle and during its development (Motte et al 2014; Duclerq et al 2011).

The mechanism of auxin and cytokinin interaction on shoot growth has been reported by Che et al (2008). In their study, shoots were regenerated from root explants

through two stages: preincubation on medium-rich auxin or callus induction medium (CIM) and then cultured into medium-rich cytokines or shoot induction medium (SIM). During the CIM preincubation stage, the pericycle cell of explants undergoes cell division and gain the ability to respond cytokinin signals to form shoots. Cell division activity is needed to obtain the gene's ability to express de novo shoot formation, which occurs only in the SIM medium if root explants are first incubated into CIM media (Che et al 2008; Che et al 2006; Cary et al 2002). After being transferred into SIM medium, explant will develop independent shoots without the presence of cytokinin (shoot commitment). At that stage, the apical meristem would develop shoots, and the expression of Cup shaped cotyledon 2 (CUC2) gene would also increase. CUC1 and CUC2 are thought to encode the NAC1-domain transcription factors that are also needed to express Shootmeristemless (STM) genes and the formation of meristem shoots (Aida et al 1997; Takada et al 2001). STM and Wuschel (WUS) are also required for the formation of meristem shoots. The role of cytokinin and genes Knotted-1 like in *Arabidopsis thaliana* (KNAT1) on shoot development is also associated with the STM gene (Lincoln et al 1994).

Shemer et al (2015) reported that shoot formation of root explants cultured on the CIM medium and then on the SIM medium is also influenced by the DNA methylation. In that study, the chromomethylase (CMT3) mutant showed high capacity to regenerate shoots in SIM-direct medium, without any treatment into a CIM medium, through direct organogenesis. The CMT3 mutants are plants that can reduce CHG methylation. The results showed that shoots were formed directly on the CMT3 explant placed in the SIM-direct medium. It might be caused by the cytokinin signal response in decreasing the level of DNA methylation of explant allowing some related genes such as WUS to be expressed in shoot formation.

Conclusions. The current study has successfully documented the effect of IAA and BAP combination on shoot induction and multiplication of the *G. verrucosa* in vitro. The best results were demonstrated in the MS medium supplemented with 0.5 mg L^{-1} BAP alone. In this treatment, 56% of explants displayed shoot formation. Meanwhile, the highest shoot number was also obtained in this treatment (2.64 shoots $explants^{-1}$). Further studies should be continuously carried out in order to enhance shoot multiplication of the *G. verrucosa*.

Conflict of interest. The authors declare no conflict of interest.

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Authors:
Muhammad Evan Nurrahmawan, Sepuluh Nopember Institute of Technology, Department of Biology, Jl. Teknik Kimia, Keputih, Kec. Sukolilo, Kota SBY, 60111 Jawa Timur, Indonesia, e-mail: muhammadevan12@gmail.com
Dwi Oktafitria, PGRI Ronggolawe Tuban University, Department of Biology, Jl. Manunggal No.61, Wire, Gedongombo, Kec. Semanding, Kabupaten Tuban, 62391 Jawa Timur, Indonesia, e-mail: dwioktafitria86@gmail.com
Hery Purnobasuki, Airlangga University, Department of Biology, Jl. Airlangga No.4-6, Airlangga, Kec. Gubeng, Kota SBY, 60115 Jawa Timur, Indonesia, e-mail: hery-p@fst.unair.ac.id
Dini Ermavitalini, Department of Biology, Sepuluh Nopember Institute of Technology, Jl. Teknik Kimia, Keputih, Kec. Sukolilo, Kota SBY, 60111 Jawa Timur, Indonesia, e-mail: dinierma@bio.its.ac.id
Nurul Jadid, Sepuluh Nopember Institute of Technology, Department of Biology, Jl. Teknik Kimia, Keputih, Kec. Sukolilo, Kota SBY, 60111 Jawa Timur, Indonesia, e-mail: nuruljadid@bio.its.ac.id

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