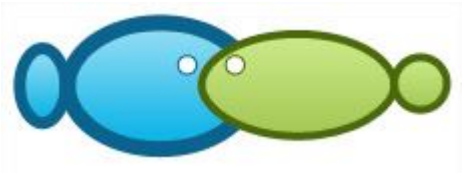

Caulerpa racemosa (Chlorophyta, Caulerpaceae) extract increases growth and biomass production of *Gracilaria verrucosa* (Rhodophyta, Gracilariaceae)

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Abstract. Low production of *Gracilaria verrucosa*, a seaweed widely cultivated in tropical countries, is often due to slow growth caused by disease called ice-ice. Green algae *Caulerpa racemosa* has been utilized as fertilizer and bactericide in horticulture and farming. The objectives of this study were to determine the effectiveness of *C. racemosa* extract in preventing ice-ice, stimulating growth and promoting biomass productivity of *G. verrucosa*. The Analysis of Variance showed no significant difference in daily growth rate in *G. verrucosa* treated with 62.5 ppm and 125 ppm *C. racemosa* extract, however daily growth rate increased significantly with increased soaking time. Daily growth rate was highest (2.47%) with 5 h soaking time, followed by 4 h (2.16%), 3 h (1.93%) and 2 h (1.80%). Extract concentration of 125 ppm resulted in higher biomass productivity (273.89 g m^{-2}) compared to 62.5 ppm (229.50 g m^{-2}). The control (soaked in distilled water) suffered from ice-ice caused by *Pseudomonas* sp. infection, resulting in loss of biomass from an initial weight of 177g m^{-2} to 103.72 g m^{-2}. Biomass productivity was highest (325.22 g m^{-2}) for the 5 h soaking time treatment, followed by the 4 h, 3 h and 2 h treatments (259.2 g m^{-2}, 220.33 g m^{-2}, and 200.93 g m^{-2}, respectively). These findings showed that exposure to *C. racemosa* metabolites could improve *G. verrucosa* productivity and hence increase production. Since both species are cultured in brackishwater fishpond, we recommend polyculture trials of *G. verrucosa* with *C. racemosa* in brackishwater fishponds and in controlled environments to investigate whether bactericidal metabolites exuded by *C. racemosa* can be absorbed by *G. verrucosa*.

Key Words: bactericide, *Caulerpa racemosa*, *Gracilaria verrucosa,* growth, ice-ice.

Introduction. The seaweed *Gracilaria verrucosa* is an economically important agarophyte red algae widely cultivated in Southeast Asia, including Indonesia. Low productivity, mainly due to disease has affected many production areas. Decreases up to 70-100% in seaweed production have been attributed to ice-ice, a disease for which up to now there is no known cure or means of eradication (Vairappan et al 2008). Many pathogenic bacteria can infect seaweed, including the genera *Vibrio, Staphylococcus, Bacillus, Photobacterium, Pseudomonas, Flavobacterium, Micrococcus., Flexibacter*, and *Alcaligenes* (Austin & Austin 1993), however, *Pseudomonas* in particular has been implicated in ice-ice on *G. verrucosa* (Nasmia et al 2014). While such pathogens are commonly present, changes in environmental factors which exceed tolerance limits can also cause seaweed to become susceptible to ice-ice (Trono 1974).

Caulerpa racemosa is a green seaweed with antibacterial activity (Kandhasamy & Arunachalam 2008), and also contains nutrients such as nitrogen, magnesium, and iron (Gabrielsen 1996) and hormones which act as growth regulators (Montano & Tupas 1990). As a source of natural antibacterial compounds (Vallinayagam et al 2009), secondary metabolites (Castro et al 2004) and nutrients, extracts or exudate from this seaweed could be expected to provide protection from bacterial disease, stimulate growth

and increase biomass production in *G. verrucosa*. The objectives of this study were to determine the effectiveness of *C. racemosa* extract in eradicating ice-ice associated with pathogenic *Pseudomonas* infection and enhance the growth and biomass production of *G. verrucosa.*

Material and Method

Isolation, identification and pathogenicity test of bacteria. Ice-ice infected *G. verrucosa* thallus were collected from farms in Takalar Regency, 35 miles south of Makassar City, Indonesia. The symptoms of ice-ice disease, such as changes in thallus colour and texture (Figure 1) have been widely described (Lobban & Harrison 1994; Lundsor 2001; Directorate General of Aquaculture 2004).

Figure 1. Ice-ice infected *G. verrucosa*.

Bacteria were isolated and identified at the Marine Microbiology Laboratory, Faculty of Marine Sciences and Fisheries, Hasanuddin University. To isolate the bacteria, Tryptic Soy Agar (TSA) and Thiosulphate Citrate Bile Sucrose (TCBS) media were incubated at 30^{o}C in an autoclave, since pathogenic bacteria on farmed marine organisms are mesophylic with an optimum temperature of 10-30^{o}C. Physiological and biochemical characteristics of the bacteria were determined. Based on the pathogenicity test, among the identified bacteria infecting *G. verrucosa* (*Acinetobacter, Pseudomonas, Vibrio, Bacillus, Flavo-cytophaga, Micrococcus*)*, Pseudomonas* was the most pathogenic bacteria, causing the change in thallus colour and texture (begun to occur on the third day of infection test). This motile bacteria (Bergey et al 1974) is a plant pathogen (Misaghi 1982) widely distributed in both fresh water and marine environments with an optimum temperature range for growth and development of 30-37^{o}C (Bergey et al 1974), overlapping the optimum range for *G. verrucosa* growth.

Extraction and detection of C. racemosa flavonoids. Flavonoids were extracted from the green alga *C. racemosa* in the Marine Microbiology Laboratory, Faculty of Marine Science and Fisheries, Hasanuddin University using the agar diffusion method (Brooks et al 2001). Distilled water was used as solvent to extract flavonoids with ratio of 300 mL water to 50 g dry *C. racemosa* powder. The presence of flavonoids in the extract was indicated by orange and yellow colours under ultra violet (UV) light (wave length 254 nm and366 nm) with Rf values of 0.56 and 0.54 (Harborne 1984), respectively using Thin Layer Chromatographic technique (Stahl 1985; Gritten et al 1991).

Application of C. racemosa extract to G. verrucosa. This experiment was conducted at the Wet Laboratory, Faculty of Marine Sciences and Fisheries, Hasanuddin University, using *G. verrucosa* thalli with an initial weight of 50 g. Treatment factors were extract concentration and soaking time. Two extract concentrations were prepared by applying the diffusion method (Schlegel & Schmidt 1994): the lowest flavonoid concentration

(62.5 ppm) which inhibited *Pseudomonas* activity and a higher extract concentration (2 x 62.5 = 125 ppm). In addition to a control (soaking within distilled water only), for each concentration, soaking time treatments were 2, 3, 4 and 5 h with 3 replicates (27 experimental units). Each treated thallus was further transferred to a culture basin containing 5 litres of *Pseudomonas*-infected seawater and provided with aeration for a culture period of 42 days for the observation on effectiveness in inhibiting *Pseudomonas* in *G. verrucosa* and enhance its growth rate and biomass production. A Randomized Complete Design (RCD) experimental design was used.

Growth rate. Weight of the *G. verrucosa* thalli was recorded for six consecutive weeks. Daily growth rate (DGR) was determined using the following formula (Dawes et al 1993, in Hurtado et al 2001):

$$DGR = [L_n (W_t/W_o)]^t \times 100$$

Where: DGR = daily growth rate; L_n = natural logarithm; Wt = fresh weight at day t (g); Wo = initial fresh weight (g); t = time interval of measurement (7 days).

Biomass production. Biomass production was computed at the end of experiment and expressed as fresh weight of the seaweed per unit culture area (g m^{-2}), and computed with the following formula:

$$Y = (W_t - W_0)/A$$

Where: Y = biomass productivity; W_t = fresh weight at day t; W_0 = initial fresh weight (50 g/basin = 177 g m^{-2}); A = area of 1 m^2 culture basin.

As a fraction of biomass, chlorophyll a content of each thallus was determined following the procedure of Arnon (1949) in Thirumaran & Anantharaman (2009):

$$\text{Chlorophyl a (mg g}^{-1}) = \frac{12.7\, A_{663} - 2.69\, A_{645}}{A \bullet 1000 \bullet W} V$$

Where: A = absorption of the specified wave length; V = volume of solvent material (mL); W = fresh weight of seaweed (g).

Data analysis. The Randomized Complete Design (RCD) enabled the application of a factorial Analysis of Variance (ANOVA) to determine significance between treatments. The Tukey test was used to evaluate and compare the significance and influence of different *C. racemosa* extract concentrations and different soaking times on the growth and biomass productivity of *G. verrucosa*.

Results. Without *C. racemosa* extract (control), *G. verrucosa* thalli was suffered on ice-ice disease due to *Pseudomonas* infection. Ice-ice symptoms generally appeared on the third day after exposure to the infecting agent. Several thalli were yellowish or white in colour by the fourth and fifth days of the treatment. Examples of the ice-ice symptoms observed are shown in Figure 2.

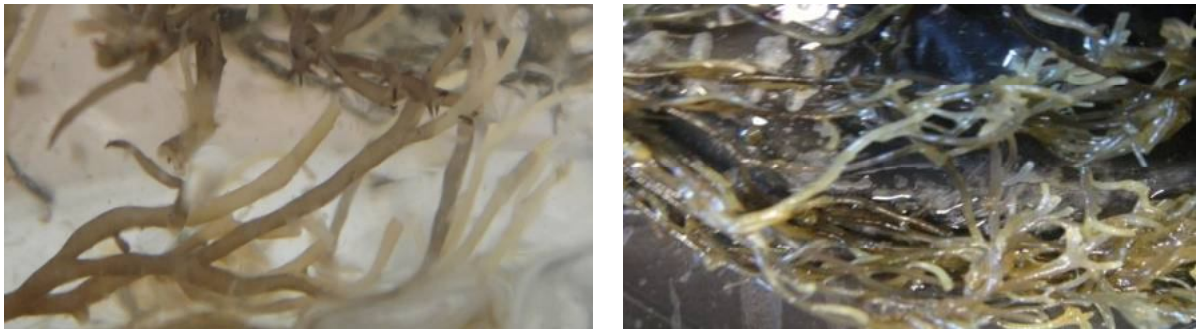
The statistical analysis showed no interaction between the two factors (*C. racemosa* extract concentration and soaking time) with respect to the DGR and biomass productivity of *G. verrucosa*. There was no growth of *G. verrucosa* in the control thalli (with distilled water without *C. racemosa* extract treatment) which experienced negative growth and a reduction in biomass, from the initial weight of 177 g m^{-2} to an average of 103.72 g m^{-2} at the end of the experiment.

The DGR (1.98%) of *G. verrucosa* treated with 62.5 ppm *C. racemosa* extract was not significantly different from the DGR (2.20%) of the thalli treated with 125 ppm *C. racemosa* extract ($p > 0.05$). However, DGR was significantly ($p < 0.05$) positively influenced by soaking time. The highest DGR (2.47%) was obtained with 5 h soaking time, followed by 4 h (2.16%), 3 h (1.93%), and the lowest (1.80%) for 2 h soaking time.

Each factor (*C. racemosa* extract concentration and soaking time) was significantly ($p < 0.05$) correlated with *G. verrucosa* biomass productivity. Similar to growth rate, the highest biomass production (325.22 g m^{-2}) was recorded for 5 h soaking time, followed by 4 h, 3 h, and 2 h (259.2 g m^{-2}, 220.33 g m^{-2}, and 200.93 g m^{-2}, respectively). The 125 ppm *C. racemosa* extract concentration resulted in significantly ($p < 0.05$) higher average biomass production (273.89 g m^{-2}) than the 62.5 ppm extract concentration (229.50 g m^{-2}).

Chlorophyll a content as a proportion of biomass increased from 0.028%-0.030% at the beginning of the experiment to 0.05% up to 0.104% at the end of the experiment in the *G. verrucosa* thalli soaked in *C. racemosa* extract. However in the control, chlorophyll a content initially increased only from 0.028% up to 0.05%.

Changes on thallus such as yellowish, whitish, excessive mucus production, and finally senescence begin to occurred on the tips.



Whole thallus was whitening, soft, fragmented, and excess of mucus release into surrounding water.

Figure 2. Condition and colour changes in *G. verrucosa* thalli due to ice-ice in the controls (without *C. racemosa* extract treatment).

Discussion. White spots, excessive mucus production, loss of turgidity and finally fragmentation of the thallus are symptoms observed in seaweed with the ice-ice disease. These symptoms are caused by toxic substances released by the infecting bacteria which are broken down the host plant cells (Christie 2003; Beattie 2007). The increase in thallus fragmentation over time (Lobban & Harrison 1994) is thought to be due to the ongoing reproduction (division) of bacterial cells in the host cells and tissue (Pelczar et al 1975), using nutrients and organic substances of the thallus (Pelczar et al 1975; Largo et al 1995).

The high daily growth rate of *G. verrucosa* soaked in flavonoid-rich *C. racemosa* extract can be attributed to the increased resistance of the soaked seaweed to ice-ice caused by *Pseudomonas* infection. Even at a low concentration and low soaking time, the flavonoids from *C. racemosa* were able to protect *G. verrucosa* from the pathogen. Flavonoids have been reported effective as a bactericide to prevent *Pseudomonas* infection (Izzati 2007; Pelczar et al 1975; Siregar et al 2012); they suppress activity and kill bacteria by destroying proteins, destabilize cell membrane structures and cytoplasm of the bacteria (Harborne 1984). Flavonoids are antibacterial compounds soluble in water (Harborne 1984) which can then enter into bacterial cells and inhibit their activity (Black & Jacobs 1993; Blunden et al 1996; Sabir 2005) as well as cell division (reproduction)

(Harborne 1984; Evans 1989; Kandalkar et al 2010), causing defects in the microsome, lysosome (Sabir 2005) and cytoplasm (Harborne 1984; Evans 1989; Kandalkar et al 2010). Thus, the protective effect might not only be due to residual flavonoids in or on the thalli tissues or surface, but also any flavonoids released into the water from the soaked thalli.

Algal extracts such as the *C. racemosa* extract have also been reported to empower the immune system of plants (Lopez et al 1995) and protect the cell membrane of seaweeds from oxidation (Blunden et al 1996). Flavonoids in green alga extracts can stimulate photosynthesis (Middleton & Teramura 1993) by protecting plants from photo-oxidation (Reddy & Raghavendra 2006) due to the extremely high light intensity exposure which is considered an important causal factor in ice-ice outbreaks during the peak of the dry season. Algal growth can be expressed as function of external or intracellular nutrient (Borchardt 1996). Activation of physiological functions of plants through algal extracts (Blunden et al 1996) could include increased effectiveness of nutrient absorption from the culture media through the thallus surface of *G. verrucosa.* In addition to direct effects from the nutrient content of the *C. racemosa* extract, the nutrients are dissolved along with the flavonoids. Both factors could contribute to higher daily growth rates in *G. verrucosa*, since *G. verrucosa* has the ability to retain nutrients and store them in the intracellular pool.

The availability of nutrients in sufficient concentration may increase the resistance of the seaweed to ice-ice, promote recovery and stimulate growth (Syamsuddin & Rahman 2014). Phosphorus plays a key role in meristem formation, the stimulation of cell division, and the repair of damaged tissues (Sutedjo 2008); it is also essential for Adenosine Tri Phosphate formation in the metabolic energy biochemical reactions within plant cells (Kuhl 1974). Potassium regulates nutrient use and stimulates meristematic growth (Sutedjo 2008), activates enzymes involved in plant growth, improves physical quality, helps avoid crop diseases and improves disease resistance, increases protein content of plants, builds cellulose, reduces lodging and activates starch synthetase (Armstrong 1998). Seaweed extracts may contain phosphorus, potassium, calcium and other plant nutrients (Rathore et al 2009). Gabrielsen (1996) reports that seaweed contains micro nutrients such as 0.66% chlor, 1.513 ppm iron, 0.804% manganese, 16 ppm copper, 28 ppm zinc, and 0.485% boron. *C. racemosa* extract has been reported to contain phosphorous essential for plant metabolism (Lapointe 1987) and as component of cell protoplasm, and high concentrations of nutrients required for the synthesis of proteins, nucleates, and other organic substances (Gabrielsen 1996). Based on nutrient analyses, *C. racemosa* thalli contain 3.60% total nitrogen, 0.938% potassium, 0.614% phosphorous, 16.79% calcium, 0.75% magnesium, 0.018% sulphur, 1.05 ppm iron, 14.95 ppm copper, and 36.13 ppm zinc. These are all nutrients needed for plant growth and development. Nutrients such as these are needed by plants and can be directly and efficiently absorbed (Chapman & Chapman 1980). In particular nitrogen, essential for improving plant response to pathogens (Sutedjo 2008), can be effectively absorbed by plants (Bird et al 1979). *C. racemosa* extract also contains hormones which stimulate agar production (Montano & Tupas 1990), in particular plant growth regulating hormones such as auxin, cytokinine and gibberellin (Montano & Tupas 1990; Khan et al 2009). It is therefore likely that increased soaking time in a *C. racemosa* extract solution enables *G. verrucosa* to absorb more nutrients and growth hormones, ultimately resulting in increased growth rate.

Bacterial infection such as that observed can cause the loss of thalli, and consequently biomass, resulting in reduced seaweed yield (Vairappan et al 2014). Lost of seaweed biomass can also result from reductions in growth and organic matter (Largo et al 1995), including the break down of plant proteins (Buell et al 2003) and carrageenan content (Vairappan et al 2014) by *Pseudomonas*. During infection process, bacterial may penetrate plant cells and digest carrageenan (Vairappan et al 2014). It is probable that the flavonoids contained in the *C. racemosa* extract were able to protect the soaked *G. verrucosa* from such organic matter reduction processes.

We observed an increase in the chlorophyll a synthesis capacity of *G. verrucosa* with increased concentration of and soaking time in the *C. racemosa* extract. Blunden et

al (1996) stated that seaweed extracts can be used to avoid degradation of the chlorophyll a biomass component. Photosyntesis determine biomass production of algae. And, chlorophyll is the driving force for the light-dependent reaction of photosynthesis. Nitrogen, magnesium, and iron are essential nutrients for chlorophyll a synthesis (Lawlor 1993; Sutedjo 2008).

Conclusions. Treatment with a *C. racemosa* extract containing flavonoids had a significant positive effect on the health of the red alga *G. verrucosa,* through disease (ice-ice) prevention and higher productivity. We found that, in addition to eradicating the pathogenic bacteria *Pseudomonas*, the nutrient and hormone content of this extract stimulate *G. verrucosa* growth and biomass production. Further research is needed before these findings can be applied to improve seaweed farming productivity. In particular, trials of *G. verrucosa* polyculture with *C. racemosa* in both brackishwater ponds (since both species are cultured in brackishwater fishpond) and in controlled environments, with investigation of the physiological pathways and whether (or to what extent) the flavonoids and other bactericidal compounds exuded by *C. racemosa* are absorbed by *G. verrucosa* under various conditions.

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