Cultivation Characteristics and Biological Responses of Agarophytic Seaweed, *Gracilaria fisheri* (Rhodophyta), in Southern Thailand

Prince of Songkla University
Pattani Campus

Phi Thi Nguyen

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Fishery Technology

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I hereby certify that this work has not been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ คุณลักษณะการเพาะเลี้ยงและการตอบสนองทางชีววิทยาของสาหร่ายวุ้น *Gracilaria fisheri* (Rhodophyta) ในภาคใต้ของประเทศไทย

ผู้เขียน Miss Phi Thi Nguyen

สาขาวิชา เทคโนโลยีการประมง

ปีการศึกษา 2557

บทคัดย่อ

การศึกษาคุณลักษณะการเลี้ยงและการตอบสนองทางชีววิทยาของสาหร่ายผมนาง *Gracilaria fisheri* เพื่อรวบรวมข้อมูลที่เป็นประโยชน์กับการเพาะเลี้ยงต่อไปภายหน้า โดยการทดสองแบ่งเป็นสองส่วนคือ ส่วนที่หนึ่งเป็นการศึกษาหาสภาวะแวดล้อมการเลี้ยง และส่วนที่สองเป็นการศึกษาหาปัจจัยที่เหมาะสมในการเจริญเติบโตของต้นกล้า ในส่วนแรกได้ทำการศึกษาในสามจังหวัด ได้แก่ ปัตตานี สงขลา และสุราษฎร์ธานี ตัวอย่างอันประกอบด้วย สาหร่าย น้ำ และดิน ที่เก็บจากบ่อที่กำลังเลี้ยงสี่บ่อในแต่ละจังหวัด ปัจจัยที่ตรวจวัดในขณะเก็บตัวอย่าง ได้แก่ ความเข้มแสง ความโปร่งแสง ความเค็ม พีเอช และความลึกของน้ำ ส่วนปัจจัยเรื่อง ผลผลิตวุ้น ความชื้น การปนเปื้อนของวัชพืช ปริมาณคาโรทีนอยด์ คลอโรฟิลล์ และ อาร์-ไฟโคอิริตริน ปริมาณธาตุอาหารและโลหะหนัก ได้นำมาวิเคราะห์ในห้องปฏิบัติการ โดยได้ตรวจสอบปริมาณ Ca, Mg, K, Cu, Mn, Zn, Fe, Ni, Cr, Cd และ Pb ทั้งในสาหร่าย น้ำ และในดิน จากการศึกษาพบว่า สาหร่ายจากจังหวัดสงขลามีปริมาณผลผลิตมากกว่าจังหวัดอื่นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) ในปริมาณ 58 ตันน้ำหนักสด ต่อเฮกแตร์ ต่อปี ส่วนผลผลิตวุ้นของจังหวัดสงขลาน้อยกว่าของอีกสองจังหวัด แต่สีและสารสีของสาหร่ายทั้งสามแหล่งไม่แตกต่างอย่างมีนัยสำคัญทางสถิติ ($p>0.05$) ส่วนธาตุหลัก คือ Ca, Mg, K และ Naในน้ำ มีปริมาณสูงกว่าในสาหร่ายและในดิน ในขณะที่ธาตุรองและโลหะหนักในสาหร่ายและในดินสูงกว่าในน้ำ ปริมาณทรายและทรายแป้งของดินตะกอน ความลึก และความโปร่งแสงของน้ำ มีความสัมพันธ์ในทางบวกกับปริมาณ Cu ในสาหร่าย และยังพบว่าปริมาณ Cu และ Cr ในดินตะกอน และความกระด้างของน้ำ มีผลในทางบวกเด่นชัดกับ ปริมาณ Mn ในสาหร่าย อีกทั้งปริมาณ Fe ในสาหร่าย มีผลในทางบวกกับปริมาณ Cu และ Mn ในดินตะกอน โดยรูปแบบการสะสมของธาตุอาหารหลักคือ Mg>K>Ca>Na มีปริมาณเท่ากับ 9.52, 12.13, 9.64 และ 2.96 มก.ก.$^{-1}$ น้ำหนักแห้ง ส่วนลำดับธาตุอาหารรองในสาหร่าย คือ Mn>Fe>Zn>Cu มีปริมาณเท่ากับ 0.62. 0.55, 0.03 และ 0.02 มก.ก.$^{-1}$ น้ำหนักแห้ง ทั้งนี้ปริมาณ Ca, Mg, K และ Na จำนวนของ K และ Na ในสาหร่ายจากปัตตานีมีปริมาณของสารเหล่านั้นมากกว่าในสาหร่ายจากอีกสองจังหวัด ลำดับของโลหะหนักในสาหร่าย ในน้ำและในดินเป็น Pb>Cr>Cd ทั้งนี้โลหะหนักในสาหร่ายและใน

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น้ำแตกต่างกันเล็กน้อยคือในสาหร่ายเป็น Ni>Pb>Cr>Cd ส่วน ในน้ำเป็น Pb>Ni>Cr>Cd นอกจากนี้ Pb ในสาหร่ายพบว่ามีปริมาณสูง ที่ 11.53 มก.ก.$^{-1}$ น้ำหนักแห้ง แต่อย่างไรก็ตามมีการขึ้นลงในตามจังหวัดและตามบ่อที่เก็บ ในการศึกษาครั้งนี้พบปริมาณ Cd ในสาหร่ายในปริมาณที่ผกผันกับในน้ำ ปริมาณ Cd ในดินอยู่ในช่วง 1.0 ถึง 1.2 มค.ก.ก.$^{-1}$ น้ำหนักแห้ง ผลผลิตสาหร่ายมีความสัมพันธ์แบบผกผันกับ K, Na, Zn ฟอสเฟต-ฟอสฟอรัส และ ไนเตรท-ไนโตรเจน ในน้ำ ผลผลิตวุ้นสัมพันธ์ในเชิงบวกกับปริมาณ Mn ในน้ำ ปริมาณคลอโรฟิลล์ สัมพันธ์ในเชิงบวกกับปริมาณ Mg ในน้ำ สำหรับธาตุอาหารหลัก คือปริมาณ K และ Na ในหร่ายมีผลเชิงบวกกับ Mg, Zn, ฟอสเฟส-ฟอสฟอรัส และ ไนเตรท-ไนโตรเจน ในน้ำ นอกจากนี้ Mn, Ni, Fe, อินทรีย์คาร์บอน, อินทรียสาร, ร้อยละของทรายและทรายแป้งในดินตะกอนมีผลในเชิงบวกกับปริมาณ K ในสาหร่าย

ในการศึกษาการขยายกล้าพันธุ์ของสาหร่าย *G. fisheri* ได้ศึกษาในสี่ปัจจัยที่คัดเลือกแล้ว ว่ามีอิทธิพลต่อสาหร่าย ได้แก่ ความเค็ม ความยาวของท่อนพันธุ์ ชิ้นส่วนของสาหร่าย และความหนาแน่นของชิ้นส่วนพันธุ์ ผลของระดับที่ดีที่สุดจะนำไปใช้ในการทดลองอันต่อไป การทดลองขนาดของท่อนพันธุ์ ทำที่ สาหร่ายขนาด 1 ถึง 5 ซม. จากส่วนใต้ของยอดสาหร่าย โดยให้ช่วงขนาดต่างกัน ช่วงละ 1 ซม. ส่วนความเค็มใช้ในช่วง15-35 ส่วนในพันส่วน ให้ห่างกันอันละ 5 ส่วนในพันส่วน ส่วนของท่อนพันธุ์ใช้จากสามแหล่งได้แก่ ส่วนยอด ส่วนล่างยอด และส่วนฐาน สำหรับความหนาแน่นของท่อนพันธุ์ใช้ปริมาณ 1-8 ก. ล.$^{-1}$ โดยเพิ่มช่วงละเป็นเท่าตัว และที่ 6 ก. ล.$^{-1}$ ในแต่ละการทดลองใช้เวลา 40 วัน เลี้ยงที่ 25 µmol m^{-2} s^{-1} และให้แสงต่อมืด 12:12 ซม. ที่อุณหภูมิ 25±2ºC จากการทดลองพบว่าที่ ขนาดท่อนพันธุ์ 2 ซม. ที่ความเค็ม 20 ส่วนในพันส่วน ใช้ท่อนพันธุ์จากส่วนยอด ที่ความหนาแน่น 1 ก. ล.$^{-1}$ เนื้อเยื่อมีอัตราการเจริญเติบโตต่อวันร้อยละ 31.0 และมวลชีวภาพสาหร่ายเพิ่มขึ้นจากเริ่มต้น. 12.40 ลักษณะทางกายภาพพบสาหร่ายเพียงส่วนยอดเจริญเป็นแบบมีขั้วแบบโคนปลายยอด ส่วนการแตกแขนงของ *G. fisheri* จำนวน 7 แขนง ซม.$^{-1}$ มีความยาวเฉลี่ยแขนงละ 0.1 ซม. ต้นอ่อนของสปอร์สาหร่าย *G. fisheri* ที่เลี้ยงใต้แสงสีที่ที่จัดให้ 2 แบบ ได้แก่ 1) ในห้องปฏิบัติการใต้แสงสีจากการบังหลอดฟลูออเรสเซนต์ด้วยพลาสติกพีวีซีสีขาว เขียว น้ำเงิน และ แดง และ 2) นอกห้องปฏิบัติการโดยการเลี้ยงเนื้อเยื่อต้นกล้าพันธุ์ ภายใต้โรงเรือนที่มุงด้วยแผนพลาสติกพีวีซีมุ้งลวดสีขาว และพลาสติกซาแรน เขียว น้ำเงิน และดำ โดยในห้องปฏิบัติการเลี้ยงที่ 20 µmol m^{-2} s^{-1} ใช้ท่อนพันธุ์จากส่วนใต้ยอดขนาด 2 ซม. และท่อนพันธุ์จากสปอร์อายุ 8 สัปดาห์ จากการกระตุ้นและสปอร์จากการเลี้ยงในบ่อซิเมนต์อายุ 8 สัปดาห์ นำมาเลี้ยงในขวดพลาสติกทรงกลม ปริมาตร 3 ล. ให้แสงต่อมืด 12:12 ซม. ที่อุณหภูมิ 25±2ºC และความเค็ม 20 ส่วนในพันส่วน ระยะเวลาเลี้ยงเป็นเวลา 40 วัน ส่วนนอกห้องปฏิบัติการ ได้เลี้ยงท่อนพันธุ์สาหร่ายที่สับเป็นท่อนขนาด 2 ซม. ในถังพลาสติกขนาด 0.8x0.5x0.3 ม^{3} ที่วางเลี้ยงใต้โรงเรือน

Prince of Songkla University Pattani Campus

ความสูง 2 ม. ที่คลุมด้วยพลาสติกซาแรนสีต่างๆ สีละ 4 ถัง โดยใช้ต้นพันธุ์หนาแน่น 250 ก. ม.$^{-2}$ เลี้ยงกลางแจ้งในที่มีแสงสูงสุดเท่ากับ 110±10 μmol m^{-2} s^{-1} อุณหภูมิ 26.0±2.5°C ความเค็ม 20 ส่วนในพันส่วน เป็นเวลา 8 สัปดาห์ พบว่า แสงจากการผ่านพลาสติกพีวีซีสีแดงทำให้สาหร่ายมีอัตราการเจริญเติบโตต่อวันสูงสุดทั้งเนื่อเยื่อและต้นกล้าสปอร์ เท่ากับร้อยละ 1.4 และ 2.8 ตามลำดับ สำหรับสารสีในเนื้อเยื่อสาหร่ายจากการเลี้ยงใต้สีที่ต่างกันไม่มีความแตกต่างอย่างมีนัยสำคัญ ($p>0.05$) แต่ปริมาณคลอโรฟิลล์ในต้นอ่อนสปอร์ใต้สีเขียว ปริมาณ 348 มค.ก.ก.$^{-1}$ น้ำหนักสด มีความแตกต่างกับที่สีอื่นอย่างมีนัยสำคัญ ($p<0.05$) ส่วนใต้พลาสติกซาแรนสีเขียวนั้นเนื้อเยื่อสาหร่ายมีอัตราการเจริญเติบโตต่อวันและมีปริมาณสารสีสูงสุดแตกต่างอย่างมีนัยสำคัญ ($p>0.05$) คือมีอัตราการเจริญเติบโตร้อยละ 2.9 และมีสารสีสูงสุด คือมีคลอโรฟิลล์ และคาโรทีนอยด์ 32.6±1.1 และ 25.4±4.8 มค.ก.ก.$^{-1}$ น้ำหนักสด ตามลำดับ และใต้แสงสีดำวัชพืชเกิดขึ้นน้อยที่สุดเพียงร้อยละ 15

ผลผลิตของสาหร่าย *G. fisheri* ขึ้นอยู่กับคุณภาพน้ำ ในขณะที่โลหะหนักที่สะสมในสาหร่ายขึ้นอยู่กับคุณลักษณะของน้ำ โดยต้นกล้าจากเนื้อเยื่อสาหร่าย *G. fisheri* แสดงออกว่าเป็นสาหร่ายที่ทนต่อความเค็มในช่วงแคบ ซึ่งความเค็มจะมีผลต่อสาหร่ายมากกว่าปัจจัยอื่น ทั้งนี้คุณภาพของแสงที่ผ่านวัสดุที่ต่างและสีต่างกันจะมีสปกตรัมที่ต่างกัน และมีอิทธิพลมากต่อการเจริญเติบโต แต่มีผลน้อยต่อรงควัตถุ การศึกษาครั้งนี้พบว่าการเลี้ยงได้ดีและประสบผลสำเร็จทั้งในสปอร์และเนื้อเยื่อ

Prince of Songkla University Pattani Campus

Thesis Title Cultivation Characteristics and Biological Responses of Agarophytic Seaweed, *Gracilaria fisheri* (Rhodophyta), in Southern Thailand

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ABSTRACT

The study about cultivation characteristics and biological responses of *Gracilaria fisheri* aimed to contribute data for the future cultivation. The study was done in two parts: the first was investigation on cultivation environments and the second was investigation on optimal conditions for seedling propagation. The first part was conducted at three provinces, Pattani, Songkhla and Surat Thani. The samples including water and sediment were collected from four cultivating ponds of each province for the analysis. The parameters of light intensity, salinity, pH, water depth and transparency were directly measured at the ponds. Agar yield, moisture content, contaminants of the seaweed and pigment constituents including carotenoids, chlorophylls, r-phycoerythrin, mineral and heavy metal contents were analyzed in the laboratory. The amounts of Ca, Mg, K, Cu, Mn, Zn, Fe, Ni, Cr, Cd and Pb in seaweed, water and sediment were determined. The seaweed in Songkhla provided the significant higher ($p<0.05$) yield than other provinces with 58 ton FW ha^{-1} $year^{-1}$. However, the agar yield from Songkhla province showed lower than those in the other two provinces. There was no significant difference ($p>0.05$) on color and pigment content of the seaweed in three provinces. The major elements: Ca, Mg, K and Na in the water were much higher than those in the sediment and seaweed whereas trace elements and heavy metals in the sediment and seaweed were higher than those in the water. The percentage of sand and silt of soil sediment, water depth and transparency of water showed the positive relation to Cu amount in the seaweed. The amount of Cu and Cr in the sediment and water hardness related absolutely to the amount of Mn in the seaweed. The amount of Fe in the seaweed showed positive relation with Cu and Mn in the sediment. The major elements in seaweed showed in order of Mg>K>Ca>Na with the amounts of 9.52, 12.13, 9.64 and 2.96 mg g^{-1} DW, respectively and trace elements in the seaweed showed in the order of Mn>Fe>Zn>Cu with the amounts of. 0.62, 0.55, 0.03 and 0.02 mg g^{-1} DW, respectively. The amounts of K and Na in the seaweed at Pattani showed significantly higher ($p<0.05$) than those at the other two provinces. The sequence of Cr, Pb and Cd in the seaweed showed the same pattern of Pb>Cr>Cd in the water and sediment. Heavy metal in the seaweed of Ni>Pb>Cr>Cd showed slightly different sequence with Pb>Ni>Cr>Cd in water. Besides, the amount of Pb in *G. fisheri* was found as high as 11.53 mg g^{-1} DW; however, it fluctuated among the provinces and ponds. In this study, it was found that the amount of Cd in the seaweed relates inversely to salinity in the water. Cd concentration in the sediment ranged from 1.0 to 1.2 µg L^{-1} DW. The seaweed yield inversely related to K, Na, Zn,

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phosphate-phosphorus and nitrate-nitrogen in the water. Agar yield of the seaweed showed positive relation with concentration of Mn in the water. The chlorophyll a related positively with Mg in the water. For major elements, the concentration of K and Na in the seaweed showed the positive relation with the concentration of Mg, Zn, phosphate-phosphorus and nitrate-nitrogen in the water. Besides, Mn, Ni, Fe, organic carbon, organic matter, percentage of sand and silt in the sediment provided positive relation to the amount of K in the seaweed.

For optimal conditions of seedling propagation, the study was done under indoor and outdoor conditions. In indoor condition, seedling of *G. fisheri* was conducted in four selected influential factors: fragment length, salinity, part of thallus and propagule density. Each experiment was subsequently done with three replications and used the best result in the next study. The experiment on fragment length was conducted from 1 to 5 cm of sub-apical segments. The study on different salinity levels was done in the range of 15-35 ppt with 5 ppt of the interval. Different zones of tissues were selected apical, sub-apical and basal fragments for the next study. The study on density was conducted from 1 to 8 g L^{-1} with doubling of the prior and 6 g L^{-1}. Each experiment was done for 40 days, under 25 µmol m^{-2} s^{-1} of light intensity and 12L:12D of photoperiod at temperature of 25±2ºC. The result showed that optimal conditions for *G. fisheri* tissues were at 2 cm of segment length under 20 ppt salinity and part of tissue from apical zone and 1 g L^{-1} density. Under optimal condition, the relative growth rate of *G. fisheri* tissues was 31.0 % day^{-1} and the final biomass was increased 12.4 times comparing to the initial biomass. New finding on physical performance of *G. fisheri* tissue was found that only apical tissues grew with apicobasal polarity. The number and length of branch were 7 branches per cm and 0.1 cm, respectively. *Gracilaria fisheri* sporeling and tissue propagation was conducted to investigate the optimal shading color for the cultivation. The study was conducted with two experiments: 1) indoor experiment under different shading colors: white, green, blue and red of tissue and sporeling and 2) outdoor experiment on tissue culture under different Saran colors of hoop house: green, blue and black and PVC window screen in white color. For indoor experiment, fluorescent lights were used and wrapped by different PVC color sheets. All treatments were set in the average light intensity of 20 µmol m^{-2} s^{-1}. Sub-apical cleaned tissues of 2 cm were stocked and eight-week age sporelings were carefully cleaned and cultured at 0.3 g L^{-1} in 3 L spherical bottles. Other conditions were maintained under 12L:12D of photoperiod, 25±2ºC temperature and 20 ppt salinity for indoor experiments for 40 days Outdoor experiment was conducted in four plastic tanks of 0.8x0.5x0.3 m^3 under different colors of hoop houses with 2 m high and the maximum light intensity of 110 µmol m^{-2} s^{-1}. The excised tissues which were chopped into 2 cm length and then stocked at the density of 250 g·m^2 under conditions of 26.0±2.5ºC temperature, 20 ppt salinity. The experiment was conducted for 8 weeks with 4 replications. The highest growth rate of *G. fisheri* tissues and spores were found under red light with 1.4 and 2.8 % day^{-1}, respectively. There was no significant difference ($p>0.05$) on pigment content of tissues indoor; whereas it was found that chlorophyll a concentration of the sporelings under green light was significant higher ($p<0.05$) with 348 µg g^{-1} fresh weight. However, under green Saran house, tissues showed the highest growth rate 2.9 % $day^{-1}$with the relative high pigment content of 32.6±1.1 µg g^{-1} FW chlorophyll and 25.4±4.8 µg g^{-1}

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FW carotenoids. Besides, the study found that *G. fisheri* cultivation was less impacted by epiphytes under back Saran house with 15%.

The yield of *G. fisheri* depended on nutrient in water while heavy metal accumulation in seaweed mainly depends on water characteristics. Seedling from tissue of *G. fisheri* expressed as a stenohaline species that was rather affected by salinity than the other factors. Light shading strongly influenced on growth of *G. fisheri* sporelings and tissues but little affected on pigment constituents. Red light is more effective light for the growth of *G. fisheri* tissues and spores in laboratory condition. The study showed the feasible and successful cultivation for *G. fisheri* sporelings and tissues.

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CONTENTS

Page

ABSTRACT (Thai Version) ....(5)
ABSTRACT ....(8)
ACKNOWLEDGEMENT ....(11)
CONTENTS ....(12)
LIST OF TABLES ....(14)
LIST OF FIGURES ....(16)
LIST OF APPENDICES ....(18)
APPENDIX 1 METHODOLOGY ....(18)
APPENDIX 2 DATA CONTENTS ....(18)
APPENDIX 3 ANALYSIS OF VARIANCE (ANOVA) ....(18)
CHAPTER 1 1
INTRODUCTION 1
1.1 Statement of the Problem 1
1.2 Related Literature 2
1.2.1 Taxonomy of *Gracilaria* 2
1.2.2 Life history of *Gracilaria* 3
1.2.3 General distribution and habitat of *Gracilaria* 4
1.2.4 Harvesting and cultivation of *Gracilaria* 6
1.2.5 Exploitation of natural populations 7
1.2.6 Cultivation of *Gracilaria* 7
1.2.7 Biotechnology of *Gracilaria* 13
1.2.8 Utilization of *Gracilaria* 17
1.3 Objectives 21
CHAPTER 2 22
RESEARCH METHODOLOGY 22
2.1 Method 23
2.1.1 Study on environmental characteristics, water and sediment 23
2.1.2 Some biological characteristics of *G. fisheri* in pond from different locations 25
2.1.3 Tissue culture under different conditions: initial length, salinity, part of thallus, density and light color 27
2.1.4 Upper scale of tissue culture under optimal conditions in laboratory and outdoor 29
2.1.5 Data analysis 30
2.2 Materials and Equipment 30
2.2.1 Materials 30
2.2.2 Equipment 30
CHAPTER 3 32
RESULTS 32
3.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment. 32
3.1.1 Elements, heavy metals and other parameters in seaweed 32

Prince of Songkla University Pattani Campus

CONTENTS (Continued)

Page

3.1.2 Water parameters and elements in various habitat of *Gracilaria fisheri* from Pattani, Songkhla and Surat Thani Provinces. 34
3.1.3 Sediment composition and characteristics from cultivated ponds....37
3.1.4 Correlation analysis of biological characteristics of seaweed and physical, chemical parameters of environment. 41
3.2 Tissue culture in different conditions....46
3.2.1 Tissue culture under different initial lengths....46
3.2.2 Tissue culture under different salinity levels....48
3.2.3 Tissue culture under different parts of thallus....51
3.2.4 Tissue culture under different propagule densities....53
3.2.5 *G. fisheri* tissue under different colors in laboratory condition....57
3.2.6 Growth of *G. fisheri* sporelings on laboratory....62
3.3 Upper scale cultivation of *G. fisheri*....68
3.3.1 *G. fisheri* tissue upper scale indoor condition....68
3.3.2 *G. fisheri* tissue at outdoor condition....69
CHAPTER 4....73
DISCUSSIONS....73
4.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment. 73
4.2 Tissue culture under different conditions....74
4.3 Tissue culture under different shading color....76
CHAPETER 5....78
CONCLUSION....78
5.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment. 78
5.2 Tissue culture under different conditions....78
5.3 Tissue culture under different shading color....79
5.4 Suggestions....79
REFERRENCES....80
APPENDICES....87
APPENDIX 1....88
METHODOLOGY FOR ELEMENT ANALYSIS....88
APPENDIX 2....93
CULTURE MEDIUM....93
APPENDIX 3....94
ANALYSIS OF VARIANCE (ANOVA)....94

Prince of Songkla University
Pattani Campus

LIST OF TABLES

Table		Page
1.1	Seaweed species from which tissue culture have been accomplished	15
3.1	Yield and biological characteristics of *G. fisheri* in Pattani, Songkhla and Surat Thani provinces..…………………………………………..	32
3.2	Element concentrations in *Gracilaria fisheri* collected from Pattani, Songkhla and Surat Thani………………………..………………...	33
3.3	Water parameters from Pattani, Songkhla and Surat Thani provinces	35
3.4	Element concentrations in the water collected from Pattani, Songkhla and Surat Thani…………..…………………………….................	36
3.5	Sediment parameters collected from Pattani, Songkhla and Surat Thani provinces………………..……………………………………..	38
3.6	Element concentrations in the sediment collected from Pattani, Songkhla and Surat Thani……………………………………………	39
3.7	Correlations for *G. fisheri* characteristics and its surrounding environment…………………………………………………………...	42
3.8	Growth rate and branch formation of *Gracilaria fisheri* tissues at different initial lengths of thallus…………………….……………...	47
3.9	Growth rate and branch formation of *Gracilaria fisheri* tissues at different salinity levels……………………………….……………...	50
3.10	Growth rate and branch formation of *Gracilaria fisheri* tissues from different parts of thallus……………………………..…………………	52
3.11	Growth rate and branch formation of *Gracilaria fisheri* tissues at different propagule densities………………………………………	54
3.12	Growth type and branch formation of *G. fisheri* tissues…………….	57
3.13	Growth and branch formation of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red………………………………………………………………..	59
3.14	Pigment contents of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red………………...	62
3.15	Growth and branch formation of *Gracilaria fisheri* sporelings cultured indoor condition under different shading colors of white, green, blue and red………………………………………….…………..	64
3.16	Pigment contents of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red…………………………………………………………………	67
3.17	Absorbance from chlorophyll and R-PE extract of *Gracilaria fisheri* sporelings cultured indoor condition under different shading colors of white, green, blue and red…………………………………….....	67
3.18	Growth of biomass, main thallus and new branches of *Gracilaria fisheri* cultured under optimal conditions at 40 days………………...	69
3.19	Growth rate of *Gracilaria fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black……...	69

Prince of Songkla University Pattani Campus

LIST OF TABLES (Continued)

Table		Page
3.20	Pigment contents of *Gracilaria fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black…………………………………………………………………..	71
3.21	Absorbance from chlorophyll and R-PE extract of *Gracilaria fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black…………………………………………..	72

Prince of Songkla University
Pattani Campus

LIST OF FIGURES

Figure		Page
1.1	*Gracilaria fisheri*. (A) Wet specimen. (B) Natural thalli. (C) Cross section of thallus with cystocarps. (D) Cross section of thallus and stain……………………………………………………………..	3
1.2	The *Gracilaria* life cycle………………………………....................	5
1.3	*Gracilaria* buried with soft substrata. (A, B) Plant fragments buried in sediment. (C) Fragments increase its size and torn free during storms. (D) fragment of thallus become embedded in the substrate…	7
1.4	(A) Transplantation attached *Gracilaria* in rocky substrata. (B) *Gracilaria* adhered to rocks, with rubber bands. (C) *Gracilaria* inserted into soft sediments by using fork. (D) *Gracilaria* adhered to sand-filled plastic tubes...	8
1.5	(A) Seedling ropes in natural *Gracilaria* populations. (B) Released spores from the fertile plants and settle onto the seed-ropes. (C) Juvenile plants attached to the seed-rope. (D) Juvenile plants removed new areas………...	10
1.6	Tissue culture of *Gracilaria changii*……………………………...	14
1.7	Protoplast regeneration of *G. changii*. (A) Cell division of protoplast after 5 days. (B) Plants regenerated from protoplast after 60 days. (C) Plant formation after 135 days…………………………………..	16
1.8	*G. changii* tissue culture in spherical photobioreactor………………	17
1.9	Shape of red algae pulp fibers in handsheet (300 x)…………………	21
2.1	Map of experimental ponds in Pattani, Songkhla and Surat Thani Provinces..	22
2.2	Cultivated pond habitat in the experimental provinces. (A, B) Cultivated ponds in Pattani Province. (C) Cultivated pond in Songkhla Province. (D) Cultivated pond in Surat Thani Province.....	23
2.3	*G. fisheri* color analysis by HunterLab Color Measurement………..	26
2.4	*G. fisheri* tissue culture in 250 mL flask……………………………	27
2.5	*G. fisheri* tissue culture under different shading colors, white, green, blue and red………………………………………………………..	29
3.1	Element concentration in *G. fisheri* collectd from Pattani, Songkhla and Surat Thani provinces. (A) Major elements. (B) Trace elements. (C) Heavy ..………………………………………………..................	34
3.2	Element concentration in water collected from Pattani, Songkhla, Surat Thani Provinces. (A) Major elements. (B) Trace elements. (C) Heavy metals………………………………………………………	37
3.3	Element concentration in sediment collectd from Pattani, Songkhla, Surat Thani Provinces. (A) Major elements. (B) Trace elements. (C) Heavy metals………………………………………………………	40
3.4	*G. fisheri* tissue culture under different lengths after 40 days. (A) Initial fragments. (B) Tissues at 1 cm initial length. (C) Tissue at 2 cm initial length. (D) Tissues at 3 cm initial length. (E) Tissues at 4 cm initial length. (F) Tissues at 5 cm initial length………………….	48

Prince of Songkla University Pattani Campus

LIST OF FIGURES (Continued)

Figure		Page
3.5	*G. fisheri* tissue culture at different salinity levels. (A) Mother plant. (B) Culture flask. (C) Tissues at 20 ppt after 20 days. (D) Tissues at 20 ppt after 40 days. (E) Tissues at 35 ppt after 40 days……………..	49
3.6	Growth rate and number of new branch (bar graphs) and RGR and branch length (line graphs) of *G. fisheri* tissues under (A, B) different initial lengths. (C, D) different salinity levels………………	51
3.7	*G. fisheri* tissue culture from different part of thallus. (A) Initial fragment. (B) Apical segments after 40 days. (C) Sub-apical segments after 40 days. (D) Basal segments after 40 days…………	53
3.8	Tissue culture of *G. fisheri* under different propagule densities. (A) Seedling plant. (B) Tissue at 1 gL^{-1}. (C) Tissue at 2 gL^{-1}. (D) Tissue at 4 gL^{-1}. (E) Tissue at 6gL^{-1}. (F) Tissue at 8 gL^{-1}…………………....	55
3.9	Growth rate and number of new branch (bar graphs) and RGR and branch length (line graphs) of *G. fisheri* tissues under (A, B) different part of thallus. (C,D) different propagule densities………..	56
3.10	*G. fisheri* tissue culture under (A) white color (B) green color (C) blue color (D) red color…………………………………………….....	58
3.11	*G. fisheri* tissue culture under different shading colors. (A) Increased biomass. (B) RGR. (C) Number of new branches……………………	60
3.12	*G. fisheri* tissue culture under different shading colors. (A) Branch length. (B) Increased length. (C) Biomass…………………………....	61
3.13	Pigment content of *G. fisheri* tissue under different shading colors…	62
3.14	*G. fisheri* spore appearance under different shading colors. (A) Sporelings attached in rope. (B) Seedling. (C) Spore under white color. (D) Spore under green color. (E) Spore under blue color. (F) Spore under red color……………………………………………….....	63
3.15	*G. fisheri* tissue culture under different shading colors. (A) Increased biomass. (B) RGR. (C) Number of new branches……………………	65
3.16	*G. fisheri* tissue culture under different shading colors. (A) Branch length. (B) Increased length. (C) Biomass…………………………....	66
3.17	Pigment content in *G. fisheri* spores under different shading colors	68
3.18	Spectrum absorbance of *G. fisheri* spore under different shading colors (A) From chlorophyll extract. (B) From R-PE extract………..	68
3.19	Growth of *G. fisheri* tissue under different shading color outdoor condition. (A) Increased biomass. (B) RGR………………………….	70
3.20	Percentage of epiphytes in *G. fisheri* tissue outdoor condition……....	70
3.21	Pigment *G. fisheri* tissue outdoor under different shading color…….	71
3.22	Spectrum absorbance of *G. fisheri* tissue under different shading colors outdoor condition. (A) From chlorophyll extract. (B) From R-PE extract………………………………………………………………..	72

Prince of Songkla University Pattani Campus

LIST OF APPENDICES
APPENDIX 1 METHODOLOGY

Page

1.1. Element analysis in seaweed and sediment (AOAC, 2000) 88
1.2. Element analysis in water 88
1.3. Sediment particle size analysis (Pipette method; Sheldrick and Wang, 1993)....91
1.4. Sediment organic matter analysis (Walkley-Black Procedure; Nelson and Sommers, 1982) 92

APPENDIX 2 CULTURE MEDIUM

Table		Page
1	The nutrient component of Modified Grund Medium....................	93
2	The component of vitamins stock solution................................	93

APPENDIX 3 ANALYSIS OF VARIANCE (ANOVA)

Table		Page
1	ANOVA of elements in *Gracilaria fisheri* from the cultivated ponds	95
2	ANOVA of pigment content and other characteristics of *Gracilaria fisheri*..................	96
3	ANOVA of color analysis of *G. fisheri*..................................	97
4	ANOVA of elements and other characteristics in the water of cultivated Ponds..................	98
5	ANOVA of elements in sediment of the cultivated ponds................	100
6	ANOVA of sediment charateristics..	101
7	ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different initial lengths..................	101
8	ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different initial lengths..................	102
9	ANOVA of increased length (%) of *G. fisheri* tissue culture under different initial lengths..................	102
10	ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different initial lengths..................	103
11	ANOVA of number of new branch per cm of *G. fisheri* tissue culture under different initial lengths..................	103
12	ANOVA of branch length (cm) of *G. fisheri* tissue culture under different initial lengths..................	104
13	ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different salinity levels..................	104
14	ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different salinity levels..................	105

Prince of Songkla University Pattani Campus

APPENDIX 3 (Continued)

Table		Page
15	ANOVA of increased length (%) of *G. fisheri* tissue culture under different salinity levels………………………………………………...	105
16	ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different salinity levels………………………………………………………..	106
17	ANOVA of number of new branch per cm of *G. fisheri* tissue under different salinity levels………………………………………………...	106
18	ANOVA of branch length (cm) of *G. fisheri* tissue under different salinity levels………………………………………………………..	107
19	ANOVA of increased biomass (%) of *G. fisheri* tissue culture from different parts of thallus……………………………………………….	107
20	ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture from different parts of thallus………………………………………………………	108
21	ANOVA of increased length (%)of *G. fisheri* tissue culture from different parts of thallus………………………………………………..	108
22	ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture from different parts of thallus………………………………………………………	109
23	ANOVA of number of new branch per cm of *G. fisheri* tissue from different parts of thallus………………………………………………..	109
24	ANOVA of branch length (cm) of *G. fisheri* tissue from different parts of thallus………………………………………………………	110
25	ANOVA of increased biomass (%) of *G. fishei* under different density levels..	110
26	ANOVA of RGR (% day^{-1}) of *G. fishei* under different density levels	111
27	ANOVA of increased length (%) of *G. fishei* under different density levels………………………………………………………………...	111
28	ANOVA of biomass (g L^{-1}) of *G. fishei* under different density levels	112
29	ANOVA of number of new branch per cm of *G. fishei* under different density levels..	112
30	ANOVA of branch length (cm) of *G. fishei* under different density levels..	113
31	ANOVA of pigment content (µg g^{-1} FW) of *G. fisheri* tissue culture under different shading colors indoor experiment................................	113
32	ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different shading colors indoor experiment...	114
33	ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different shading colors indoor experiment……………………………………..	114
34	ANOVA of increased length (%) of *G. fisheri* tissue culture under different shading colors indoor experiment……………………………	115
35	ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different shading colors indoor experiment……………………………………..	115
36	ANOVA of number of new branch per cm of *G. fisheri* tissue culture under different shading colors indoor experiment................................	116

Prince of Songkla University Pattani Campus

APPENDIX 3 (Continued)

Table		Page
37	ANOVA of branch length (cm) of *G. fisheri* tissue culture under different shading colors indoor experiment…………………………..	116
38	ANOVA of increased biomass (%) of *G. fisheri* spore culture under different shading colors indoor experiment…………………………..	117
39	ANOVA of RGR (% day^{-1}) of *G. fisheri* spore culture under different shading colors indoor experiment……………………………………..	117
40	ANOVA of increased length (%) of *G. fisheri* spore culture under different shading colors indoor experiment.......................................	118
41	ANOVA of biomass (g L^{-1}) of *G. fisheri* spore culture under different shading colors indoor experiment……………………………………..	118
42	ANOVA of number of new branch per cm of *G. fisheri* spore culture under different shading colors indoor experiment…………………….	119
43	ANOVA of branch length (cm) of *G. fisheri* spore culture under different shading colors indoor experiment..	119
44	ANOVA of pigment content (µg g^{-1} FW) of *G. fisheri* spore culture under different shading colors indoor experiment…………………….	120
45	ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different shading colors outdoor condition……………………………………..	121
46	ANOVA of epiphytes (%) and pigment content (µg g^{-1} FW) of *G. fisheri* tissue culture under different shading colors outdoor condition	122
47	ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different shading colors outdoor condition……………………………	123
48	ANOVA of biomass (g m^{-2}) of *G. fisheri* tissue culture under different shading colors outdoor condition……………………………	124

Prince of Songkla University Pattani Campus

CHAPTER 1

INTRODUCTION

1.1 Statement of the Problem

Seaweeds have long been consumed as a natural source of food and medicines in Asian countries (Besada *et al*., 2009, Khan *et al*., 2015) and western countries (Almela *et al*., 2006) due to high fiber and mineral concentration and low fat content (Almela *et al*., 2006; Rodenas de la Rocha *et al*., 2009). Seaweeds are popularly used as daily products, food products, adhesives, pharmaceutical products, etc (Murty and Banerjee, 2012). *Gracilaria fisheri* belongs to the genus *Gracilaria* (Rhodophyta) which *Gracilaria* accounts for 60-80% in the global agar production (Yeong *et al*., 2014). *G. fisheri* is a commercial species that is commonly extracted for agar production (Chirapart *et al*., 2006). In Thailand, this alga also used as fresh vegetable and dried products (Benjama and Masniyom, 2012). Cultivation of this alga was done in natural earthen ponds (Chirapart and Lewmanomont, 2004) and in abandoned shrimp ponds (Ruangchuay *et al*., 2010).

Unfortunately, seaweeds exhibit the high metal pollution accumulation capacity (Almela *et al*., 2006). It is well known that metals in water are directly absorbed by seaweed (Muse *et al*., 1999); thus, the concern of heavy metals in seaweed and surrounding environment was stated (Hashim and Chu, 2004). There are a strong relation of heavy metal content in seaweed and their environmental parameters i.e. salinity, temperature, pH, light and nutrients, oxygen, etc (Besada *et al*., 2009; Rodenas de la Rocha *et al*., 2009). Therefore, seaweeds are recently known as a bio-indicator for marine environmental contamination (Almela *et al*., 2006; Besada *et al*., 2009; Misurcova, 2012). However, there is little information about nutrient content and cultivation characteristic on this species. Therefore, the study was aimed (1) to evaluate the major elements (Ca, Mg, K, Na), trace element (Cu, Mn, Zn, Fe) and heavy metal accumulation (Ni, Cr, Cd, Pb) in *G. fisheri*, thus it might condition for the seaweed as an edible safety food (2) to investigate the general surrounding characteristics in *G. fisheri* cultivation pond that could to provide the database for expanding the seaweed cultivation.

Seaweed tissue culture is basically established from higher plant tissue culture (Baweja *et al*., 2009; Yeong *et al*., 2014). For certain biotechnological application, seaweed tissue culture might be useful for propagation of marine seed stock (Baweja *et al*., 2009) and improve the quality of phycocolloids due to the fast growing strain of seaweeds (Kaliaperumal, 1998). Many species of red seaweed have been applied tissue culture techniques to provide the desired strains (Yeong *et al*., 2014), especially for *Gracilaria* including *Gracilaria verrucosa* and *Gracilaria chorda* (Choi *et al*., 2006), *Gracilaria vermiculophylla* (Yokoya *et al*., 1999), *Gracilaria edulis* and *Gracilaria tenuistipitata* (Yu *et al*., 2013). To increase the industrial scale production of seaweed biomass, the recent studies are needed to develop techniques for consistent supply of high-quality seed stock, strain improvement and efficient mass culture of high yielding commercial strains (Yeong *et al*., 2014). However, there have been few reports on seedling maintenance of the species. Therefore, it shows the importance to investigate the optimal conditions for

Prince of Songkla University Pattani Campus

G. fisheri tissue maintenance in laboratory and it may be a database for seedling maintenance in further cultivation in tanks or other systems.

Light plays an important role in controlling plant morphology. There have long been reported that light quantity significantly influence on growth morphology and pigmentation of macroalgae (Figueroa *et al*., 1995). Han *et al*. (2003) mentioned that light wavelengths influence macroalgal reproduction. Nevertheless, there is lacking of light quality on algal growth and pigmentation. So far, it was mentioned that white light has important role in the overall biomass and on the photomorphogenesis; green light affects the spore germination (Kim *et al.,* 2015); blue light stimulates pigment and protein synthesis and red light favoured thallus expansion, cell division and carbon accumulation with high photosynthetic efficiency (Figueroa *et al*., 1995). Therefore, the study was aimed to investigate the effective shading color for the growth of *G. fisheri* spores and tissues. The success of this study might further make the culture system independent from wild stock. Besides, the finding from environmental chacteristics of *G. fisheri* cultivation could benefit for *G. fisheri* tissue culture indoor as well as outdoor condition; therefore this might support more information for *G. fisheri* cultivation and increase the production of the seaweed.

1.2 Related Literature

1.2.1 Taxonomy of *Gracilaria*

Phylum: Rhodophyta
Subphylum: Rhodophytina
Class: Florideophyceae
Order: Gracilariales
Family: Graclariaceae
Genus: *Gracilaria*
Species: *fisheri*

Gracilaria fisheri (Figure 1.1A and 1.1B) (Xia et Abbott) Abbott, Zhang et Xia is described as bushy thallus composition with brown to dark brown color. The mature height ranges from 12 to 20 cm. The branches are cylindrical and irregularly have 2-3 orders. The main axis branch is 1.1-1.5 mm in diameter and 1.7-2.2 mm for the base (Ruangchuay *et al*., 2007).

A general description of *Gracilaria* was described as having a “bushy” thallus, which may be rigid, with relatively short branches, being long in respect to their diameter. *Gracilaria* commercial forms were described as being less than 30 cm in height, with a slender thallus having terete axes (usually less than 2 mm in diameter). *Gracilaria* has more than 150 species which had been named and been described as “chaotic” (Critchley, 1997b). Unfortunately, *Gracilaria* species are difficult to identify based on morphological characteristic in many cases. New possibilities of precise identification by molecular biology techniques were required towards these given problems. It is important to create silica-gel, air-dried and/or herbarium presses as voucher specimens to avoid future taxonomic confusion.

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Therefore, the correct identification can be confirmed by using morphological and molecular analysis (Thomsen *et al.,* 2005).

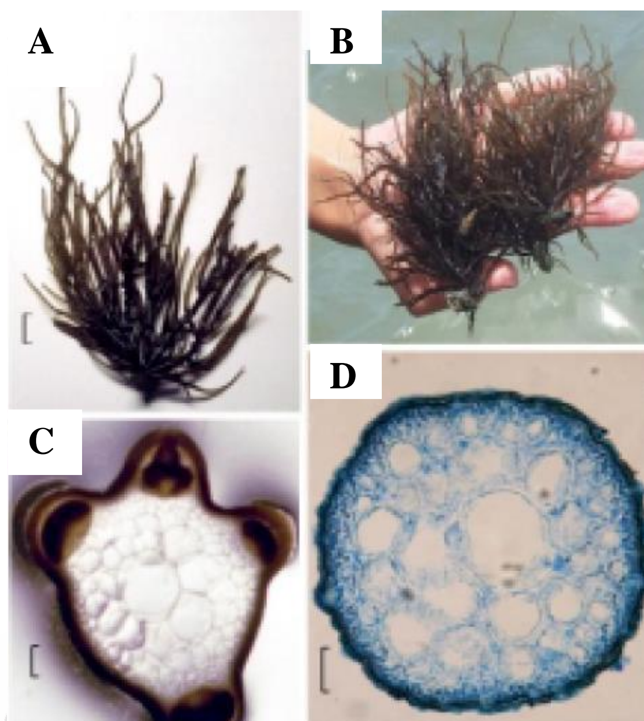

Figure 1.1 *Gracilaria fisheri*. (A) Wet specimen (scale bar = 2 cm). (B) Natural thalli. (C) Cross section of thallus with cystocarps (scale bar = 0.5 mm). (D) Cross section of thallus and stain (scale bar = 0.5 mm).
Source: Ruangchuay *et al*. (2007)

Molecular techniques have been used to detect *G. vermiculophylla* in the eastern Atlantic and Pacific, western Atlantic and in its native range in the western Pacific. Besides, maintaining constant collections and sequencing standards across distinct institutions are necessary for *Gracilaria* taxonomy (Gulbransen *et al.,* 2012). For seaweed taxonomy, some researchers based on anatomy, supporting a subgeneric scheme for genus *Gracilaria* (subgenera *Gracilariella*, *Textoriella*, *Gracilaria*, and *Hydropuntia*) while other researchers based on molecular evidence, suggesting the taxonomic independence of genus *Gracilariopsis*, *Gracilaria*, and *Hydropuntia.* There are four species of *Gracilaria* (*Gracilaria*ceae, Rhodophyta) with *textorii*-type spermatangial conceptacles (subgenus *Textoriella* Yamamoto) that are habited in the Gulf of Mexico and the Mexican Caribbean. They are *Gracilaria blodgettii*, *G. cervicornis*, *G. mammillaris*, and *G. tikvahiae* (Dreckmann and Senties, 2009).

1.2.2 Life history of *Gracilaria*

The cystocarp of *G. fisheri* (Figure 1.1C and 1.1D) has conical shape which normally ranges from 0.5 to 0.7 mm in diameter (Ruangchuay *et al*., 2007). *Gracilaria* has a typical red algal which is triphasic life history (*Polysiphonia*-type) (Figure 1.2). The morphology of the inseparable (isomorphic) genetically distinct

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generations (haploid and diploid phases) is yet alternative with the sexes separated (unisexual, dioecious) in the gametophyte phase (Critchley, 1997b). In *Gracilaria*, the Polysiphonia type of life-cycle pattern may employ either carpospores or tetraspores as a seed material. The carpospore derived tetrasporophytes and preferred due to their diploid nature (Mantri *et al*., 2009; Yu *et al*., 2013).

Critchley (1997b) also mentioned that female thalii (if present) can be recognized, without the aid of microscope, by the presence of cystocarps which appear as distinct, hemispherical lumps variously distributed along the thallus. The cystocarp is the product of the successful union of male and female gametes. The large numbers of non-motile male gametes (spermatia) are liberated from the haploid, male, parent plant. The female gamete (egg or carpogonium) is retained on the female thallus and fertilized on the site. Engel and Destombe (2002) mentioned that the number of cystocarps in high-shore pools was significantly higher at low tide than that at high tide whereas in low shore cystocarp yield did not differ between high and low tide periods. Almost red seaweeds are dioecious and only male plants release their gametes (spermatia) since the fertilization and subsequent zygote development takes place on the female plant whereas sperm limitation might be reduced in species that retain female gametes, several characteristics of the reproductive biology of red seaweeds suggested that spermatia could be limited.

1.2.3 General distribution and habitat of *Gracilaria*

Gracilaria genus is composed of more than 150 species and considered as one of the largest genera. *Gracilaria* species are usually found along the Atlantic, Pacific and Indian Ocean. There are more than 65 species of *Gracilaria* from the Indian Ocean. In some countries such as Indonesia, Chile, Malaysia and Thailand, there are *Gracilaria* cultivation practicing. This genus is also selected to culture in Namibia and South Africa with small scale. Recently, the over-exploitation of *Gracilaria* for the market demand is a consideration (Ganesan *et al*., 2011).

G. fisheri is an economically important species and commonly found as growing on snails (Ruangchuay *et al*., 2007). *G. fisheri* is commonly found along the coast of south-east Asian countries (Kanjana *et al*., 2011). In Thailand, *Gracilaria* spp. are commonly found in several parts of the country such as Pattani, Songkhla and Trat Provinces. Especially, *G. fisheri* and *G. tenuistipitata* are intensively cultured and used as a commercial source of agar and abalone feed (Chirapart *et al*., 2006). *Gracilaria* sp. is now cultivated on a large scale in several countries such as Chile, China and Taiwan. *Gracilaria verrucosa* is reported among 8 species found in Korean coast in the upper intertidal zone and from estuaries to open sea around Korean Peninsula. While *Gracilaria chorda* commonly distributed in the lower intertidal zone of south western coast of Korea (Choi *et al*., 2006).

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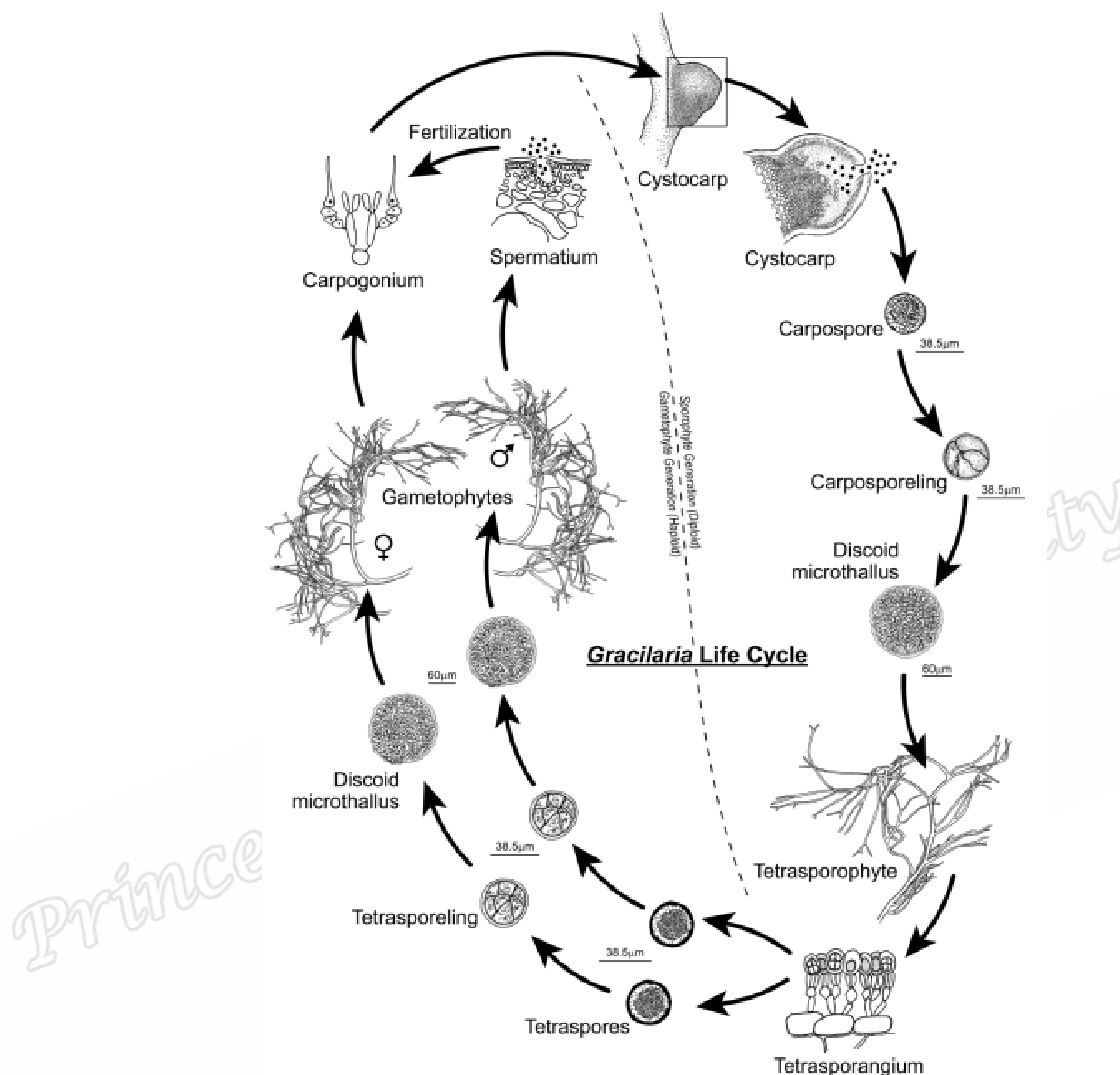

Figure 1.2 The *Gracilaria* life cycle
Source: Yarish *et al.* (2012)

The geographic distribution of *Gracilaria* is wide; the majority of species are reported to be for warm water, tropical regions. *Gracilaria* distribution is limited when requirements for seawater temperatures are 20°C or higher for at least three months of the year apparently. The number of *Gracilaria* is reduced at higher latitude, with only one or two species represented in those floras (Critchley, 1997b). *Gracilaria* genus comprises more than 100 species with a worldwide distribution except in Arctic Ocean. This genus includes more than half of the world agarophyte production. Gracilarioids are commercially cultivated in large scale in Chile, South Africa, China, Taiwan and Israel (Raikar *et al.,* 2001).

In the world, the major production areas for *Gracilaria* are Chile, Malaysia, Thailand, New Zealand, Philippine, Indonesia, China, Taiwan and Southern Africa. The considerable variation in ecological conditions can affect the areas occupied.

The convenient environment for *Gracilaria* growth requires freshwater dilutions, high inputs of nutrients and low water-motion, in combination with high temperature and even burial by mobile sediments. Thus gracilarioid populations are rarely presented to extreme wave action. *Gracilaria* may be attached into the substrate or frequently "free-living". Free-living populations are usually more productive than those that are attached (Critchley, 1997b).

There were several producing red seaweeds such as *Geledium pusillum, Gelidiella acerosa and Gracilaria* spicies which grew along the coast of the Gulf of Thailand and Andaman Sea. *Gracilaria fisheri* and *G. tenuistipitata* were usually harvested in large quantity as commercial source of agar and abalone feed (Chirapart *et al.,* 2006).

Attached (benthic) plants can be found in areas of even strong water motion and are commonly attached to shells and small stones, stabilized by loose sediments. The plants can be adhered to calcareous tube-worms, mussel byssal threads or attached with other algae and/or marine angiosperms. *Gracilaria* accumulations majorly occur in sheltered sites where substrata are soft and unstable, including sand-silt mixtures. These populations are not permanently adhered but may include free-living or only temporarily attached thalli. Portions of whole plant or fragments of thalli may become entrapped under deposits of sediment. The buried thallus may germinate laterally to anchor the plant and then grow new erect shoots, which can be temporarily stabilized but probably washed out during heavy swells and storms. Regeneration of the population begins from the remaining buried thalli (Figure 1.3); the free biomass is then deposited on the shore (Critchley, 1997b).

1.2.4 Harvesting and cultivation of *Gracilaria*

The dried *Gracilaria* production was 25,000-30,000 tonnes globally in 1989 (Anderson *et al*., 1998; Critchley, 1997b). The annual production of *Gracilaria* excessed of 37,000 dry tons which accounted for one-third of aquaculture in Chile (Mantri *et al*., 2009). The leading producer of *Gracilaria* comes from Chile that caused to the great interest in *Gracilaria* farming in Chile (Anderson *et al*., 1998). The rest minor amounts were harvested in Brazil. Only from the shore (deposited by wave action) in Southern Africa, small quantities of *Gracilaria* were collected, legislation prohibits direct harvesting from the natural beds there. The total volume of *Gracilaria* farm in the world was not well known but approximately 50-60 % (15,000 dry tonnes) of the total production might be provided in this way. The major farming areas were Taiwan, China, Chile, and Hawaii. In Chile, annual production of *Gracilaria* was of 70,000 wet tonnes. There was 90% of total production from cultivated as sterile vegetative thalli, on intertidal or subtidal soft bottoms (Halling *et al*., 2005). Development of cultivation techniques proceeded in Thailand, Malaysia, Indonesia and Philippine while demonstrations of pilot scale production had been made in the USA, Caribbean, Brazil and Namibia (Critchley, 1997b).

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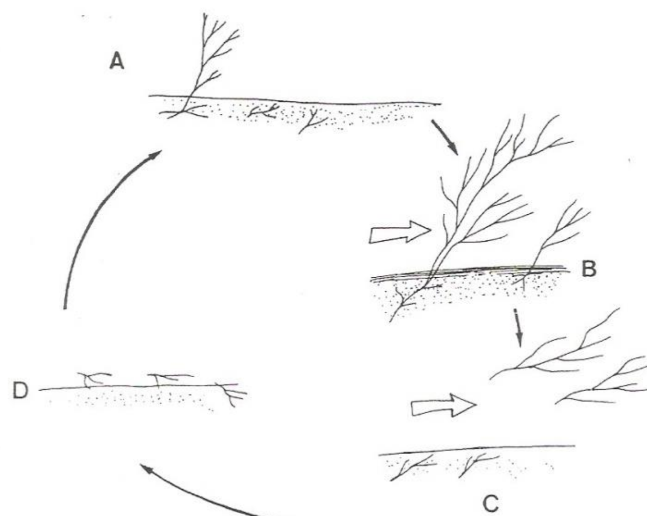

Figure 1.3 *Gracilaria* buried with soft substrata. (A, B) Plant fragments buried in sediment. (C) Fragments increase its size and torn free during storms. (D) Fragment of thallus become embedded in the substrate.
Source: Critchley (1997b)

1.2.5 Exploitation of natural populations

The production of gracilarioids usually got maximum with 5 tonnes.ha^{-1}.y^{-1} in natural populations. Wild crop production are influenced by seasonal factors, summer production may be high but cease altogether during winter. Management and development of harvesting strategies for natural crops requires a clear understanding of biology, productivity and the impact of environmental factors and harvesting pressures on production (Critchley, 1997b). Besides, the overexploitation of the wild biomass of economically important agarophytes results in the development of cultivation technique of *Gracilaria* for supplying the market demand (Raikar *et al.,* 2001).

Raking in various forms is the method most commonly used to harvest *Gracilaria*. This method may change bottom sediment characteristics and decrease bed productivity. Passive harvesting of gracilarioids deposited on beaches had been practiced successfully in southern Africa. *Gracilaria* is collected from the beach, spread thinly in the desert and dried within two to three days. The harvested gracilarioid supported an export industry and until recently a local agar extraction plant (Critchley, 1997b).

1.2.6 Cultivation of *Gracilaria*

1) Open water cultivation of *Gracilaria*

There are three basic methods used for the cultivation of gracilarioids in open waters such as bays, estuaries, upwelling sites, reef flats and others, these are bottom stocking, attachment to ropes and nets and floating rafts and cage culture (Bezerra and Marinho-Soriano, 2010; Critchley 1997b; Mantri *et al*., 2009).

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1.1) Bottom stocking

Critchley (1997b) mentioned that many techniques had been developed to duplicate the natural field conditions of vegetative thalli entrapped in soft sediments. The simplest method is to transfer vegetative thalli, which are naturally adhered to small stones and shells, to areas where plants can grow and increase density (Figure 1.4A). Other methods comprise securing *Gracilaria* to rocks with rubber bands in order to stabilize the thalli in soft sediment (Figure 1.4B). Various forked planting tools were used to push bundles of thalli into soft sediment (Figure 1.4C). Alternative anchorage systems had been conducted for the establishment of subtitle beds all sandy systems. Sand filled plastic tubes are used for *Gracilaria* anchored (Figure 1.4D).

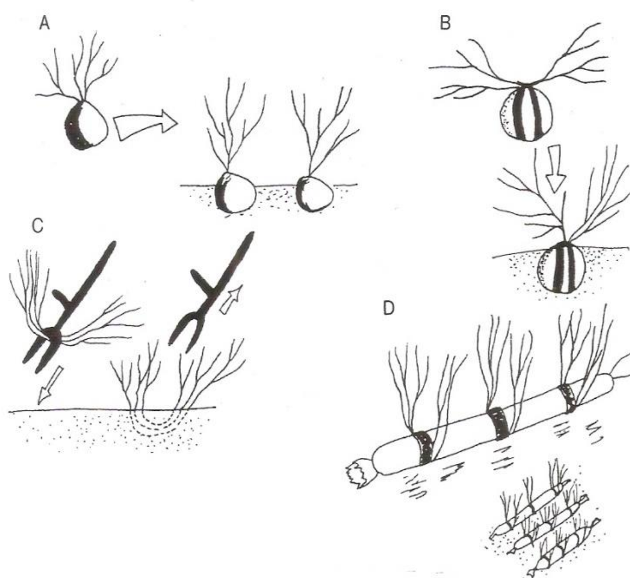

Figure 1.4 (A) Transplantation attached *Gracilaria* in rocky substrata. (B) *Gracilaria* adhered to rocks, with rubber bands. (C) *Gracilaria* inserted into soft sediments by using fork. (D) *Gracilaria* adhered to sand-filled plastic tubes.
Source: Critchley (1997b)

Bottom cultivation type of *Gracilaria* may face some restrictions such as limited suitable cultivation areas by the competition of salmon and mollusk farming. Another restriction is the decrease in biomass production caused by repeated removal of the apical meristems. Besides, *Gracilaria* production may also be affected by epiphytes, mussel fouling, grazing and sedimentation (Halling *et al*., 2005). Harvest season is determined by monthly biomass determination; for instance, when two continuous months do not differ by more than 15% or a biomass of 15 kg m^{-2} is achieved. However, crop production may be poor if the stocking site is environmentally different to that from where the plants originated. During harvest season or storms, thalli may tear loose from their substratum. In recent years, the problem of beach pollution caused by the plastic degradation, thus the plastic tube method has become less popular (Critchley, 1997b).

1.2) Rope farming

Two forms of out-planting using ropes are used. The first form includes vegetative thalli which are tied to, or inserted within a rope. The second form comprises reproductive material which is utilized as a source of spores which are settled onto the surface of the rope. Ropes may be located and suspended in the sea in a number of ways (Critchley, 1997b). Vegetative rope cultivation is also applied in Chile. This system is considered as labor-and-time-consuming and inefficient by the unproductive rope. The alternative system of spore and seedling ropes has been applied to avoid the aging effect when harvesting the mature thallus (Halling *et al*., 2005). With 1 meter length of rope, *Gracilaria* can produce 3.5 to 4 kg fresh weight depending on the species. *G. edulis* can produce the highest yield of 4 kg fresh weight m^{-1} length rope. However, the declining growth rates of *Gracilaria* may be met by the repeated and continuous farming from the same stock as an effect of aging or senescence (Mantri *et al*., 2009).

In the first form, selected cuttings or whole thalli may be tied onto or inserted within ropes. The lines must be locally available and cost effective and uses pieces of tape which are tied at intervals to the main planting rope. Gracilarioid thalli can also be inserted at the lay of the rope where can be opened. Another technique is called "super-rope", a plastic netting tube which has hollow tube meshing where *Gracilaria* is tied and held in location. In all cases, plant seed must be kept cool and moist while they are being attached to the line. When the gracilarioid is tied, ropes are then suspended, stretched, under tension, between stakes buried in the sediment or supported at different levels by buoys or rafts. Ropes are horizontally held at different depths or may even be allowed to hang vertically. A limiting factor to the growth of gracilarioids on ropes is water column transparency; sunlight can be harmful to crop growth in surface waters. Thus the optimum depth range for suspension needs to be investigated (Critchley, 1997b).

The second form that uses lines, nets or ropes can be designed as substrata for spore in natural populations of fertile *Gracilaria* (Figure 1.5). Recruitment of spores may be useful when the gracilarioid thallus is slender to be manually tied to or threaded into a rope (Critchley, 1997b). Spore culture is a successful planting method in many species of *Gracilaria*, but with limit species. The spore planting method has the advantage as producing large quantity of uniform seedlings and reproductive tissue (Mantri *et al*., 2009). Spore-seeding method only uses a small amount of reproductive *Gracilaria* to produce a large quantity of seeded ropes (Halling *et al*., 2005). Spore culture has been reported to be successful in a number of species of *Gracilaria* such as *G. parvispora*, *G. chilensis*, *G. edulis* and *G. dura* (Yu *et al*., 2013).

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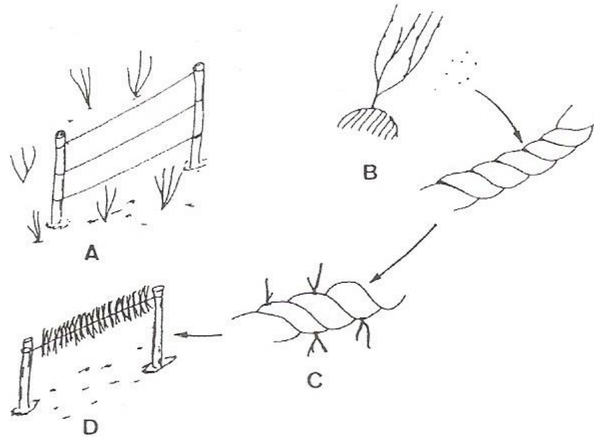

Figure 1.5 (A). Seedling ropes in natural *Gracilaria* populations. (B). Released spores from the fertile plants and settle onto the seed-ropes. (C). Juvenile plants attached to the seed-rope. (D). Juvenile plants removed new areas.
Source: Critchley (1997b)

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1.3) Cage culture system

In brackish water ponds, *Gracilaria* grew well in low density and non-fertilization in bamboo cages. Cage system was reported as containing low nutrient contents and should be fertilized to produce a high growth rate of seaweed. In tank culture system, *Gracilaria* species supply an adequate fertilization regime that ensures to produce high growth of algae (Nagler *et al*., 2003).

2) Pond farming and polyculture of *Gracilaria*

Gracilaria cultivation is commonly found in tanks or ponds. *Gracilaria* grows well at specific sites (Glenn *et al*., 1999). It is selected to be cultured in shrimp farms as wastewater treatment (Kanjana *et al*., 2011; Wongprasert *et al*., 2014). *G. chilensis* is reported as suitable species for integration with salmon in both land-based tanks and in suspended cages. *Gracilaria* growth in poly-culture system is more productive in comparison with monoculture due to fertilization effects (Halling *et al*., 2005). In Malaysia, *G. changii* has been cultivated in the small scale in shrimp ponds, mangrove ponds and irrigation canals. Besides, *G. edulis* is abundantly found on intertidal mudflats, fish cages, mangroves, corals and mangrove estuary (Yu *et al.,* 2013).

Critchley (1997) reported that *Gracilaria* is one of the largest seaweeds to grow well in man-made impoundments although it does not naturally grow in ponds. It can grow both in low salinity and water motion. In China, 1500 ha ponds were reported to provide 2,000 tonnes.year^{-1} dry *Gracilaria* for agar production. Individual ponds are 0.7-1.0 ha in size, 60-70 cm depth, with ranges in temperature and salinity of 15-30°C and 10-20 ppt, respectively, that are relatively high production of 40 tonnes.ha^{-1} of dry material over a 150-day growing period; it is achieved with a stocking density of 0.6 kg.m^{-2} and frequent cropping. Approximately 12,000 tonnes (fresh weight) of *Gracilaria* were produced from 300 ha of farm ponds with the yield of 16-43 tonnes ha^{-1} year^{-1} in 1971. The most limitation of this

cultivation system might be that only elatively low value, food-grade agar was produced. In ponds, increased summer temperatures in ponds might be a factor responsible for the low gel strength values of the agar produced from the crop.

Gracilaria ponds are generally situated in area not exposed to strong prevailing winds but located near to the source of both freshwater and seawater. Water depth plays an important role in modifying temperatures changes in pond. In summer with air temperatures of 32°C the pond depth may be increase to 50-60 cm; when air temperatures are below 10°C, depth of the pond may be as low as 20-30 cm. The pond depth is usually kept at 30-40 cm. Water is exchanged every 2-3 days, but can up to every 6-15 days that adjusts for salinity and mineral nutrient supplies for algal growth. Fresh thalli are seeded approximately 5-6 $tonnes.ha^{-1}$ into the ponds. Gracilarioids is harvested by using nets or rakes removing 1/3 or 1/2 of the total biomass every 30-35 days in the summer and every 45 days in the winter. Ponds may be fertilized at the rate of 3 $kg.ha^{-1}$ (or 1 gL^{-1}) urea or ammonium sulphate or 120-180 $kg.ha^{-1}$ pig or chicken manure. The optimal water pH for *Gracilaria* is in the range of 7.0-8.0. To stabilize pH, ponds can be drained and applied calcium carbonate ($CaCO_3$) at 1,000 kg per 3 ha in the winter. Under conditions of reduced water movement, epiphytes develop on thalli and may become serious pests. Taiwanese operators have developed a polyculture system that combines culture of several economically important marine species at the same time. By selective grazing tilapia (*Oreochromis mossambicus*) and the more valuable milkfish (*Chanos chanos*) can control epiphytes on the *Gracilaria*. Prawns and crabs may also be cultured in the polyculture system. Owning to the advent of abalone (*Haliotis)* cultivation in Taiwan, the *Gracilaria* is used as the sole source of food for these gastropods and is now more profitable for the farmers (Critchley, 1997b).

In recent years, the coastal aquaculture has been increasing emphasis on the sustainable development. The organisms are cultured at different water levels that are the basic of environmentally friendly aquaculture. Thus the integrated systems combining fish culture and seaweed culture was conducted. In this system, the wastes of one resource consumer become a resource (fertilizer or food) for others in the system that makes a balanced ecology (Zhou *et al.,* 2005).

3) Tank production of *Gracilaria* (Critchley, 1997b)

Tank culture of gracilarioids capable to use all available techniques, thus provides the greatest productivity per unit area, which is greater than by any other types of farming. This culture system can be precisely controlled and several steps may be mechanized for decreasing labor input. However, this is also the most expensive form of cultivation.

Experiments have explained the effects of a number of variables in tank system that growth may be limited by nutrient and carbon dioxide (CO_2) supply with low water flow. In spite of energy expensive, regular aeration is required to maintain the seaweed in circulation, mixing the biomass to the surface, into the light and reducing diffusion gradients at the surface of the crop. Aeration period can be adjusted to promote the maximum economic profit because there were no effects of daily aeration periods on gel characteristic. Water temperature is extremely fluctuated; tanks heat up due to solar heating and loose heat during colder periods

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due to radiation. The salinity of the water may be affected by evaporation or precipitation which is increased in the strong-aeration systems.

The nutrient provision is required in gracilarioid tank cultivation, but generally a nitrogen-based fertilizer is only required. The tank cultivation should be monitored nutrient status of the medium. Under conditions of nutrient depletion or too high levels of radiance, the crop will be lost color. In small tank (55 L) that had vigorous aeration and rapid water exchanges (20-30 volumes per day with low nutrient enrichments (10-100 µM nitrogen and 10 µM phosphorous), with stocking density of 2-3 kg m^{-2} (fresh) and weekly harvesting back to initial weight, the algal growth produced 35 $g.m^{-2}.day^{-1}$ dry weight (or 127 $tonnes.ha^{-1}.year^{-1}$. Specific growth rate of algae was determined up to 60 $\%.day^{-1}$ in short term experiments. *Gracilaria* intensive cultivation had successfully conducted in systems as large scale as 24,000 L with the growth of 22-25 $g.m^{-2}.day^{-1}$ dry weight and can be maintained for several years.

To maintain a high level of productivity, the gracilarioid crop was periodically harvested to avoid self-shading. Crop densities were recommended of 2-5 kg m^{-2} wet weight. Stocking density may affect to agar production with a productivity of 127 ± 72 $g.m^{-2}.day^{-1}$ in tanks with 4 kg m^{-2} fresh weight. At lower stocking density, epiphytes may also occur. In the Chilean system, the gracilarioid yield produced the greatest productivity (8 kg m^{-2}) in summer comparing to other seasons around year.

4) Selection of *Gracilarioids* for cultivation (Critchley, 1997b)

In convenience for the purpose of mass cultivation, gracilarioids easily regenerate when they were cut. Therefore, *Gracilaria* farming does not need to involve the whole triphasis life history. Vegetative propagation of gracilarioids can utilize the simplest material which can be seen in the selection of healthy, fast growing frond in rope cultivation in the Caribbean. Besides, genetic selection in other areas has been practiced and propagated strains by vegetative means. The suitability for the cultivation of both native and exotic gracilaioids had been conducted in Israel. Native species might not always be the most suitable or economic species for cultivation; isolates from elsewhere might grow faster under the same conditions. Alternative crops was necessary to be applied during the year using strains which are better adapted to either low or high temperatures in order that a production plant might economically keep operation throughout the year.

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5) Environmental factors on *Gracilaria* growth

Temperature, salinity and light intensity are the most important factor on benthic marine algae growth (Raikar *et al*., 2001). Growth rate of *Gracilaria* spp. increased when light intensity increased. Low growth rate of *G. textorii* observed at 10 umol $m^{-2}s^{-1}$ with 1.55% whereas *G. corticata* got 12.78% at 10 µmol m^{-2} s^{-1} (Raikar *et al*., 2001). Yu *et al*. (2013) mentioned that light and salinity significantly affect *G. edulis* and *G. tenuistipitata* growth. *Gracilaria* species can growth both in tropical and temperate water; it was abundantly found at water of 20-27°C (Raikar *et al*., 2001). Different species of *Gracilaria* require different optimal temperature and salinity (Raikar *et al*., 2001). Choi *et al*. (2006) reported that *G. verrucosa* and *G. chorda* grew well in the range of 10-30°C and 5-35 ppt whereas the optimal range was found at 17-30°C and 15-30 ppt. *G. chorda* got maximum growth rate at 30°C, 25 ppt with 4.95% day-1 while 25°C and 25 ppt is optimal for *G. verrucosa* with 4.47% day^{-1} (Choi *et al*., 2006).

Either too low or too high sanity might limit the growth of *Gracilaria* species; for example, *G. edulis* reduced growth at at 5 and 40 ppt with bleaching occurred at 5 ppt within one week (Yu *et al*., 2013). Raikar *et al*. (2001) mentioned that *G. corticata* died at salinity lower than 15 ppt, and the seaweed died after 2 days at less than 10 ppt. The earlier study reported that optimal salinity for *G. fisheri* was 20-25 ppt (Prud'homme van Reine and Trono, 2001). Ruangchuay *et al*. (2010) reported that the temperature range for *G. fisheri* growth was 20-30°C and the water depth for *G. fisheri* cultivated ponds ranged from 25.0 to 61.7 cm. Water depth plays an important role in modifying temperatures changes in pond. In summer with air temperatures of 32°C the pond depth may be increase to 50-60 cm; when air temperatures are below 10°C, depth of the pond may be as low as 20-30 cm. The pond depth is usually kept at 30-40 cm. Water is exchanged every 2-3 days, but can up to every 6-15 days that adjusts for salinity and mineral nutrient supplies for algal growth. Ponds may be fertilized at the rate of 3 $kg.ha^{-1}$ (or 1 $g.L^{-1}$) urea or ammonium sulphate or 120-180 $kg.ha^{-1}$ pig or chicken manure. The optimal water pH for *Gracilaria* is in the range of 7.0-8.0. To stabilize pH, ponds can be drained and applied calcium carbonate ($CaCO_3$) at 1,000 kg per 3 ha in the winter. Under conditions of reduced water movement, epiphytes develop on thalli and may become serious pests (Critchley, 1997b).

1.2.7 Biotechnology of *Gracilaria*

1) Tissue culture *Gracilaria*

In the 1970s, the application of plant cell and tissue culture in seaweeds (Figure 1.6) began to exploit the benefits that multidimensional technique has provided in higher plants in response to the requirements of the seaweed market and cultivation practices (Baweja *et al.*, 2009). Baweja *et al.* (2009) also mentioned that the seaweed consumption has grown as predicted with prospects to go even further. The demand was increasing day-by-day for the raw materials in food and

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phycocolloid industries. Subsequently, concentrated work on new strain selection and improvement of an efficient mass culture system was obviously needed.

In addition, the interest in the application of tissue culture technique in seaweed had been growing for genetic manipulation and micro propagation both in order to improve the aqua culture crops and to increase the knowledge about the process of differentiation, morphogenesis and regeneration. Seaweed tissue culture may be already useful for certain biotechnological applications, such as clonal propagation of seed material for mariculture. However, the investigation of growth and development applied in higher plant tissue culture is lacking, and it is required for more complex biotechnological applications in seaweeds (Baweja *et al.*, 2009). Plant tissue culture is set up a basic powerful tool for the exploitation of seaweed at the cellular level. Seaweed tissue culture techniques were expected to be developed enough in the near future. Combination with molecular genetics, it might give supporting to the same biotechnological applications as in higher plants in the genomic age, a field in which seaweed was also far behind higher plants (Baweja *et al.,* 2009).

Figure 1.6 Tissue culture of *Gracilaria changii*
Source: Yeong *et al.* (2014)

Reddy *et al.* (2008b) mentioned that there were more than 85 species of seaweeds, including nine species of *Gracilaria* (Table 1.1) for which tissue culture aspects have been reported. Recently, the scope of these techniques has been extended for use in bioprocess technology for production of high value chemicals of immense importance in the pharmaceutical and nutraceutical sectors, although the initial aim of these techniques focuses mostly on genetic improvement and clonal propagation of seaweeds for marine culture. For *Gracilariopsis persica* tissue culture, the healthy plants is chosen and used the middle parts of the thallus. Then the thallus were cleaned and sterilized by using brush and autoclaved seawater. The axenic thallus were cut into 3 cm fragments and cultured in PES (Provasoli’s Enriched Seawater) medium. *Gracilariopsis persica* tissues are cultured at the condition of 3,000 Lux light, 12 dark: 12 light of photoperiod and temperature of 24ºC (Matinfar *et al.*, 2013).

Table 1.1 Seaweed species from which tissue culture have been accomplished

Species	Status of success	Reference
Gracilaria acuminate	CI	Huang and Fujita (1997)
G. chilensis	CI	Collantes *et al.* (2004)
G. corticata	CI & PR	Rajakrishna Kumar *et al.* (2007)
G. papenfussii	PR	Polne-Fuller and Gibor (1987)
G. perplexa	CI	Yokoya *et al.* (2004)
G. tenuistipitata	CI	Yokoya *et al.* (2004)
G. tenuifrons	PR	Yokoya (2000)
G. textori	CI	Huang and Fujita (1997)
G. verrucosa	CI & PR	Gusev *et al.* (1987); Kaczyna and Megnet (1993)

CI, callus induction; PR, plant regeneration
Source: Reddy *et al.* (2008b)

Matinfar *et al.* (2013) found that *Gracilariopsis persica* tissue grew well at the conditions of 2 cm initial length with the growth rate of 5.4 % day^{-1}, density of 5 piece L^{-1} and apical part of thallus with the same growth rate of 6.6% day^{-1}.

2) Protoplast culture of *Gracilaria*

Protoplasts are living plant cells without cell walls which offer uniform single cell system; thus it facilitates several aspects of modern biotechnology, especially genetic transformation and metabolic engineering (Reddy *et al*., 2008b; Huddy *et al*., 2013). Extraction of cell wall lytic enzymes from different phycophages of red and brown seaweed species and microbial sources has improved protoplast isolation and their yield. Recently, reliable procedure is now available to isolate and culture protoplasts from various groups of seaweed. Up to 2008, there were 89 species belonging to 36 genera of green, red and brown seaweeds having successful protoplast isolation and regeneration (Reddy *et al*., 2008b; Huddy *et al*., 2013).

Protoplast is serving excellent experimental materials for biochemical and morphogenetic studies. There are nine genera of red seaweeds applied for protoplast isolation, *Porphyra*, *Kappaphycus*, *Grateloupia*, *Bangia*, *Gelidium*, *Solieria*, *Palmariam*, *Chondrus* and *Gracilaria*. There are several species of *Gracilaria* genus isolated protoplast by enzymatic method such as *G. asiatica*, *G. changii* (Figure 1.7) (Yeong *et al.,* 2008), *G. chilensis*, *G. tikvahiae* and *G. gracilis* (Huddy *et al*., 2013).

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Figure 1.7 Protoplast regeneration of *G. changii*. (A) Cell division of protoplast after 5 days. (B) Plants regenerated from protoplast after 60 days. (C) Plant formation after 135 days.
Source: Yeong *et al*., (2008)

To date, there are more than 89 seaweed species that have protoplast isolation and regeneration. The yield, viability and regeneration rate of protoplast regeneration depends on the culture conditions such as enzyme constituents and their concentration, pH, osmotic conditions, incubation temperature, physiological state and age of donor plant and culture medium (Reddy *et al*., 2008a; Huddy *et al*., 2013).

Seaweed protoplasts are extracted mainly by enzymatic methods. These methods require a fair understanding of chemical composition of cell walls because the seaweed cell walls are structurally complex and different from land plant cell walls. The yield, viability and regeneration rate of protoplasts depends on several factors such as the enzyme constituents and their concentration, pH, osmotic conditions and ionic strength of protoplast isolation medium, incubation temperature, physiological state and age of donor plant, protoplast culture medium and its culture conditions (Reddy *et al*., 2008a).

The development of tissue and protoplast studies showed a great potential for the development of improved strains of greater economic importance whereas the advances in macroalgal biotechnology lagged far behind land plant studies. Because the commercially utilized stocks of Gracilarioids persisted as poor population, traditional hybridization techniques via sex cells were unavailable. Moreover, protoplast isolation, culture and somatic hybridization had had some limited success in *Gracilaria* studies (Critchley, 1997a).

3) Photo-bioreactor cultivation of *Gracilaria*

The maximization of biomass productivity in photo-bioreactors is an important step for the development of microalgae biotechnology. In microalgal cultivation, light availability inside the reactor is often the bottleneck for algal growth. Many models have been developed to predict growth and volumetric productivities in photo-bioreactors by stimulating light intensity. Each type of the model is used with different conditions such as reactor type, strain, cultivation conditions (Barbosa *et al*., 2003).

Tsoglin *et al*. (1996) mentioned that some specific problems must be solved to develop high performance photo-bioreactors for algal cultivation. First of all, the reactor design should be universal and allow the cultivation of various

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unicellular photosynthesizing organisms. Besides, the cultivator design must provide for the uniform illumination of the culture surface and the fast mass transfer of CO_2 and O_2 to ensure a high efficiency of light use by the culture.

G. changi tissue culture has been applied in photo-bioreactor (Figure 1.8) with the biomass increase 1gL^{-1} after 72 days (0.69% day^{-1}) in 500mL spherical photo-bioreactor (Yeong *et al.*, 2014). High rates of mass transfer must be reached by means that neither damage cultured cells nor suppress their growth. Moreover, the photo-bioreactor must normally work under conditions of intensive foaming as generally occurs in reactors with high rates of mass transfer. The energy consumption for mass transfer and the arrangement of the light receiving surface of the algal suspension must be reduced for the industrial scale production of biomass (Tsoglin *et al.*, 1996).

Figure 1.8 *G. changii* tissue culture in spherical photobioreactor
Source: Yeong *et al.* (2014)

1.2.8 Utilization of *Gracilaria*

Gracilaria is also used for the food industry and a number of biological and biotechnological applications (Critchley, 1997b).

1) Seaweeds for phycocolloids

Most of the world's commercial agar was extracted from gracilarioids. The word "agar" comes from the Malay term "agar-agar" and was originally used for the gel extracted from *Eucheuma* (Critchley, 1997b; Pereira, 2012). The main product of *Gracilaria* is agar. Some 5,000 tonnes of agar was annually produced from 25,000-30,000 tonnes of dry weight gracilarioids. In addition, the market demands for gracilariods have obviously increased in recent years, so that during the period 1976-1983, the export of *Gracilaria* was increased

eleven folds from Chile due to increasing in the free on board price which in 1984 was seven and half times the 1972 value of $ 1,500 per dry tone (Critchley, 1997b).

The agars, carrageenans, and alginates, collectively termed hydrocolloids, are a major source of industrially important algal products. Agar and agaroses are used in medical and biological sciences for culture media and for gel electrophoresis. Agars are also used in many other products, including ion-exchange and affinity chromatography, pharmaceutical products, and fruit fly foods. Carrageenans are used as binders and thickeners in a wide variety of pastes, lotions, and water-based paints. Alginates are used to bind textile printing dyes, to stabilize paper products during production, to coat the surfaces of welding rods, to serve as binders and thickeners in numerous pharmaceutical products, and to act as binders in animal feed products (Abdallah, 2012).

Agar is polysaccharide derived from red seaweed families, *Gracilaria*ceae and Gelidiellaceae (Ahmad *et al*., 2011; Coppen and Nambiar, 1991; Ganesan and Subba-Rao, 2004; Istini *et al*., 1994; Pereira, 2012). The basic structure of agar is an alternated sequence of 3-linked-P-D-galactopyranose and 4-linked 3,6-anhydro-cr-L-galactopyranose (Istini *et al*., 1994). *Gracilaria* agar structure consists of repeating units of (1,3) linked-D-galactose and (1,4) link 3,6-anhydro-L-galactose. However, *Gracilaria* spp. comprise some structure with different substituents like sulfate esters, methoxyls and pyruvic acid (Ahmad *et al*., 2011). Agar presents great gelling power with the wide range of conditions. It is used in the preparation of jellies, dairy products and bakery products (Coppen and Nambiar, 1991; Critchley, 1997b; Brownlee *et al*., 2012; Pereira, 2012). Japan is the main consumer and producer (Coppen and Nambiar, 1991; Critchley, 1997b), but the biggest suppliers included Chile (63 %), Philippines (15 %), South Africa (10 %) and Brazil (6 %) (Coppen and Nambiar, 1991). The quality of agar depends on its physical properties such as gel strength, gelling and melting temperature (Buriyo and Kivaisi, 2003; Chirapart *et al*., 2006; Coppen and Nambiar, 1991; Ganesan and Subba-Rao, 2004). Ganesan and Subba-Rao (2004) metioned that the genus *Gracialria* generally produces low quality of agar due to high sulphate content that called agaroides. Fortunately, alkali treatment can be used to transform agaroides into real agar by converting L-galactose-6-sulphate to 3,6 anhydro L-galactose which improves gel forming ability (Coppen and Nambiar, 1991; Critchley, 1997b; Ganesan and Subba-Rao, 2004).

Red algae are mainly utilized as a raw material for agar, extraction or the food industries or in the production of tissue culture media. In Malaysia, they are popularly cultured for agar production (Norziah and Ching, 1999). Dawes *et al*. (1999) mentioned that the red seaweeds *Gracilaria, Eucheuma*, and *Hypnea* are the most important genera of commercial, subtropical and tropical macroalgae in terms of the production of economically valuable phycocolloids. In 1991, the agar production was annually supplied for more than 53% (1,000 tonnes agar) from *Gracilaria* seaweed, imported to the US market.

Chirapart *et al.* (2006) said that changes in agar yield and property depend upon species, season and environmental parameters. Gel properties of agar can also be changed depending on stage of the life cycle. In Thailand, *G. fisheri* and *G. tenuistipitata* are two commercial agarophyte species that are generally harvested from natural stock for agar extraction.

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Bacteriological (microbiological), sugar-reactive and food grades are three principle grades of agar. In the world market, the highest grade agar for bacteriological grade was 600 tonnes, with a market value of $20 million in 1989. Sugar-reactive agar is obtained mainly from some *Gracilaria* species from the eastern Pacific (*Gracilaria lemaneiformis)* which retains its gel strength with the addition of sugar (at least 75 g per 100 mL in a 1% agar solution) and the gel becomes elastic. International prices of agar increased its value as the traditional market for microbiological agars has been expanded by new uses. Similarly, the market for sugar-reactive agars has expanded (Critchley, 1997b).

Gracilaria spp. is the largest sources of agar extraction that contributes about 60 % of the world agar production (Ahmad *et al*., 2011; Buriyo and Kivaisi, 2003; Yeong *et al*., 2014). It was reported that *Gracilaria* and *Gelidium* were the most important agarophytes that contribute about 53 % and 44 % to the world's agar production. Buriyo and Kivaisi (2003) mentioned that the agar yield of *Gracilaria cornea*, *G. corticata* and *G. fergusoni* were in the range of 28-48 % dry weight. It was also reported that agar yield varied under different seasons (Buriyo and Kivaisi, 2003). In addition, the genus *Gelidium* is considered to produce the best quality of agar, but it is high cost and insufficient from wild stock. Therefore, *Gracialria* has intensively been studied due to the excellent substitute for *Gelidium* agar (Ahmad *et al*., 2011).

Gracilarioids is known as a main source to give good yields of agar but with poor gel strength value (Critchley, 1997b). Generally, values of gel strength derived from *Gracilaria* are around 120-150 g.cm^{-1} whereas that values derived from *Gelidium* or *Gelidiella* are around 300-350 g.cm^{-1} (Coppen and Nambiar, 1991). Traditionally, *Gelidium* and *Pterocladia* are the sources of bacteriological grade due to gel strength (nearly 600 g cm^{-2}) and the difference between melting and gelling temperatures. Their melting and gelling temperature are melting at 85-86°C and gelling at 32-36°C (Critchley, 1997b).

2) Seaweeds as food

Some seaweeds are appreciated of high mineral content such as iron in *Himanthalia elongata*, calcium in *Undaria pinnatifida* and *Chondrus crispus*. In Japan, daily seaweed consumption is accounted for more than 10% in the Japanese diet (Pereira, 2012). Norziah and Ching (1999) mentioned that the red algae such as *Porphyra, Palmaria, Gracilaria, Gelidium and Eucheuma* are among the major edible seaweeds. The certain edible seaweeds are reported that contain amounts of protein, vitamins and mineral essential for human nutrition. In the coastal areas, fresh and dried seaweeds are popularly consumed as a source of food. Seaweed is generally suitable for simple food processing such as making cool, gelatinous dishes or concoctions depending on the type of species (Norziah and Ching, 1999). The nutrients composition of seaweed is varied and influenced by species, geographic area and season of the year and water temperature. The nutrient composition of these sea- vegetables interests in human consumption due to their low calorie food, but rich in vitamins, minerals and dietary. *Gracilaria* species was also reported to contain carotenoid pigments which are important in shrimp and fish diets (Norziah and Ching, 1999). Japan and China are countries that use seaweed as a source of

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food commonly. Approximately 25% of all food consumed in Japan consists of seaweed prepared and served in many forms. It has become the main source of income for the Japanese fishermen (Norziah and Ching, 1999).

3) Other applications from seaweed

In recent years, Gelidialian red algae was recorded that contain rhizoidal filaments which are processed to make bleached pulps and used as raw materials for papermaking, except the family Gelidiellaceae. Red algae were mostly used from the Gelidiaceae family, which contains three genera in Korea, *Gelidium, Pterocladiella,* and *Acanthopeltis*. The rhizoidal filaments, cortical cells are usually reddish in color and medullary cells filled with mucilaginous carbohydrates. High brightness of red algae pulp (Figure 1.9) can be derived from extracting mucilaginous carbohydrates after heating the algae in an aqueous medium and treating the extracted with bleaching chemicals (Seo *et al.*, 2010).

Besides, seaweeds also contain a tremendous diversity of oxidant compounds because they usually live in the intertidal zone and survive in a highly competitive environment (Ruperez *et al*., 2012). There are many medicinal uses from seaweeds such as cardiovascular disease prevention, cholesterol lowering, anti-diabetes, anti-coagulative, anti-inflammatory immunomodulating and anti-cancer effects. Moreover, some tropical seaweeds contain the nutrients composition, vitamin C, tocopherol, dietary fibers, minerals, fatty acid and amino acid profiles. Seaweeds are rich in macro minerals such as Ca, Mg, N, P and K and trace elements like Zn, I and Mn. The contents of calcium and phosphate-phosphorus in seaweeds are higher than those in apples, oranges, carrots or potatoes. The regular consumption of seaweed positively relates to a reduced risk of human breast cancer. The seaweed anti-cancer compounds include water soluble polysaccharides such as laminarans, sulphated polysaccharides and fucoidans (Mohamaed *et al*., 2012).

Seaweeds are evaluated as low calories content, rich in soluble dietary fiber, proteins, minerals, vitamin, antioxidants, phytochemical and polyunsaturated fatty acids. Recently, they are applied as gelling agent and stabilizers in the food and pharmaceutical industries (Abdallah, 2012; Kongkiattikajorn and Pongdam, 2006; Mohamaed *et al*., 2012).

Moreover, seaweeds produce large quantities of halometabolites that is held within algal membrane bound vesicles and resulted from the catalytic activity of haloperoxidases. In biological defense mechanisms, these halometabolites probably act as hormones or as repellents (Kongkiattikajorn and Pongdam, 2006).

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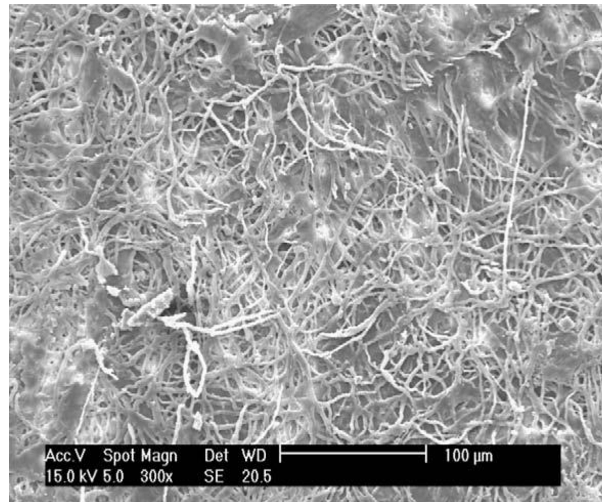

Figure 1.9 Shape of red algae pulp fibers in handsheet (300 x)

Source: Seo *et al.* (2010)

Vanitha and Chandra (2012) said that some species of red algae such as *Gracilaria corticata, Grateloupia lithophila, Gelidium* sp. and *Bryocladia thwaitesia* contain phycobilin pigments which are used in food stuffs including the coloring of fermented milk products, ice creams, chewing gums, soft drinks, desserts, sweet cake decoration and milk shakes. Beside, algal pigments have a great commercial value as natural colorants in nutrient pharmaceutical and cosmetic industry. In addition, Abdallah (2012) mentioned that seaweeds can be applied to reduce the nitrogen and phosphorus content of effluents from sewage treatments.

1.3 Objectives

1.3.1 To examine some biological characteristics: contaminant, color, pigment, mineral and agar contents of *G. fisheri* from different locations

1.3.2 To study on the relation of seaweed characteristics and some environmental factors: sediment organic matters, sediment texture and water quality

1.3.3 To examine the growth rate and development of *G. fisheri* tissues in different conditions of salinity, initial length, density, part of thallus and light color in laboratory

1.3.4 To study on upper scale of tissue culture under optimal conditions and seedling stock at indoor and outdoor conditions

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CHAPTER 2

RESEARCH METHODOLOGY

The sampling was done in *G. fisheri* cultivating earthen ponds in the Gulf of Thailand side at three provinces, namely Pattani (P1), Songkhla (P2), and Surat Thani (P3) (Fig. 1). The pond sizes were in the ranges of 0.16-0.48 ha at P1; 0.16-0.64 ha at P2; 0.16-0.48 ha at P3. The distances among ponds in each site were 0.5-4.0 km at P1; 0.1-5.0 km at P2 and 0.5-5.0 km at P3 (Fig. 2). The samples including the seaweed at the harvesting stage, water and sediment were randomly collected from four cultivating ponds of each site during July 2013 to November 2014. The samples were put in plastic bags and brought back to analyze.

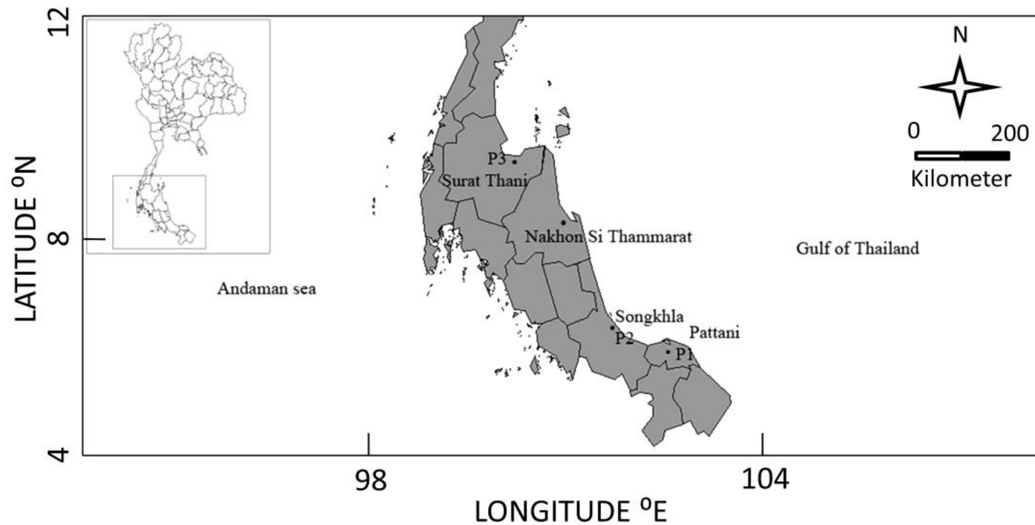

Figure 2.1 Map of experimental ponds in Pattani, Songkhla and Surat Thani Provinces

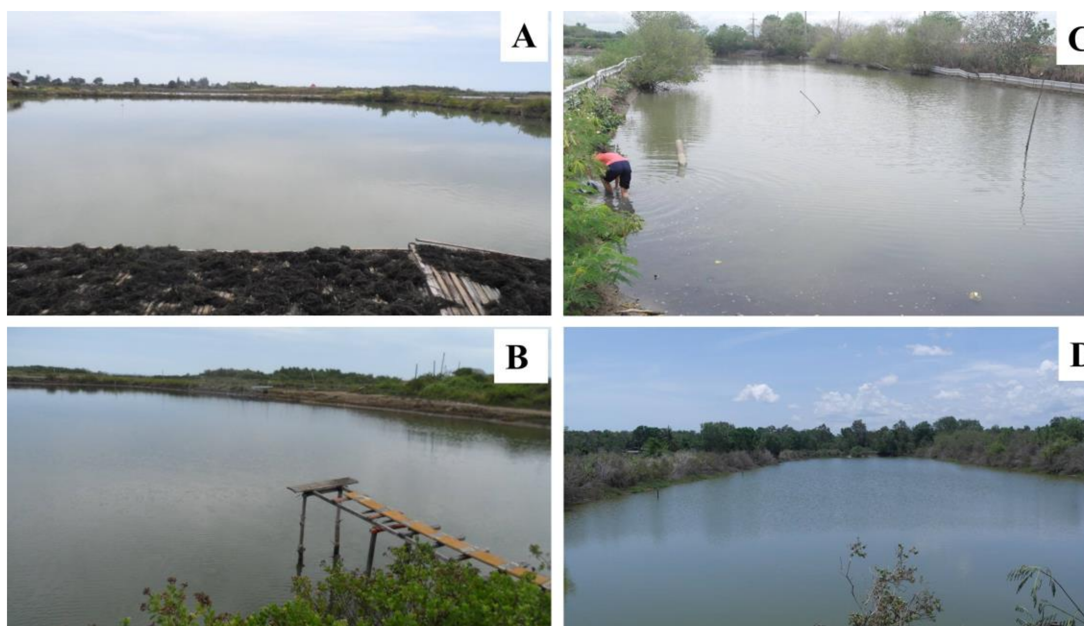

Figure 2.2 Cultivated pond habitat in the experimental provinces. (A, B) Cultivated ponds in Pattani Province. (C) Cultivated pond in Songkhla Province. (D) Cultivated pond in Surat Thani Province.

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2.1 Method

2.1.1 Study on environmental characteristics, water and sediment

1) Measurement of some parameters at the filed

At the field, some parameters were measured comprising temperature, light intensity, salinity water depth and transparency by using thermometer, Lux meter, Hand-held Refractometer and Secchi disk, respectively.

2) Measurement of some parameters in laboratory

Other parameters were also analyzed in laboratory including pH, alkalinity, hardness, nitrate-nitrogen and phosphate-phosphorus in water; metals, texture and organic matter in sediment. The pH was measured by using pH meter. Besides, alkalinity, hardness, phosphate-phosphorus and nitrate-nitrogen concentration were analyzed in laboratory by using the methods of Laongsiriwong and Predalumpaburt (2003).

3) Water analysis

3.1) Alkalinity analysis (Titrimetric method)

The water sample used of 50 mL and was contained in the 50 mL flasks. Three drops of Phenolphthalein were added into these flasks. Water sample in the flasks were then titrated by 0.02 N H_2SO_4. After titrating, they were added three

drops of Methyl red and titrated again by 0.02 N H_2SO_4. The total alkalinity in water sample was calculated by using the formula,

Total alkalinity ($mg.L^{-1}$) = [(A+B)*0.02*50000]/V
Where A = the endpoint of the first titration (mL)
B = the endpoint of the second titration (mL)
V = volume of water sample (mL)

3.2) Hardness analysis (EDTA titrimetric method)

This analysis used 50 mL flasks to contain 5 mL water sample and 45 mL distill water. After that, 1 mL buffer solution and 0.05 g of indicator was be added into these flasks. At this time, water in the flasks turned to violet color. Finally, water samples were titrated by 0.01 N EDTA. Then, the water sample color turned from violet to blue color within one drop. The volume of used EDTA was recorded to calculate the amount of water hardness as the formula,

Hardness ($mg.L^{-1}$) = (A*1000)/V
Where A = the endpoint of the titration (mL)
V = volume of water sample (mL)

3.3) Phosphate-phosphorus analysis (Photometric method)

The 100 mL flasks were used to contain 50 mL of water sample and standard solution. The flasks were then added 8 mL of prepared solution which included 50 mL of 5 N H_2SO_4, 5 mL of Potassium Ar, 30 mL of Ascorbic acid solution and 15 mL of Ammonium molipdate. After that, the flasks were added 1-2 drops of Phenolphthalein and 1 drop of 1 N H_2SO_4. Finally, water samples were analyzed by using spectrophotometer (at λ = 880 nm).

3.4) Nitrate-nitrogen analysis (Brucine colorimetric method)

For this analysis, 10 mL of water sample and standard solution was used. The flask was added 2 mL of NaCl, 10 mL of H_2SO_4 and 0.5 mL Brucin and shake. Next, these flasks were put into thermomachine at 95°C for 20 minutes. The samples were analyzed by using spectrophotometer (at λ = 410 nm) after cooling.

4) Elements in water

Atomic Absorption Spectrophotometric method was used to determine the concentration of Cd, Cr, Ni, Cu, Fe, Pb, Mg, Ca, Mn, and Zn (method 974.27 of AOAC, 2000), K (method 973.53 of AOAC, 2000) and Na (method 973.54 of AOAC, 2000).

5) Elements in sediment

The concentration of Ca, Mg, Cu, Mn, Zn, Fe, Ni, Cr, Cd and Pb were determined by using Atomic Absorption Spectrophotometric method (method 965.09 of AOAC, 2000) while K and Na were determined by using Flame Photometric method (method 983.02 of AOAC, 2000). Besides, organic carbon, organic matter

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were measured according to Walkley-Black Procedure (Nelson and Sommers, 1982), sediment texture was tested by using Pipette method (Sheldrick and Wang, 1993).

2.1.2 Some biological characteristics of G. *fisheri* in pond from different locations

The seaweed was collected about 1 kg from each pond. The sample was conducted for contaminant, chlorophyll and r-phycoerythrin concentration, thalus color, agar and metal content separately. Seaweed metal analysis was conducted as the same manner with sediment metal analysis, but 1 gram of seaweed was be used instead of using sediment.

1) Contaminant measurement

The contaminants include mollusk and epiphytes were separated from the seaweed sample and recorded the weigh to determine the percentage in seaweed.

2) Chlorophyll concentration analysis

Seaweed was cleaned and weighted 0.5 grams with three replications for each seaweed sample from each pond. Seaweed was then grinded and added acetone (90%) in small increments until seaweed is more homogenous up to 10 mL of acetone. Seaweed solution was poured into the 15 mL test tubes and centrifuged at 8,000 rpm for 5 minutes. Finally, total chlorophyll in seaweed was measured by using spectrophotometer at wavelength of 750, 664, 647, 630, 510 and 480 nm (Parson *et al*., 1992).

3) Concentration of r- phycoerythrin analysis

The method for r-phycoerythrin analysis is similar with chlorophyll analysis, but 10 mL of distill water were used instead of using acetone. The concentration of r-phycoerythrin in seaweed was be also measured by using spectrophotometer at wavelength of 650, 615 and 565 nm (MacColl and Guard-Friar, 1987).

4) Color analysis (Dutta *et al*., 2013)

Seaweed samples were carefully cleaned and removed the contaminants. Then, the samples were cut and put into the same size of bag (5×8 cm) with the weight of 2 g per bag (seaweed should fill all the bag, Figure 2.3). The visual color was measured by using a HunterLab Color Measurement System. The measurement was determine L*, a* and b* that are the brightness, redness and yellowness, respectively. The instrument (10 observer, Illuminant D-65) was calibrated against a standard white reference tile.

The total color difference (ΔE) is determined by the formula:

$$\Delta E = ((\Delta a)^2 + (\Delta b)^2 + (\Delta L)^2)^{1/2}$$ (Dutta *et al*., 2013)

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Figure 2.3 *G. fisheri* color analysis by HunterLab Color Measurement

5) Agar analysis (Chirapart *et al*., 2006; Praipoon *et al*., 2006)

5.1) Raw material was collected from three locations and each location has four seaweed ponds collected to analyze agar content and washed under tap water over night to remove salt and epiphytes.

5.2) Seaweed was rinsed by distill water and cut into small pieces of 1-2 cm.

5.3) The sample then was dried in oven temperature at 60 ^{o}C for several hours untill completely dried.

5.4) Thirty gram of seaweed was used to analyze agar content in 2-liter flask with 900 mL of distill water.

5.5) The samples were boiled for 2 hours in water bath.

5.6) The solution was filtered by muslin clothes.

5.7) The filtrate was gelled in room temperature condition.

5.8) Then gelled agar was freezed at -20 ^{o}C for 24-48 hours.

5.9) The freezed agar was thawed under tap water and pressed to remove water content.

5.10) Agar was then dried at 60 ^{o}C for 24 hours.

5.11) Dried agar was weighted to determine agar yield.

The percentage of agar yield is determined by:

$$\text{Agar yield } (\%) = \frac{Weight\,dried\,agar(g)}{Weight\,of\,seaweed\,used\,(g)} \times 100$$

(Marinho-Soriano and Bourret, 2003)

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2.1.3 Tissue culture under different conditions: initial length, salinity, part of thallus, density and light color

1) Tissue culture in different initial length

1.1) Seaweed was cleaned by marine water for several times to remove the contamination such as mud, other algae. They were stocked back to culture tank for acclimation. Healthy thallus was cleaned and cut into pieces of 1, 2, 3, 4 and 5 cm length.

1.2) Tissue was weighed at 2 gL^{-1} and put to culture in 250 mL flasks (Figure 2.4) under different salinity of 15, 20, 25, 30 and 35 ppt. Each treatment was done with three replications.

1.3) The flasks were cultured under 25 $\mu mol.m^{-2}.s^{-1}$ of light intensity and 12L:12D of photoperiod at temperature of 25±1°C. Modified Grund Medium (MGM) (Mensi *et al.*, 2011) was supplied to the flask every 10 days while the water exchange.

1.4) The tissues were cultured for 40 days. The tissue was measured weight, length of main tissue and branches and number of branch every 10 days.

1.5) Growth rate of the tissue was calculated and the best result was applied for the next experiments.

2) Tissue culture in different salinity

2.1) Healthy thallus was selected to clean and cut into pieces with optimal lengths.

2.2) The tissue was measured weight at 2 gL^{-1} to stock into the culture flask. Tissues were cultured for 40 days with 3 replications.

2.3) The tissue was measured weight, length of main tissue and branches and number of branch every 10 days.

2.4) Growth rate of the tissue was calculated and the best result was applied for the next experiments.

Figure 2.4 *G. fisheri* tissue culture in 250 mL flask

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3) Tissue culture in different part of thallus

3.1) The healthy thallus was cleaned and cut into the optimal length from different zones of thallus: apical, sub-apical and closed root zone.

3.2) The tissue was cultured for 40 days under the conditions as above with the optimal length and saliniy. The tissue was measured weight, length of main tissue and branches and number of branch every 10 days

3.3) Growth rate of the tissue was calculated and the best result was applied for the next experiments.

4) Tissue culture in different density

4.1) Healthy thallus was selected the optimal part of thallus, cleaned and cut into the optimal length.

4.2) Thallus was stocked at different density of 1, 2, 4, 6 and 8 gL^{-1}.

4.3) The tissues were cultured for 40 days under the conditions as above at the optimal salinity and initial length. The tissue was measured biomass, length of main tissue and branches and number of branch every 10 days.

4.4) Growth rate of the tissue was calculated and the best result was applied for the next experiments.

5) Tissue culture under different light

5.1) The healthy thallus was cleaned and cut into the optimal length from optimal zone.

5.2) The tissue was stocked at 1 gL^{-1} and cultured in 3 L of the round plastic bowl and put under different light color of white, red, blue and green (Figure 2.5) at the light intensity of 20±1 $\mu mol.m^{-2}.s^{-1}$ at the optimal salinity and density. The culture was conducted with three replications for 40 days.

5.3) The tissue was measured weight, length of main tissue and branches and number of branch every 10 days.

5.5) Growth rate of the tissue was calculated.

5.5) The tissue was cultured under the conditions as above at the optimal salinity.

6) Spore culture under different light

6.1) Eight weeks age sporelings were collected and cleaned before stocking the experiment.

6.2) The spores were cultured in 3 L of the round plastic bowl and put under different light colors of white, red, blue and green of PVC sheets at the light intensity of 20±1 $\mu mol.m^{-2}.s^{-1}$ at 20 ppt salinity and 1 g/ 3L density.

6.3) The spore was measured weight, length of the longest thallus, number of new branches and branch length on the longest thallus every 10 days.

6.4) Growth rate of the tissue was calculated.

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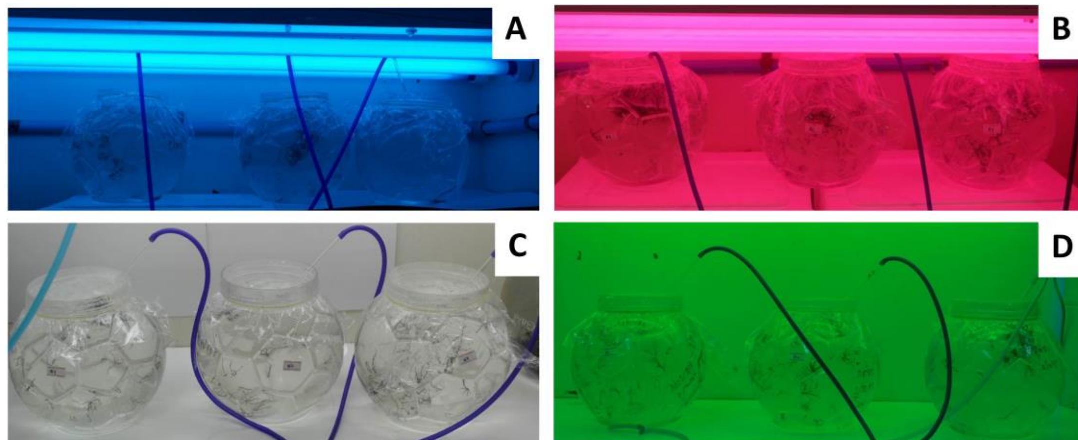

Figure 2.5 *G. fisheri* tissue culture under different shading colors: (A) Blue, (B) Red, (C) White, (D) Green

2.1.4 Upper scale of tissue culture under optimal conditions in laboratory and outdoor

1) Upper scale of issue culture under optimum conditions

1.1) The healthy thallus was cleaned and cut into the optimal length from optimal zone.
1.2) The tissue was cultured in 6 L of volumetric flask for 40 days under the optimum conditions from the result of above.
1.3) The tissue was measured weight, length of main tissue and branches and number of branch every 10 days.
1.4) Growth rate of the tissue was calculated.

2) Culture of seedling stock at outdoor condition

2.1) The seedling from tissue with initial density of 250 g m^{-2} was reared in 0.8×0.5×0.3 m^2 plastic tank and put in greenhouse at outdoor of Division of Fishery Technology.
2.2) The house was covered by 50% commercial light protection of Saran plastic in different colors of; white from PVC window screen and red, blue and green from polyamides sheets. Aeration tubes were connected to the tanks. Each house has four tanks; light intensity was 110±10 μmol m^{-2} s^{-1}. MGM was enriched while water very week and seedling was measured weight.
Relative growth rate (RGR) of the seaweed tissues was calculated by

using the formula of Matinfar *et al*. (2013) as follow

%RGR= [(Wf−Wi)/Wi]x100}/t
(Wf = final weight; Wi = initial weight; t = time (day))
Percentage of increased biomass was calculated by increased biomass (%) = $[(W_f-W_i)/W_i]$ x100. Similarly, the percentage of increased length was determined by increased length (%) = [(final length-initial length)/initial length] x100.

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2.1.5 Data analysis

Correlations between biological parameters: yield, agar, pigment and element and physical parameters: water depth, transparency, light intensity, temperature, sediment composition and chemical parameters: elements in water and in sediment were analyzed. The significant differenc was observed at 95%.

One-way ANOVA was used to compare the mean of *G. fisheri* tissue growth rate including weight and length growth in different conditions separately. The variance mean of tissue growth rate in each parameter was analyzed the significant difference ($p<0.05$) by using Tukey HSD[a].

One-way ANOVA was used to compare the mean of element accumulation in seaweed and environment surrounding. The variance mean of element concentration in seaweed, water and sediment was analyzed the significant difference ($p<0.05$) by using Tukey HSD[a].

2.2 Materials and Equipment

2.2.1 Materials

1) Seaweed tissues
2) Sodium nitrate ($NaNO_3$) (Analytical reagent, Ajax Finechem)
3) Ferrous sulfate heptahydrate ($FeSO_4.7H_2O$) (Laboratory reagent, Lobachemie)
4) Manganese (II) chloride ($MnCl_2.4H_2O$) (Analytical reagent, Ajax Finechem)
5) Ethylene Diamine Tetra Acetic Acid Disodium Salt ($Na_2EDTA.2H_2O$) (Laboratory reagent, Lobachemie)
6) Hydrogen peroxide (H_2O_2, 30 or 50%) (Laboratory reagent, Fisher Scientific)
7) Hydrochloric acid (HCl, 1M) (Baker analyzed reagent, Avantor)
8) Sodium chloride (NaCl) (Analytical reagent, Ajax Finechem)
9) Ammonium iron (II) sulfate hexahydrate ($(NH_2)_2Fe(SO_4)_2.6H_2O$, 0.5N)
10) Potassium dichromate ($K_2Cr_2O_7$, 1N) (Analytical reagent, Fisher Chemicals)
11) Sulfuric acid (H_2SO_4, concentrated) (Analytical reagent, Ajax Finechem)
12) Perchloric acid ($HClO_4$, 60%) (Analytical reagent, Ajax Finechem)
13) Nitric acid (HNO_3) (Laboratory reagent, Lobachemie)

2.2.2 Equipment

1) Grape paper, Paper-ruler, Laboratory knife, Dropper
2) Filter candles, Shakers, Sieves, Thermometer
3) Flasks (50, 250 and 500 mL) (Schott Duran, Germany)
4) Hand-held Refractometer (Atago, Japan)
5) Lux meter (Tenmars, Taiwan)
6) Air pump (Resun, AC-9908)
7) Modified Grund Medium (Mensi *et al.*, 2011)

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8) Electric balance (Denver, Thailand)
9) Volume metric (50, 100, 500 and 1,000 mL) (Witeg, Germany)
10) Cylinder (LabFocus, Thailand)
11) Beakers (100, 200, 500 and 1,000 mL) (Pyrex, Vista)
12) pH meter (Metrohm, Harisau Switzerland)
13) Spectrophotometer (Genesys, China)
14) Water bath (Edelstahl, Thailand)
15) HunterLab (UltraScan XE, Virginia)
16) Centrifuge (Hettich Universal, Tuttlingen)
17) Autoclave (Hiclave-HVE50, Thailand)
18) Vortex (Gene 2, USA)

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CHAPTER 3

RESULTS

3.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment.

3.1.1 Elements, heavy metals and other parameters in seaweed

The yield from Songkhla province showed the significant higher (<0.05) than those of the other provinces with 58 ton FW ha^{-1} $year^{-1}$ (Table 3.1). Besides, the pond in that province produced the cleaned seaweed which showed significant low contaminants (<0.05) comparing to those from the other two provinces, Pattani and Surat Thani. However, the agar yield from seaweed from this province showed lower agar yield comparing to those in two other provinces. The highest agar yield and lowest moisture content in seaweed were found at Surat Thani with 28.76 and 80.45%, respectively.

Table 3.1 Yield and biological characteristics of *G. fisheri* in Pattani, Songkhla and Surat Thani provinces

Parameters	Province		
	Pattani	Songkhla	Surat Thani
Yield (ton FW ha^{-1} $year^{-1}$)	20.25 ± 4.03^{a}	58.00 ± 6.98^{c}	38.00 ± 2.58^{b}
Total color difference	60.94 ± 4.22^{a}	59.61 ± 2.10^{a}	62.30 ± 1.98^{a}
Pigment (µg g^{-1} FW)			
Carotenoids	54.69 ± 41.19^{a}	56.97 ± 9.45^{a}	40.24 ± 16.91^{a}
Chl. a	131.63 ± 91.60^{a}	163.74 ± 11.28^{a}	180.37 ± 63.32^{a}
Chl. b	8.00 ± 10.37^{a}	6.77 ± 4.62^{a}	5.38 ± 1.40^{a}
Chl. c	12.13 ± 6.94^{a}	10.64 ± 6.69^{a}	14.82 ± 7.64^{a}
R-PE	34.67 ± 22.21^{a}	50.22 ± 14.69^{a}	58.32 ± 10.23^{a}
Agar (%)	23.49 ± 3.61^{ab}	20.02 ± 4.41^{a}	28.76 ± 4.78^{b}
Moisture (%)	83.75 ± 0.81^{b}	83.12 ± 0.40^{b}	80.45 ± 1.05^{a}
Contaminant (%)	13 ± 2^{b}	7 ± 2^{a}	14 ± 3^{b}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at p<0.05.

The carotenoids, Chlorophyll a (Chl. a) and r-phycoerythrin (R-PE) were in the ranges of 40.24-56.97, 131.63-180.37 and 34.67-58.32 µg g^{-1} FW, respectively. There was no significant difference on color and pigment content of the seaweed in three provinces.

For major elements, the ranges of Ca and Mg in seaweed were 9.22-10.01 and 11.40-13.40 mg g^{-1} DW (dry weight) (Figure 3.1A). There was no significant difference on Ca and Mg concentration in seaweed of three provinces. The concentrations of K and Na in seaweed at Pattani were 23.35 and 4.96 mg g^{-1} DW whereas they were 3.71 and 1.60 mg g^{-1} DW in seaweed at Songkhla and 1.85 and

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2.3 mg g^{-1} DW in seaweed at Surat Thani. The amount of K and Na in seaweed of Pattani province showed the significantly higher than those in the other two provinces (Table 3.2).

Trace element content in seaweed was not significantly different among three provinces. The concentration of Cu was in the range of 0.000-0.003 mg g^{-1} DW which showed lower than other trace elements. Mn, Zn and Fe amount in seaweed were in the ranges of 0.56-0.75, 0.03-0.04 and 0.17-0.84 mg g^{-1} DW, respectively (Figure 3.1B). There was no significant difference of Ni, Cr and Pb concentration in seaweed from three provinces. However, it showed that Cd accumulated in seaweed at Songhla was higher than those at the other two. The amount of Cd in seaweed at Pattani, Songkhla and Surat Thani provinces were 0.08, 0.13 and 0.10 µg g^{-1} DW, respectively (Figure 3.1C).

Table 3.2 Element concentrations in the seaweed collected from Pattani, Songkhla and Surat Thani

		Province		
		Pattani	Songkhla	Surat Thani
Major element (mg g^{-1} DW)	Ca	9.22±1.50^{a}	9.35±1.45^{a}	10.01±1.28^{a}
	Mg	11.59±1.26^{a}	11.40±1.41^{a}	13.40±0.41^{a}
	K	23.35±1.39^{b}	3.71±3.62^{a}	1.85±0.15^{a}
	Na	4.96±1.31^{b}	1.60±1.43^{a}	2.33±0.78^{a}
Trace element (mg g^{-1} DW)	Cu	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Mn	0.57±0.20^{a}	0.56±0.29^{a}	0.75±0.04^{a}
	Zn	0.03±0.00^{a}	0.04±0.01^{a}	0.04±0.01^{a}
	Fe	0.17±0.09^{a}	0.64±0.58^{a}	0.84±0.60^{a}
Heavy metal (µg g^{-1} DW)	Ni	6.73±1.68^{a}	6.03±1.03^{a}	8.26±2.42^{a}
	Cr	2.80±2.99^{a}	1.76±0.86^{a}	1.43±0.53^{a}
	Cd	0.08±0.01^{a}	0.13±0.02^{b}	0.10±0.01^{ab}
	Pb	5.33±3.09^{a}	4.79±1.75^{a}	6.60±3.43^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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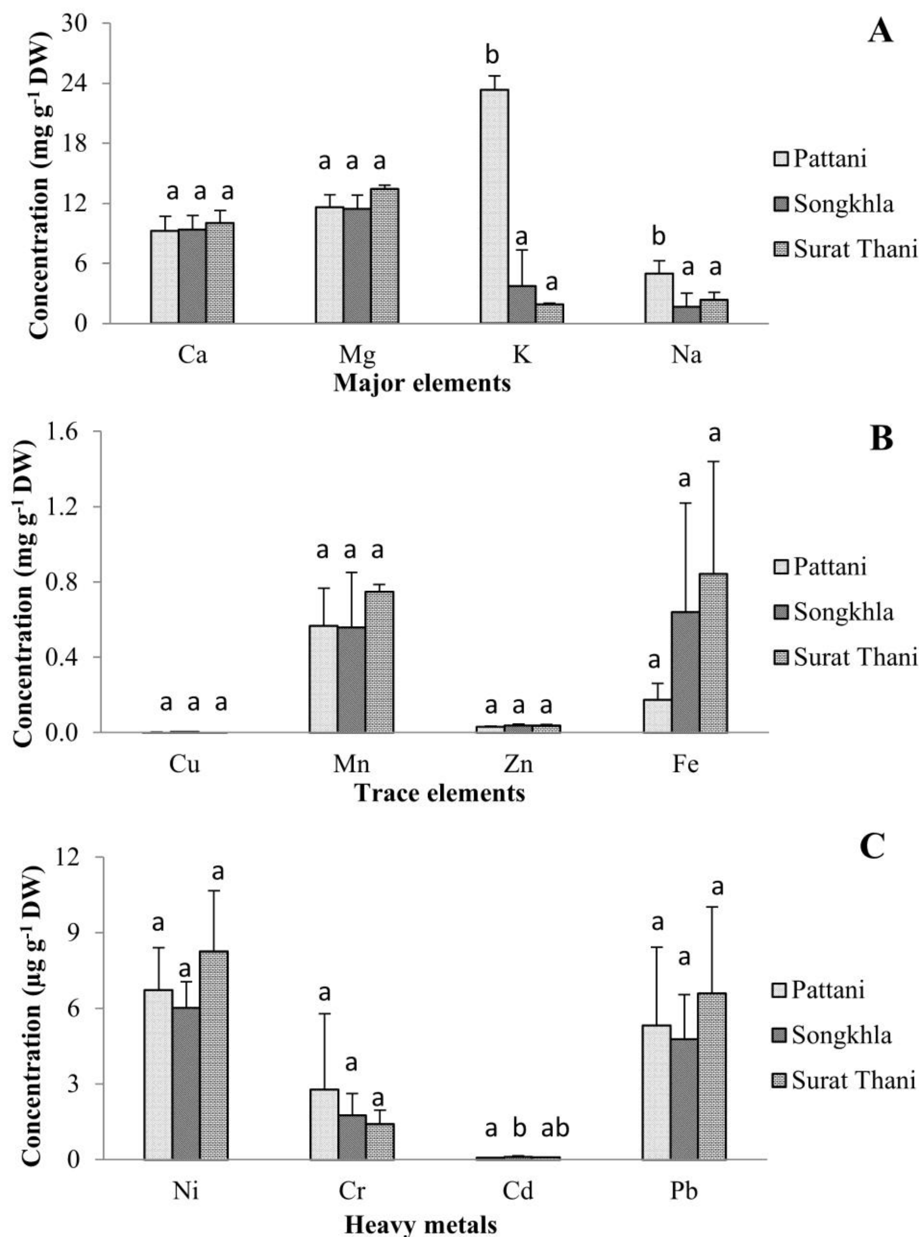

Figure 3.1 Element concentrations in *G. fisheri* collected from Pattani, Songkhla and Surat Thani provinces. (A) Major elements. (B) Trace elements. (C) Heavy metals.

3.1.2 Water parameters and elements in various habitat of *Gracilaria fisheri* from Pattani, Songkhla and Surat Thani Provinces.

There was no difference from physical characteristics, water depth, transparency and temperature and light intensity (Table 3.3). The average water depth for *G. fisheri* cultivation at provinces was in the range of 28.2-71.7 cm. Water temperature, transparency and light intensity were in the ranges of 30.1-32.7 ºC, 22.5-48.0 cm, and 534-666 µmol m^{-2} s^{-1}, respectively. Alkalinity and hardness

concentration and pH were in the ranges of 53-98, 1,938-3,370 mg L^{-1} and 7.23-7.43, respectively. However, it was found that salinity at Songkhla with 14.7 ppt and showed significantly effect ($p<0.05$) lower than those of other provinces. The concentration of phosphate-phosphorus and nitrate-nitrogen was found significantly higher ($p<0.05$) than those at the other two provinces. The amount phosphate-phosphorus and nitrate-nitrogen in water of Pattani were 0.06 and 0.29 mg L^{-1}, respectively.

Table 3.3 Water parameters from Pattani, Songkhla and Surat Thani provinces

	Province		
	Pattani	Songkhla	Surat Thani
Phosphate-phosphorus (mg L^{-1})	0.06 ± 0.04^{b}	0.01 ± 0.00^{a}	0.02 ± 0.01^{ab}
Nitrate-nitrogen (mg L^{-1})	0.29 ± 0.06^{b}	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}
Alkalinity (mg L^{-1})	81.75 ± 37.75^{a}	53.58 ± 13.04^{a}	97.83 ± 31.31^{a}
Hardness (mg L^{-1})	$2,905\pm677^{a}$	$1,938\pm1,692^{a}$	$3,370\pm1,074^{a}$
pH	7.43 ± 0.17^{a}	7.23 ± 0.34^{a}	7.38 ± 0.41^{a}
Salinity (ppt)	24.5 ± 2.1^{b}	14.7 ± 3.9^{a}	21.0 ± 2.0^{b}
Temperature (ºC)	31.7 ± 1.0^{a}	32.7 ± 2.1^{a}	30.1 ± 1.9^{a}
Depth (cm)	40.2 ± 17.3^{a}	71.7 ± 39.3^{a}	28.2 ± 11.7^{a}
Transparency (cm)	25.2 ± 13.6^{a}	48.0 ± 22.2^{a}	22.5 ± 8.6^{a}
Light (µmol m^{-2} s^{-1})	560 ± 145^{a}	534 ± 251^{a}	665 ± 463^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

The Ca, K and Na concentrations were in the ranges of 4971-5723, 1018-1261, 1408-1846 mg g^{-1} DW, respectively (Figure 3.2A). No significant difference ($p>0.05$) was found in the amount of Ca, K and Na in water at three provinces. However, Mg concentration in water at Pattani showed significantly lower ($p<0.05$) than those at the other two provinces with 625 mg g^{-1} DW (Table3.4).

There was no difference on the amount of trace elements, Cu, Mn and Zn in water at three provinces (Figure 3.2). However, Fe concentration in water at Surat Thani was significantly higher ($p<0.05$) than those at other two provinces with 0.73 mg g^{-1} DW. There was no significant difference ($p>0.05$) from the concentration of heavy metals, Ni, Cr, Cd and Pb, in water at all provinces. The ranges of Ni, Cr, Cd and Pb amount in water were 0.47-0.57, 0.10-0.12, 0.03-0.04 and 0.49-0.63 µg g^{-1} DW, respectively (Figure 3.2C).

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Table 3.4 Element concentrations in the water collected from Pattani, Songkhla and Surat Thani

Concentration (mg L^{-1})		Province		
		Pattani	Songkhla	Surat Thani
Major element	Ca	5268±1819[a]	4971±828[a]	5723±572[a]
	Mg	625±129[a]	808±8[b]	820±9[b]
	K	1208±216[a]	1018±288[a]	1261±248[a]
	Na	1846±663[a]	1814±192[a]	1408±880[a]
Trace element	Cu	0.06±0.02[a]	0.06±0.01[a]	0.06±0.01[a]
	Mn	0.10±0.05[a]	0.07±0.02[a]	0.28±0.21[a]
	Zn	0.06±0.02[a]	0.03±0.01[a]	0.04±0.01[a]
	Fe	0.18±0.09[a]	0.23±0.17[a]	0.73±0.35[b]
Heavy metal	Ni	0.48±0.19[a]	0.47±0.11[a]	0.57±0.16[a]
	Cr	0.10±0.04[a]	0.10±0.02[a]	0.12±0.03[a]
	Cd	0.04±0.01[a]	0.03±0.01[a]	0.04±0.02[a]
	Pb	0.53±0.26[a]	0.49±0.15[a]	0.63±0.20[a]

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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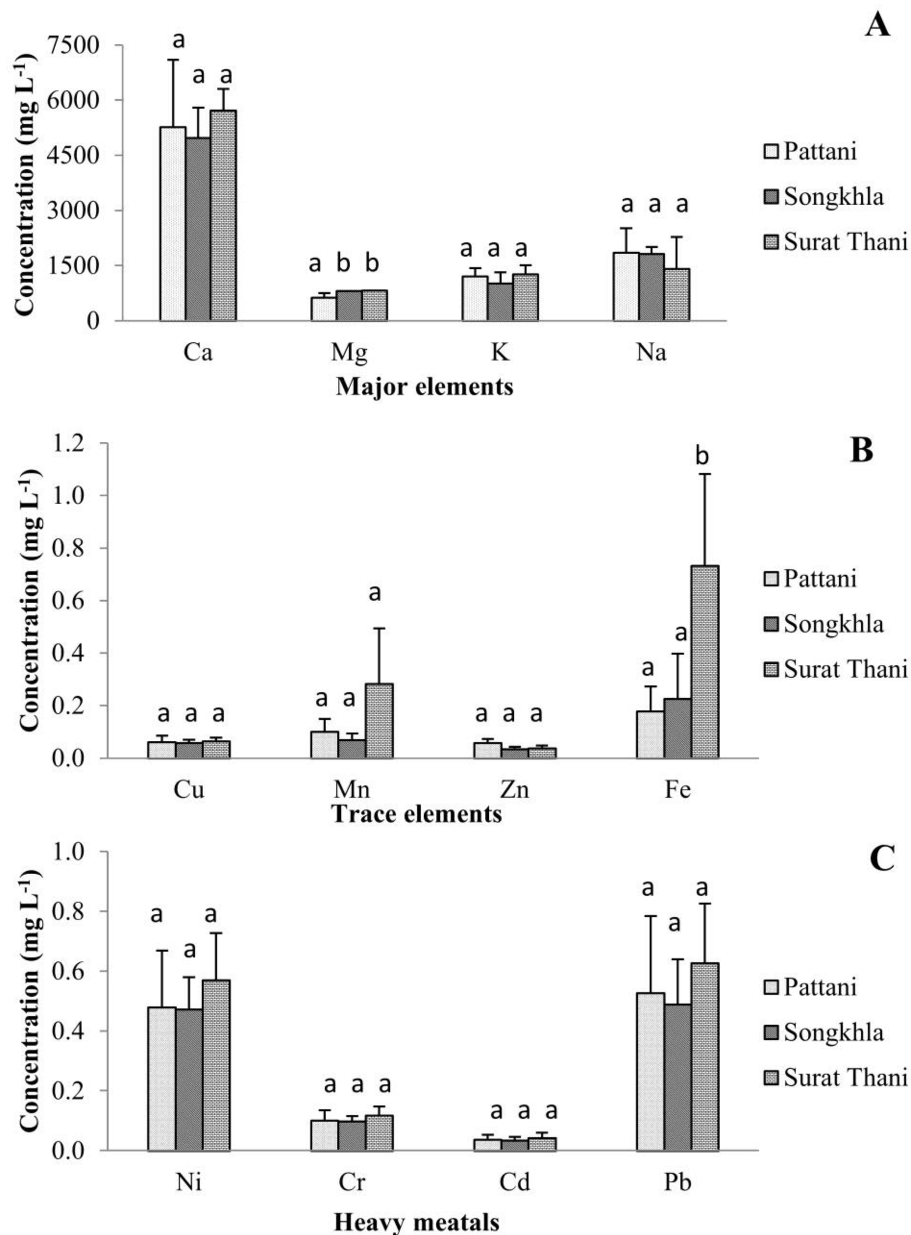

Figure 3.2 Element concentrations in the water collected from Pattani, Songkhla, Surat Thani Provinces. (A) Major elements. (B) Trace elements. (C) Heavy metals.

3.1.3 Sediment composition and characteristics from cultivated ponds

The sediment in Surat Thani province contained higher percentage of organic carbon and organic matter than the other two provinces which were 2.31 and 3.97%, respectively. Generally, there was no difference affact ($p>0.05$) of sediment texture among provinces. The sediment compositions of all ponds showed the highest percentage of silt (Table 3.5).

Table 3.5 Sediment parameters collected from Pattani, Songkhla and Surat Thani provinces

	Province		
Percentage (%)	Pattani	Songkhla	Surat Thani
Organic carbon	0.61 ± 0.17^{a}	1.63 ± 0.82^{ab}	2.31 ± 0.67^{b}
Organic matter	1.05 ± 0.29^{a}	2.81 ± 1.42^{ab}	3.97 ± 1.14^{b}
Sand	40.85 ± 45.50^{a}	2.42 ± 0.80^{a}	6.88 ± 12.32^{a}
Clay	1.66 ± 1.32^{a}	0.73 ± 0.13^{a}	0.77 ± 0.61^{a}
Silt	57.49 ± 44.49^{a}	96.85 ± 0.73^{a}	92.35 ± 12.78^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

The concentrations of Mg, K and Na in sediment of three provinces were not significantly different ($p>0.05$) (Figure 3.3A) and showed in the ranges of 134-162, 75-87 and 34-48 mg g^{-1} DW, respectively. The amount of Ca in sediment at Songkhla showed the significant higher ($p<0.05$) than those of the other two provinces with 278 mg g^{-1} DW (Table 3.6).

The concentrations of Zn and Fe in sediment from three provinces were not significantly different ($p>0.05$) which showed in the ranges of 0.84-0.99 and 11.19-12.45 mg g^{-1} DW, respectively (Figure 3.3B). However, there were significant differences ($p<0.05$) in Cu and Mn amount in from three provinces. The highest Cu in sediment was found at Surat Thani province with 0.21 mg g^{-1} DW while the highest Mn showed at Songkhla with 10.04 mg g^{-1} DW. The amount of heavy metal accumulation in sediment at three provinces showed no significant difference ($p>0.05$). The concentration of Ni, Cr, Cd and Pb were in the ranges of 212-298, 12.72-16.17, 0.99-1.23 and 24.29-34.03 μg g^{-1} DW (Figure 3.3C).

Table 3.6 Element concentrations in the sediment collected from Pattani, Songkhla and Surat Thani

		Province		
		Pattani	Songkhla	Surat Thani
Major element (mg g^{-1} DW)	Ca	82.1±53.5[a]	277.8±100.4[b]	130.7±52.0[a]
	Mg	133.6±41.9[a]	162.2±5.8[a]	152.6±21.2[a]
	K	75.5±64.7[a]	75.5±10.7[a]	86.9±20.6[a]
	Na	34.3±30.0[a]	38.2±7.5[a]	48.2±9.9[a]
Trace element (mg g^{-1} DW)	Cu	0.07±0.03[a]	0.11±0.11[ab]	0.21±0.04[b]
	Mn	5.46±2.89[a]	10.04±1.09[b]	9.45±1.88[ab]
	Zn	0.99±0.21[a]	0.95±0.18[a]	0.84±0.23[a]
	Fe	11.19±1.29[a]	12.42±0.20[a]	12.45±0.34[a]
Heavy metal (µg g^{-1} DW)	Ni	212.50±65.98[a]	297.25±24.09[a]	288.83±48.27[a]
	Cr	12.72±3.04[a]	16.17±2.09[a]	15.45±1.03[a]
	Cd	0.99±0.65[a]	1.23±0.07[a]	1.14±0.21[a]
	Pb	24.29±16.14[a]	34.03±6.99[a]	30.38±11.63[a]

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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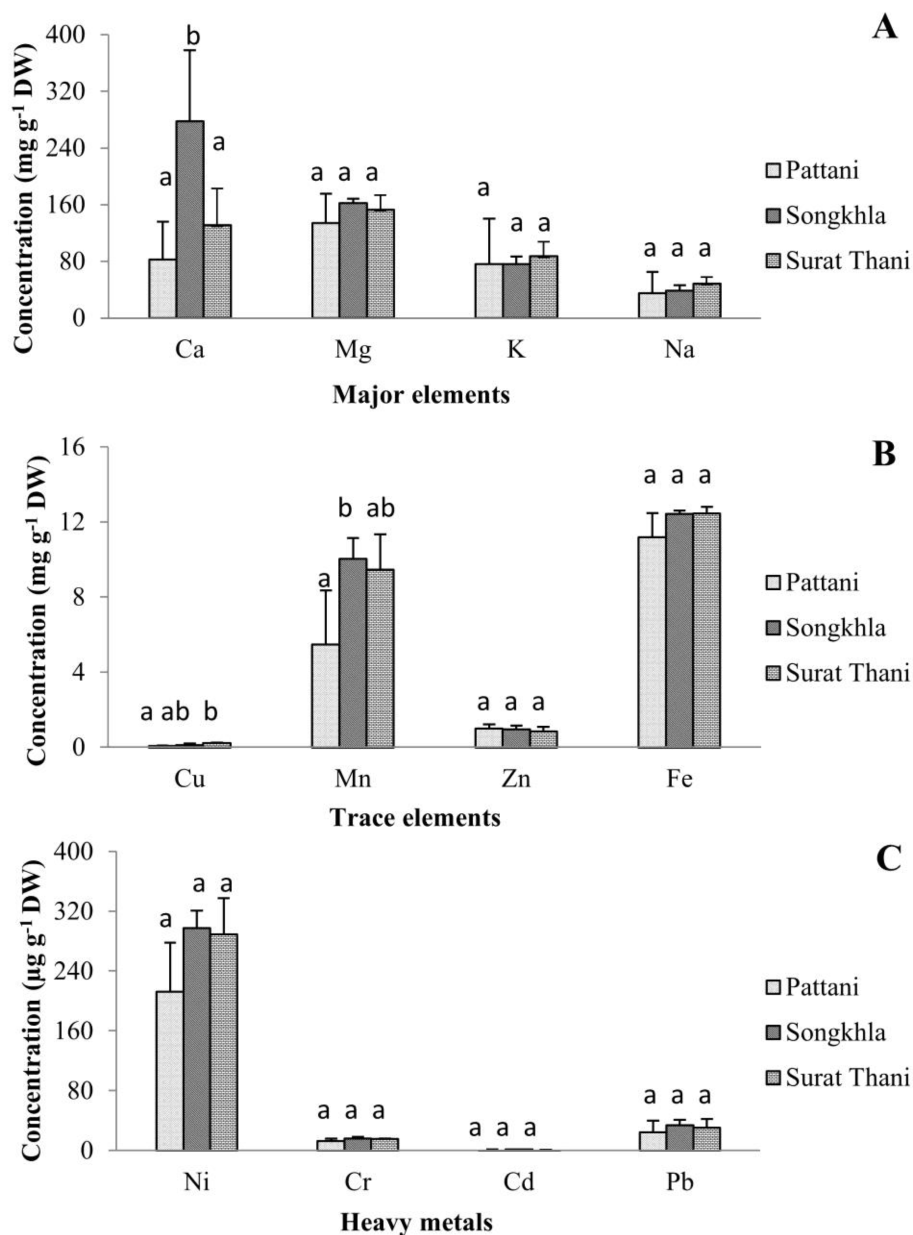

Figure 3.3 Element concentrations in sediment collected from Pattani, Songkhla, Surat Thani Provinces. (A) Major elements. (B) Trace elements. (C) Heavy metals.

3.1.4 Correlation analysis of biological characteristics of seaweed and physical, chemical parameters of environment.

The seaweed yield showed inverse relation to K, Na, Zn, phosphate-phosphorus and nitrate-nitrogen in water whereas, the yield showed positive relation to Mg, Mn, Cd in water and Ni and Cr of sediment. Agar provided positive realation to Mn in water Chla had positive relation to Mg in the water. The phosphate-phosphorus in water related positively with Chl.a concentration. The r-phycoerythrin showed positive relation to Ca, Ni and Cr in water, but showed negative relation to percentage of clay (Table 3.7).

Element concentration and metal accumulation in seaweed were also related positively to the water and sediment charateristics. For major elements, the concentration of K and Na in seaweed showed the positive relation with the concentration of Mg, Zn, nitrate-nitrogen and phosphate-phosphorus in water. Besides, salinity and Mn, Ni, Fe, organic carbon and organic matter, percentage of sand and silt in sediment also related positively to the amount of K in seaweed. However, Ca in seaweed showed no relation with other factors except clay oppositly. The Na in water and Cu in sediment had the positive correlation with Mg in seaweed.

Trace elements in seaweed were mainly dependent on some parameters in sediment and few parameters in water. The percentage of sand and silt of sediment and the water depth and transparency of water showed the positive raletion to Cu amount in seaweed. The amount of Cu and Cr in sediment and water hardness showed positive relation to the amount of Mn in *G. fisheri.* The amount Fe in seaweed showed positive relation to Cu and Mn in sediment.

Heavy metal accumulation in seaweed mainly depends on water charateristics. The concentration of Ni provided the positive ralation to the amount of Mn, alkalinity and hardness in water, but showed negative relation to the amount of Na in water. The accumulation of Cr in seaweed showed positive raletion to the amount of Mg and phosphate-phosphorus in water. Besides, it was found that salinity and nitrate-nitrogen in water inversly related to the accumulation of Cd in seaweed; and only Cr accumulated in sediment showed positive relation with Pb accumulation in seaweed.

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Table 3.7 Correlations for *G. fisheri* characteristics and its surrounding environment.

	Yield[1]	Ca[1]	Mg[1]	K[1]	Na[1]	Cu[1]	Mn[1]	Zn[1]	Fe[1]	Ni[1]	Cr[1]	Cd[1]	Pb[1]	Cato[1]	Chla[1]	Chlb[1]	Chlc[1]	R-PE[1]	Color[1]	Agar[1]	Mois.[1]	Epip.[1]
Yield[1]	1.00			**-0.74**	-0.66							**0.82**										**-0.75**
Ca[1]		1.00																				
Mg[1]			1.00				**0.77**			**0.77**											-0.61	
K[1]	**-0.74**			1.00	**0.85**							-0.61									0.64	
Na[1]	-0.66			**0.85**	1.00							-0.62			-0.61							
Cu[1]						1.00																
Mn[1]			**0.77**				1.00			0.70			0.65									
Zn[1]								1.00								-0.64						
Fe[1]									1.00													
Ni[1]			**0.77**				0.70			1.00												
Cr[1]											1.00											
Cd[1]	**0.82**			-0.61	-0.62							1.00										**-0.76**
Pb[1]							0.65						1.00	**-0.59**								
Caro[1]													-0.59	1.00	**0.75**	**0.78**			-0.61			
Chla[1]					-0.61									**0.75**	1.00							
Chlb[1]								-0.64						**0.78**		1.00	0.60		**-0.71**			
Chla[1]																0.60	1.00					
R-PE[1]																		1.00				
Color[1]														-0.61		**-0.71**			1.00			
Agar[1]																				1.00	-0.66	
Mois[1]			-0.61	0.64																-0.66	1.00	
Epip[1]	**-0.75**											**-0.76**										
Ca[2]																		0.60				
Mg[2]	0.61			**-0.83**	**-0.83**						-0.61				0.65						-0.64	
K[2]																						0.63
Na[2]			-0.64							**-0.87**												
Cu[2]																						
Mn[2]										0.63										0.71	**-0.76**	
Zn[2]	-0.59			0.66	0.58																	
Fe[2]																						
Ni[2]																		0.58				

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	Yield[1]	Ca[1]	Mg[1]	K[1]	Na[1]	Cu[1]	Mn[1]	Zn[1]	Fe[1]	Ni[1]	Cr[1]	Cd[1]	Pb[1]	Cato[1]	Chla[1]	Chlb[1]	Chlc[1]	R-PE[1]	Color[1]	Agar[1]	Mois.[1]	Epip.[1]
Cr[2]																		0.60				
Cd[2]																						
Pb[2]																						
Phos.[2]	-0.61			**0.73**	**0.87**						**0.73**				-0.70							
Nitr.[2]	**-0.80**			**0.96**	**0.82**							-0.61									0.60	
Alka.[2]										0.66												
Hard.[2]							0.58			0.70												
pH[2]																						
Salin.[2]	**-0.88**			0.62	0.62							**-0.82**										0.66
Temp.[2]																						
Depth[2]						**0.87**																
Trans.[2]						**0.79**													-0.58			
Light[2]										**0.76**												
Ca[2]	**0.80**																					
Mg[2]																						
K[2]																						
Na[2]																						
Cu[2]			**0.80**				0.60		0.67												-0.65	
Mn[2]	0.60			**-0.77**	-0.69				0.60													
Zn[2]																						
Fe[2]				-0.67																		
Ni[2]	0.61			-0.64																		
Cr[2]	0.60						0.63						0.62									
Cd[2]																						
Pb[2]																						
OC[2]				**-0.78**																		
OM[2]				**-0.78**																		
Sand[2]				0.61																		
Clay[2]		-0.59																**-0.72**				
Silt[2]				-0.63		0.16																

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	Ca^2	Mg^2	K^2	Na^2	Cu^2	Mn^2	Zn^2	Fe^2	Ni^2	Cr^2	Cd^2	Pb^2	$Phos.^2$	$Nitr.^2$	$Alka.^2$	$Hard.^2$
Ca^2	1.00		**0.72**		**0.91**				**0.90**	**0.90**	**0.81**	**0.91**				
Mg^2	0.15	1.00					-0.65						**-0.91**	**-0.90**		
K^2	**0.72**		1.00		0.62				0.64	0.62	0.62	0.67				
Na^2				1.00											-0.67	
Cu^2	**0.91**		0.62		1.00				**0.96**	**0.93**	**0.90**	**0.97**				
Mn^2						1.00				0.60		0.58				
Zn^2		-0.65					1.00						0.65	0.63		
Fe^2								1.00								
Ni^2	**0.90**		0.64		**0.96**				1.00	**0.99**	**0.95**	**0.99**				
Cr^2	**0.90**		0.62		**0.93**	0.60			**0.99**	1.00	**0.93**	**0.98**				
Cd^2	**0.81**		0.62		**0.90**				**0.95**	**0.93**	1.00	**0.95**				
Pb^2	**0.91**	0.25	0.67		**0.97**	0.58			**0.99**	**0.98**	**0.95**	1.00				0.60
$Phos.^2$		**-0.91**					0.65						1.00	**0.79**		
$Nitr.^2$		**-0.90**					0.63						**0.79**	1.00		
$Alka.^2$				-0.67											1.00	
$Hard.^2$												0.60				1.00
pH^2																**0.71**
$Salin.^2$							0.67							0.59		0.64
$Temp.^2$								**-0.75**								
$Depth^2$																
$Trans.^2$																
$Light^2$				-0.72		0.64										0.68
Ca^2														-0.59		
Mg^2	-0.66				-0.63											
K^2	**-0.78**				-0.69							-0.60				
Na^2	**-0.71**				**-0.72**							-0.60			0.60	
Cu^2								0.62								
Mn^2		**0.77**												**-0.83**		
Zn^2																
Fe^2		**0.77**												**-0.79**		
Ni^2		0.62												**-0.74**		
Cr^2																
Cd^2	**-0.74**				-0.58											
Pb^2	**-0.72**				-0.69				-0.59			-0.62				
OC^2		0.59					**-0.77**	0.67						-0.68		
OM^2		0.59					**-0.77**	0.68						-0.68		
$Sand^2$		-0.67												**0.72**		
$Clay^2$							**0.84**						0.59			
$Silt^2$		0.69												**-0.74**		

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	pH[2]	Salin.[2]	Temp.[2]	Depth[2]	Trans.[2]	Light[2]	Ca[3]	Mg[3]	K[3]	Na[3]	Cu[3]	Mn[3]	Zn[3]	Fe[3]	Ni[3]	Cr[3]	Cd[3]	Pb[3]	OC[3]	OM[3]	Sand[3]	Clay[3]	Silt[3]
pH[2]	1.00	0.61																					
Salin.[2]	0.61	1.00					**-0.78**																
Temp.[2]			1.00				0.64				-0.61												
Depth[2]				1.00	0.96																		
Trans.[2]				**0.96**	1.00																		
Light[2]						1.00																	
Ca[3]		**-0.78**	0.64				1.00																
Mg[3]								1.00	**0.85**	**0.78**		**0.74**		**0.87**	**0.83**		**0.86**	**0.92**			**-0.94**		**0.94**
K[3]								**0.85**	1.00	**0.87**				0.70	0.66		**0.89**	**0.84**			**-0.79**		**0.77**
Na[3]								**0.78**	**0.87**	1.00				0.59	0.59		0.70	0.69			-0.70		0.69
Cu[3]			-0.61								1.00												
Mn[3]								**0.74**				1.00		**0.86**	**0.76**		0.58	0.62		0.58	**-0.83**		**0.84**
Zn[3]													1.00										
Fe[3]								**0.87**	0.70	0.59		**0.86**		1.00	**0.91**		**0.84**	**0.81**			**-0.98**		**0.98**
Ni[3]								**0.83**	0.66	0.59		**0.76**		**0.91**	1.00	0.68	**0.84**	**0.88**			**-0.89**		**0.90**
Cr[3]															0.68	1.00							
Cd[3]								**0.86**	**0.89**	0.70		0.58		**0.84**	**0.84**		1.00	**0.88**			**-0.88**		**0.87**
Pb[3]								**0.92**	**0.84**	0.69		0.62		**0.81**	**0.88**		**0.88**	1.00			**-0.85**		**0.84**
OC[3]																			1.00	**1.00**			
OM[3]												0.58							**1.00**	1.00			
Sand[3]								**-0.94**	**-0.79**	-0.70		**-0.83**		**-0.98**	**-0.89**		**-0.88**	**-0.85**			1.00		**-1.00**
Clay[3]																						1.00	
Silt[3]								**0.94**	**0.77**	0.69		**0.84**		**0.98**	**0.90**		**0.87**	**0.84**			**-1.00**		1.00

Superscript numbers mean: (1) in seaweed, (2) in water and (3) in sediment. Bold letters mean correlation significance at 0.01. Non-bold letters mean correlation significance at 0.05. Some abbreviated words mean Caro=carotenoids; Mois.=moisture; epip.=epiphytes; Salin.=s alinity; temp.=temperature.

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3.2 Tissue culture in different conditions

Percentage of biomass increase, growth rate, number and length of new branch of *G. fisheri* tissue showed the improvement after each successful experiment. Each factor has particular effect on *G. fisheri* tissue. Some salinity levels showed an affect on pigment loss and growth of the tissue. The growing part of thallus determined the growth polarity whereas propagule densities regulated branchlet which produced from new branch. The detail of growth and branch formation characteristics are described in the following.

3.2.1 Tissue culture under different initial lengths

The lengths of tissues had no significant effect ($p>0.05$) on the growth and branch formation of *Gracilaria fisheri* tissues (Figure 3.4). The tissues with 1 and 2 cm of initial lengths enhanced biomass with 423.3 and 480.0% (or 4.2 and 4.8 times increase comparing to the initial biomass), respectively which was significantly higher ($p<0.05$) than those at 4 and 5 cm of initial lengths. The RGR of tissues at 1 and 2 cm of initial length of the starter were 10.6 and 11.0% day^{-1}, respectively (Figure 3.6A). The increased length of tissue at 4 and 5 cm initial lengths were significantly lower ($p<0.05$) than those of the tissue in other treatments. The maximum increased length of tissues was observed at 1 cm initial length tissues with 19.3 % (Table 3.8). The tissues at 1 and 2 cm initial lengths produced 4 and 3 new branches per cm, respectively (Figure 3.6B). The maximum branch lengths were found at 4 cm of fragment length with 2.0 cm. At 40 days, the final weight of tissues in all treatments was not significantly different ($p>0.05$). The highest biomass of tissues was found at 2 cm of the length with 10.8 ± 1.3 g L^{-1} (Table 3.8). The tissues in all treatments grew with non-polarity and produced branchlets without pigment loss.

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Table 3.8 Growth rate and branch formation of *Gracilaria fisheri* tissues at different initial lengths of thallus

	Length (cm)	Days			
		10	20	30	40
Increased biomass (%)	1	30.0±12.5^{a}	57.0±22.4^{a}	164.7±36.3^{a}	423.3±48.7^{a}
	2	34.6±6.0^{a}	58.0±11.4^{a}	178.0±45.2^{a}	440.7±63.0^{a}
	3	23.3±5.3^{a}	64.8±4.2^{a}	195.3±26.6^{a}	322.0±75.6^{a}
	4	23.7±11.9^{a}	70.8±10.6^{a}	208.7±59.3^{a}	294.7±68.9^{a}
	5	24.9±11.9^{a}	76.4±21.6^{a}	188.0±22.7^{a}	294.0±62.6^{a}
RGR (% day^{-1})	1	3.0±1.2^{a}	2.8±1.1^{a}	5.5±1.2^{a}	10.6±1.2^{a}
	2	3.5±0.6^{a}	2.9±0.6^{a}	5.9±1.5^{a}	11.0±1.6^{a}
	3	2.3±0.5^{a}	3.2±0.2^{a}	6.5±0.9^{a}	8.0±1.9^{a}
	4	2.4±1.2^{a}	3.5±0.5^{a}	7.0±2.0^{a}	7.4±1.7^{a}
	5	2.5±1.2^{a}	3.8±1.1^{a}	6.3±0.8^{a}	7.3±1.6^{a}
Number of new branch per cm	1	2.0±1.0^{a}	3.0±0.0^{b}	3.0±1.0^{b}	4.0±0.0^{b}
	2	1.0±0.0^{a}	2.0±0.0^{ab}	2.0±1.0^{ab}	3.0±0.0^{b}
	3	1.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}
	4	1.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{ab}	3.0±0.0^{a}
	5	1.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}	2.0±0.0^{a}
Branch length (cm)	1	0.1±0.0^{ab}	0.4±0.0^{ab}	1.1±0.0^{b}	2.0±0.3^{b}
	2	0.1±0.0^{ab}	0.4±0.1^{ab}	0.8±0.1^{a}	1.6±0.1^{ab}
	3	0.1±0.0^{a}	0.4±0.0^{b}	1.0±0.1^{ab}	1.9±0.1^{ab}
	4	0.1±0.0^{b}	0.4±0.0^{b}	1.0±0.0^{b}	2.0±0.3^{b}
	5	0.1±0.0^{ab}	0.3±0.0^{a}	0.8±0.0^{a}	1.3±0.2^{a}
Biomass (g L-1)	1	2.6±0.2^{a}	3.1±0.4^{a}	5.3±0.7^{a}	10.5±1.0^{a}
	2	2.7±0.1^{a}	3.2±0.2^{a}	5.6±0.9^{a}	10.8±1.3^{a}
	3	2.5±0.1^{a}	3.3±0.1^{a}	5.9±0.5^{a}	8.4±1.5^{a}
	4	2.5±0.2^{a}	3.4±0.2^{a}	6.2±1.2^{a}	7.9±1.4^{a}
	5	2.5±0.2^{a}	3.5±0.4^{a}	5.8±0.5^{a}	7.9±1.3^{a}
Increased length (%)	1	10.3±2.1^{c}	19.0±2.0^{c}	13.7±2..5^{d}	19.3±5.9^{c}
	2	6.2±0.3^{b}	10.3±6.6^{b}	11.3±3.8^{cd}	9.3±4.3^{b}
	3	3.2±1.0^{a}	6.6±3.0^{ab}	7.0±3.6^{bc}	9.0±1.7^{b}
	4	3.0±0.2^{a}	3.2±1.4^{a}	3.6±1.5^{ab}	3.2±1.3^{ab}
	5	1.4±0.2^{a}	2.3±0.3^{a}	1.5±1.1^{a}	1.9±1.2^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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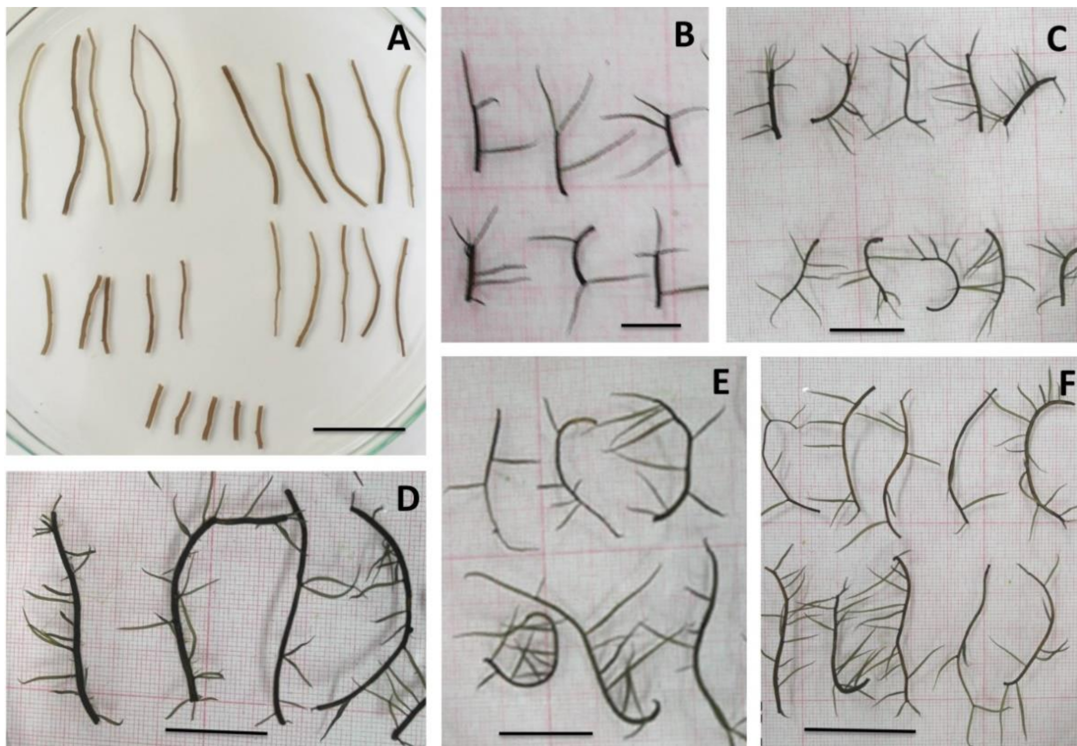

Figure 3.4 *G. fisheri* tissues cultured under different length experiment at 20 ppt salinity, 2 g L^{-1} density, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature. (A) Initial fragments. (B) Tissues at 1 cm initial length. (C) Tissue at 2 cm initial length. (D) Tissues at 3 cm initial length. (E) Tissues at 4 cm initial length. (F) Tissues at 5 cm initial length.

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3.2.2 Tissue culture under different salinity levels

The salinity level played an important role in *G. fisheri* tissue growth that might limit tissue growth or cause to lethal tissues. Excised tissues at 15, 30 and 35ppt started bleaching that caused to the slow growth after 10 days culture. Meanwhile the tissues at 20 and 25 ppt grew well without bleaching. There was a significant difference ($p<0.05$) in tissue biomass at 35 ppt comparing to the biomass of the other salinities (Figure 3.5). The maximum biomass increase of the tissue was observed at 20 ppt with 504.1% (or 5.0 times increase in biomass). The RGR of the tissues at 35 ppt was significant lower ($p<0.05$) comparing to those at the other salinities (Figure 3.6C). The excised tissues got the highest and lowest RGR at 20 ppt and 35 ppt with 12.6 and 3.4 % day^{-1}, respectively. There was no significant difference ($p>0.05$) on number of branches among the treatments (Figure 3.6D). The increased length of the tissues at 30 and 35 ppt were significantly lower ($p<0.05$) than in other salinity levels. Moreover, it presented that at 15 and 35 ppt, tissue showed the lowest branch length with 1.1 and 1.3 cm, respectively. Under different salinity levels, the final biomass of the tissues at 15, 20, 25 ppt showed the significant difference ($p<0.05$) with those at 30 and 35 ppt. The highest biomass of tissues was recorded at 20 with 12.1±1.6 g L^{-1} (Table 3.9). However, all tissues in the treatments produced branchlets and grew with non-polarity (Table 3.12).

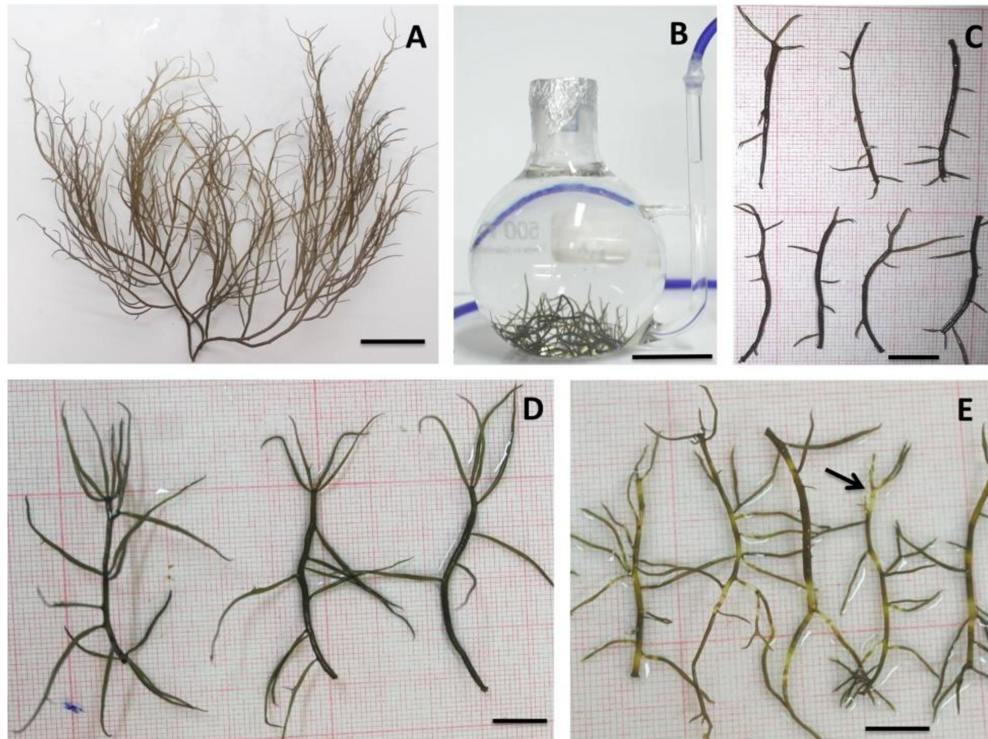

Figure 3.5 *G. fisheri* tissues cultured at different salinity level experiment at 2 g L^{-1} density, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25°C temperature. (A) Mother plant. (B) Culture flask. (C) Tissues at 20 ppt after 20 days. (D) Tissues at 20 ppt after 40 days. (E) Tissues at 35 ppt after 40 days. Scale bar = 2 cm (A, B), 1 cm (B, C, D).

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Table 3.9 Growth rate and branch formation of *Gracilaria fisheri* tissues at different salinity levels

	Salinity (ppt)	Days			
		10	20	30	40
Increased biomass (%)	15	64.4±3.6^{a}	132.9±28.8^{ab}	208.3±38.0^{ab}	397.6±52.3^{b}
	20	108.8±31.6^{a}	168.6±13.3^{b}	261.5±41.5^{b}	504.1±78.5^{b}
	25	83.4±9.5^{a}	149.8±24.5^{b}	304.7±75.3^{b}	447.0±64.7^{b}
	30	100.3±61.4^{a}	136.7±49.6^{ab}	198.1±41.0^{ab}	395.0±69.9^{b}
	35	58.8±12.2^{a}	55.9±30.4^{a}	90.9±28.5^{a}	137.7±21.5^{a}
RGR (% day^{-1})	15	6.4±0.4^{a}	6.6±1.4^{ab}	6.9±1.3^{ab}	9.9±1.3^{b}
	20	10.9±3.2^{a}	8.4±0.7^{b}	8.7±1.4^{b}	12.6±2.0^{b}
	25	8.3±0.9^{a}	7.5±1.2^{b}	10.2±2.5^{b}	11.2±1.6^{b}
	30	10.0±6.1^{a}	6.8±2.5^{ab}	6.6±1.4^{ab}	9.9±1.7^{b}
	35	5.9±1.2^{a}	2.8±1.5^{a}	3.0±0.9^{a}	3.4±0.5^{a}
Number of new branch per cm	15	1.0±0.0^{ab}	2.0±0.0^{a}	2.0±0.0^{ab}	2.0±0.0^{a}
	20	1.0±0.0^{c}	2.0±0.0^{ab}	2.0±0.0^{a}	2.0±0.0^{a}
	25	1.0±0.0^{bc}	2.0±0.0^{ab}	3.0±0.0^{ab}	2.0±0.0^{a}
	30	1.0±0.0^{abc}	3.0±1.0^{ab}	3.0±0.0^{ab}	3.0±0.0^{ab}
	35	0.0±0.0^{a}	3.0±1.0^{b}	3.0±0.0^{b}	3.0±0.0^{b}
Branch length (cm)	15	0.2±0.1^{a}	0.3±0.1^{a}	0.7±0.1^{a}	1.1±0.1^{a}
	20	0.3±0.0^{a}	0.6±0.0^{b}	1.1±0.0^{b}	1.6±0.2^{bc}
	25	0.2±0.0^{a}	0.5±0.1^{b}	1.1±0.0^{b}	1.8±0.2^{c}
	30	0.2±0.0^{a}	0.5±0.0^{ab}	1.1±0.1^{b}	1.7±0.1^{c}
	35	0.3±0.1^{a}	0.4±0.1^{ab}	0.8±0.1^{a}	1.3±0.1^{ab}
Biomass (g L^{-1})	15	3.3±0.1^{a}	4.7±0.6^{b}	6.2±0.8^{b}	10.0±1.0^{b}
	20	4.2±0.6^{a}	5.4±0.3^{b}	7.2±0.8^{bc}	12.1±1.6^{b}
	25	3.7±0.2^{a}	5.0±0.5^{b}	8.1±1.5^{c}	10.9±1.3^{b}
	30	4.0±1.2^{a}	4.7±1.0^{b}	6.0±0.8^{b}	9.9±1.4^{b}
	35	3.2±0.2^{a}	3.1±0.6^{a}	3.8±0.6^{a}	4.8±0.4^{a}
Increased length (%)	15	15.6±1.6^{c}	28.8±1.6^{b}	31.2±5.6^{c}	32.0±2.7^{b}
	20	13.6±2.2^{c}	18.0±2.0^{a}	19.7±3.1^{b}	26.4±5.6^{b}
	25	13.7±1.2^{c}	18.7±2.9^{a}	17.1±5.1^{b}	29.8±5.2^{b}
	30	7.3±0.7^{b}	19.1±7.3^{a}	13.8±2.5^{b}	15.7±5.8^{a}
	35	4.0±1.2^{a}	13.0±2.3^{a}	11.3±3.8^{a}	12.6±5.3^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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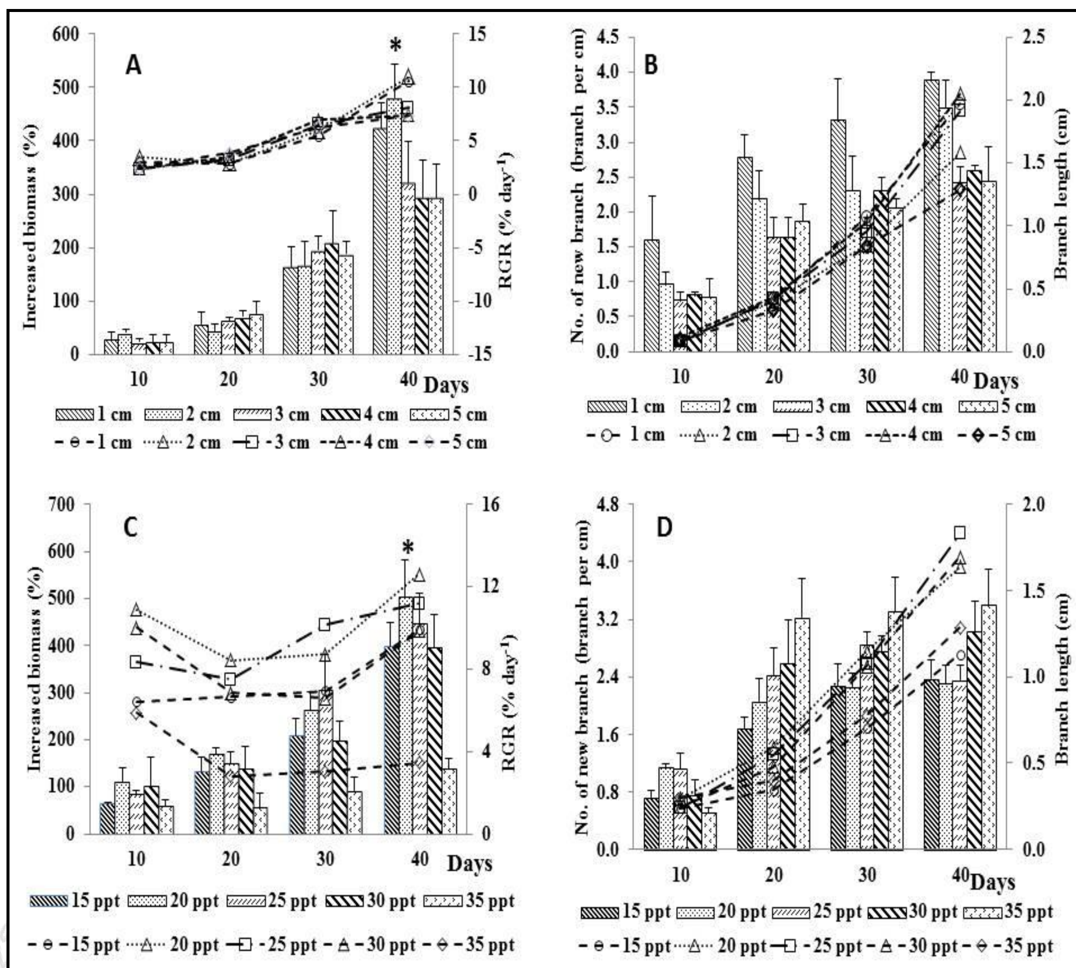

Figure 3.6 Growth rate and number of new branch (bar graphs) and RGR and branch length (line graphs) of *G. fisheri* tissues cultured at 2 g L^{-1} density, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under (A,B) different initial lengths. (C,D) different salinity levels. (*) is selected condition.

3.2.3 Tissue culture under different parts of thallus

Different parts of thallus (Figure 3.7) might strongly regulate growth and branch formation characteristics of *G. fisheri* tissues. After 40 days, tissues from apical zone showed the significant higher ($p<0.05$) biomass than those of the other two parts. The maximum increased biomass was observed in apical fragment with 506.7% (or 5.0 times enhance in biomass). Consequently, apical segments produced the highest RGR with 12.7 % day^{-1} which started extending upper to the tip in 40 days (Figure 3.9A). Hence, this caused to the significantly higher ($p<0.05$) increase in length than those of sub-apical and basal parts. The maximum increased length of tissues was found at apical part with 119.2% (1.2 times comparing to the initial length) (Table 3.10). In contrast, apical fragment produced the lowest number of branches with 2 branches per cm and branch length with 1 cm (Figure 3.9B). Basal segments produced the highest number of new branch with 4 branches per cm. The

branch length from apical segments got significantly lower ($p<0.05$) than those of other segment parts. The final biomass of apical tissues was significantly higher ($p<0.05$) than those of the other two zones. The highest biomass was found at apical fragment with 12.1±1.1 g L^{-1} (Table 3.10). It is clear to see that apical tissues grew with apicobasal polarity while sub-apical and basal tissues grew with non-polarity (Table 3.12).

Table 3.10 Growth rate and branch formation of *Gracilaria fisheri* tissues from different parts of thallus

	Part of thallus	Days			
		10	20	30	40
Increased biomass (%)	Apical	49.7±16.4^{a}	160.5±27.8^{b}	310.0±41.6^{a}	506.7±53.7^{b}
	Sub-apical	29.6±8.9^{a}	90.3±12.5^{ab}	230.7±33.1^{a}	396.7±34.1^{ab}
	Basal	57.8±11.4^{a}	116.9±33.6^{a}	275.3±76.6^{a}	390.0±42.0^{a}
RGR (% day^{-1})	Apical	5.0±1.6^{a}	8.0±1.4^{b}	10.3±1.4^{a}	12.7±1.3^{b}
	Sub-apical	3.0±0.9^{a}	4.5±0.6^{ab}	7.7±1.1^{a}	9.9±0.8^{ab}
	Basal	5.8±1.1^{a}	5.8±1.7^{a}	9.2±2.5^{a}	9.7±1.0^{a}
Number of new branch per cm	Apical	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	2.0±0.0^{a}
	Sub-apical	1.0±0.0^{a}	2.0±0.0^{ab}	2.0±0.0^{ab}	3.0±0.0^{ab}
	Basal	1.0±0.0^{a}	3.0±0.0^{b}	3.0±0.0^{b}	4.0±0.0^{b}
Branch length (cm)	Apical	0.1±0.0^{a}	0.3±0.0^{a}	0.5±0.1^{a}	1.0±0.2^{a}
	Sub-apical	0.1±0.0^{a}	0.4±0.0^{b}	1.1±0.1^{b}	1.9±0.3^{b}
	Basal	0.1±0.0^{a}	0.5±0.0^{b}	1.2±0.1^{b}	1.7±0.1^{b}
Biomass (g L^{-1})	Apical	3.0±0.3^{a}	5.2±0.6^{b}	8.2±0.8^{a}	12.1±1.1^{b}
	Sub-apical	2.6±0.2^{a}	3.8±0.3^{a}	6.6±0.7^{a}	9.9±0.7^{a}
	Basal	3.2±0.2^{a}	4.3±0.7^{ab}	7.5±1.5^{a}	9.8±0.8^{a}
Increased length (%)	Apical	34.0±3.9^{b}	66.3±18.8^{b}	119.3±13.0^{b}	119.2±30.6^{b}
	Sub-apical	10.9±3.6^{a}	6.3±3.3^{a}	15.0±1.8^{a}	5.3±4.4^{a}
	Basal	11.8±0.3^{a}	6.8±5.3^{a}	17.8±1.9^{a}	10.3±3.3^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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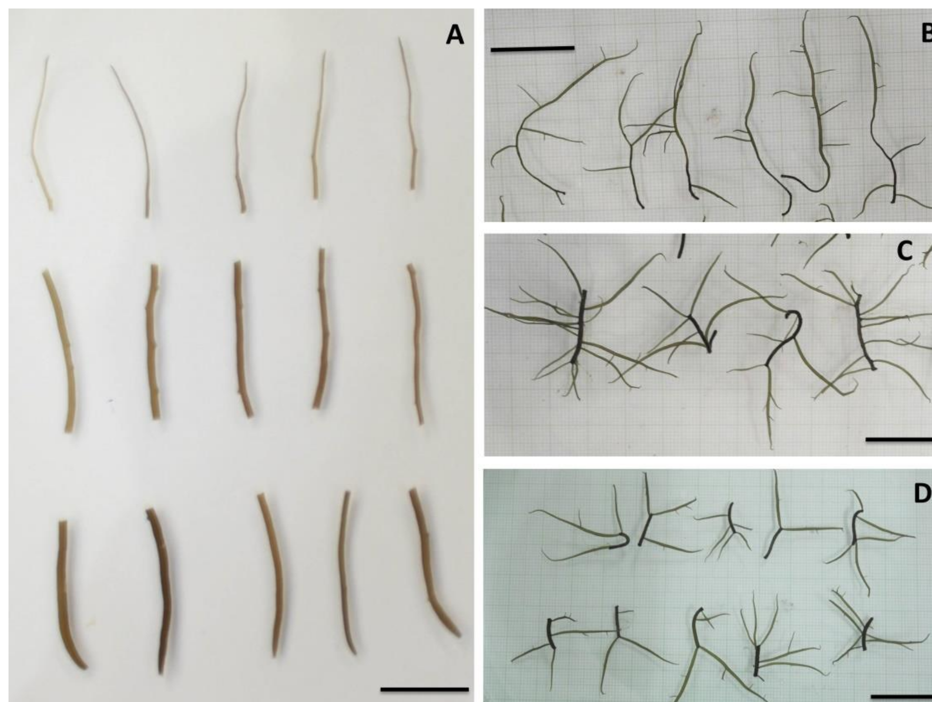

Figure 3.7 *G. fisheri* tissue culture from different part of thallus experiment at 20 ppt salinity, 2 g L^{-1} density, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature. (A) Initial fragment. (B) Apical segments after 40 days. (C) Sub-apical segments after 40 days. (D) Basal segments after 40 days. Scale bar =1cm (A), 2cm (B, C, D).

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3.2.4 Tissue culture under different propagule densities

Tissues under different propagule density grew variously (Figure 3.8). The lower propagule density produced higher increased biomass and RGR (Figure 3.9C). The increased biomass of tissues at 1 and 2 g L^{-1} were significantly higher ($p<0.05$) than those at 4, 6 and 8 g L^{-1} In this study, the maximum increased biomass was at 1 g L^{-1} with 1,238.7% (or 12.4 times increase in biomass). Tissues got the highest RGR with 31.0 % day^{-1} at 1 g L^{-1}. The lowest RGR of tissues was found at 8 g L^{-1} with 6.4 % day^{-1}. The increased length of tissue at 1 g L^{-1} was significantly higher ($p<0.05$) than that at other densities with 216.7% (or 2.2 times enhance in length) (Table 3.5). Number of branch at 1 and 2g L^{-1} density were significantly higher ($p<0.05$) than those at 4, 6 and 8 g L^{-1} density. Tissues at lowest density in the experiment (1 g L^{-1}) produced the highest number of new branch with 7 branches per cm (Figure 3.9D). It was obviously seen that the growth of new branch length dropped down after 30 days because of the higher density in the flask. After 40 days, branch length of tissues at 1, 2, 4 g L^{-1} of the density showed significant higher ($p<0.05$) than those at 8 and 6 g·L^{-1} of the density. The final weight at 1, 2, 4, 6 and 8 of the density were 13.4±2.2, 16.9±1.0, 22.1±1.9, 24.3±1.2 and 28.4±1.4 g L^{-1}, respectively (Table 3.11). All treatments grew with apicobasal polarity and no pigment loss occurrence. However, the branchlets were only produced under 1 and 2 g L^{-1} propagule densities (Table 3.12).

Table 3.11 Growth rate and branch formation of *Gracilaria fisheri* tissues at different propagule densities

	Density ($g\ L^{-1}$)	Days			
		10	20	30	40
Increased biomass (%)	1	173.3±37.0^{b}	449.3±89.5^{c}	862.7±130.9^{c}	1238.7±223.1^{c}
	2	119.3±31.8^{ab}	287.3±22.7^{b}	500.7±45.0^{b}	744.7±52.3^{b}
	4	84.3±23.0^{a}	162.3±12.7^{a}	274.0±32.9^{a}	452.0±46.5^{a}
	6	60.9±10.0^{a}	116.7±14.0^{a}	194.2±18.4^{a}	305.3±20.3^{a}
	8	69.8±5.6^{a}	111.2±10.1^{a}	161.7±6.7^{a}	254.7±17.0^{a}
RGR (% day^{-1})	1	17.3±3.7^{b}	22.5±4.5^{c}	28.8±4.4^{c}	31.0±5.6^{c}
	2	11.9±3.2^{ab}	14.4±1.1^{b}	16.7±1.5^{b}	18.6±1.3^{b}
	4	8.4±2.3^{a}	8.1±0.6^{a}	9.1±1.1^{a}	11.3±1.2^{a}
	6	6.1±1.0^{a}	5.8±0.7^{a}	6.5±0.6^{a}	7.6±0.5^{a}
	8	7.0±0.6^{a}	5.6±0.5^{a}	5.4±0.2^{a}	6.4±0.4^{a}
Number of new branch per cm	1	1.0±0.0^{b}	2.0±1.0^{c}	5.0±1.0^{b}	7.0±1.0^{c}
	2	1.0±0.0^{ab}	1.0±0.0^{b}	3.0±1.0^{a}	4.0±0.0^{b}
	4	1.0±0.0^{a}	1.0±0.0^{ab}	2.0±1.0^{a}	2.0±0.0^{ab}
	6	0.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}
	8	1.0±0.0^{a}	1.0±0.0^{a}	1.0±0.0^{a}	1.0±1.0^{a}
Branch length (cm)	1	0.1±0.0^{a}	0.4±0.1^{a}	0.7±0.1^{c}	0.8±0.1^{b}
	2	0.1±0.1^{a}	0.3±0.2^{a}	0.6±0.2^{c}	0.9±0.1^{b}
	4	0.1±0.0^{a}	0.2±0.0^{a}	0.5±0.0^{bc}	0.7±0.2^{ab}
	6	0.1±0.0^{a}	0.2±0.1^{a}	0.3±0.0^{a}	0.4±0.1^{a}
	8	0.1±0.1^{a}	0.1±0.1^{a}	0.3±0.1^{ab}	0.4±0.1^{a}
Biomass ($g\ L^{-1}$)	1	2.7±0.4^{a}	5.5±0.9^{a}	9.6±1.3^{a}	13.4±2.2^{a}
	2	4.4±0.6^{b}	7.7±0.5^{b}	12.0±0.9^{b}	16.9±1.0^{b}
	4	7.4±0.9^{c}	10.5±0.5^{c}	15.0±1.3^{c}	22.1±1.9^{c}
	6	9.7±0.6^{d}	13.0±0.8^{d}	17.7±1.1^{d}	24.3±1.2^{c}
	8	13.6±0.4^{e}	16.9±0.8^{e}	20.9±0.5^{e}	28.4±1.4^{d}
Increased length (%)	1	56.7±16.7^{c}	119.8±20.8^{d}	194.3±30.5^{c}	216.7±42.0^{d}
	2	36.5±6.5^{b}	92.8±5.3^{c}	118.0±14.9^{b}	173.8±4.0^{c}
	4	29.5±3.8^{ab}	67.2±1.5^{b}	98.2±13.8^{ab}	148.7±13.3^{bc}
	6	22.2±5.8^{ab}	58.5±10.3^{ab}	88.2±8.7^{ab}	115.2±7.3^{ab}
	8	18.5±2.6^{a}	45.8±2.3^{a}	75.2±5.0^{a}	100.5±3.5^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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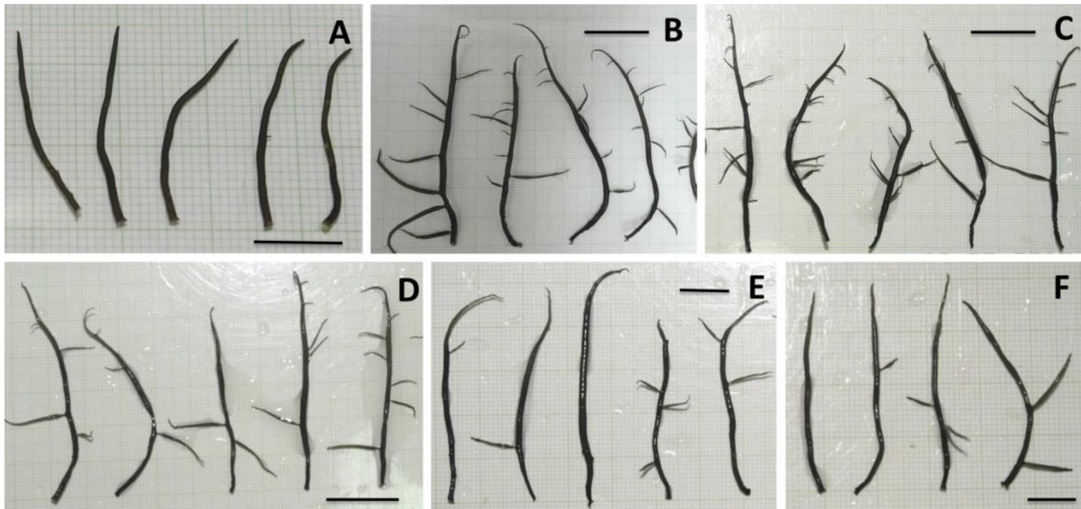

Figure 3.8 Tissue culture of *G. fisheri* of 2cm length of apical part, cultured in 20 ppt salinity, 12L:12D photopheriod, 20 μmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under different propagule density experiment. (A) Seedling plant. (B) Tissue at 1 gL^{-1}. (C) Tissue at 2 gL^{-1}. (D) Tissue at 4 gL^{-1}. (E) Tissue at 6 gL^{-1}. (F) Tissue at 8 gL^{-1}.

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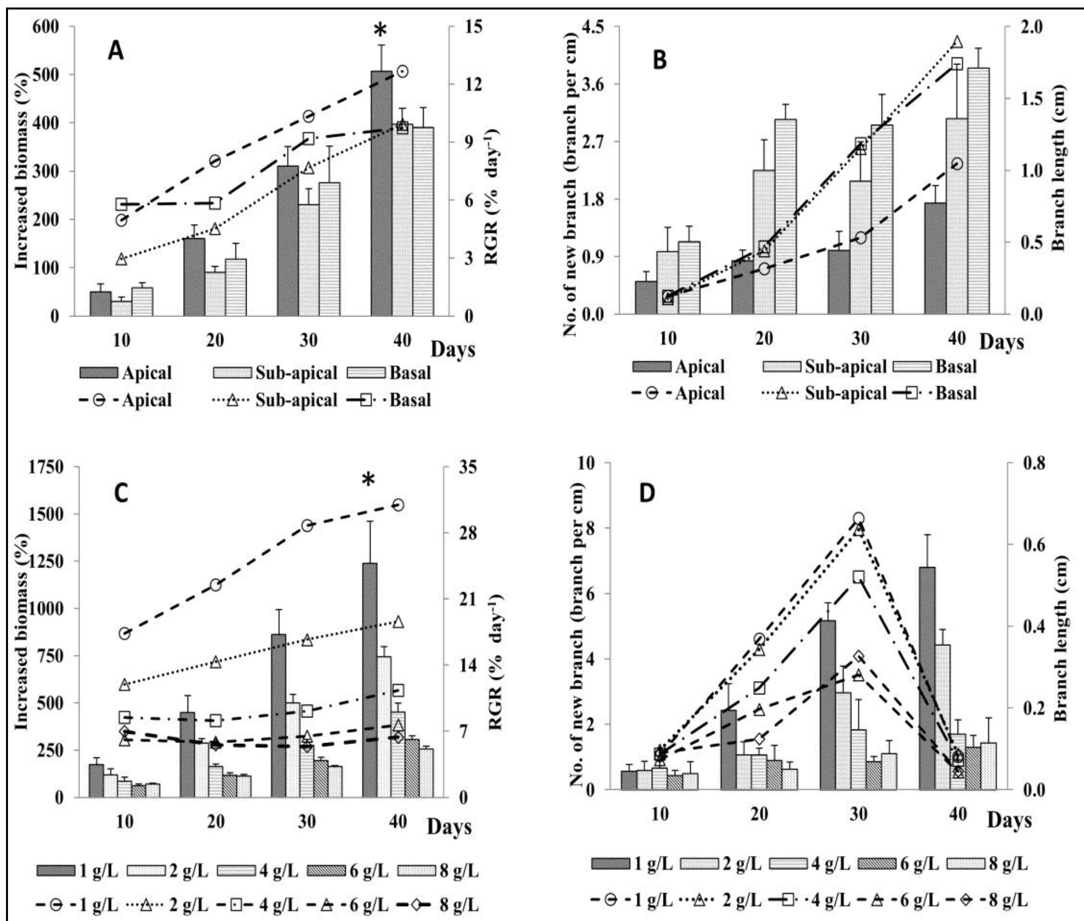

Figure 3.9 Growth rate and number of new branch (bar graphs) and RGR and branch length (line graphs) of *G. fisheri* tissues cultured at 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under (A,B) Different part of thallus. (C, D) Different propagule densities. (*) is selected condition.

Table 3.12 Growth type and branch formation of *G. fisheri* tissues

Factors	Levels	Growth type	Pigment loss	Branchlet produce
Initial length (cm)	1	Non-polarity	Absent	Yes
	2	Non-polarity	Absent	Yes
	3	Non-polarity	Absent	Yes
	4	Non-polarity	Absent	Yes
	5	Non-polarity	Absent	Yes
Salinity (ppt)	15	Non-polarity	Present	Yes
	20	Non-polarity	Absent	Yes
	25	Non-polarity	Absent	Yes
	30	Non-polarity	Present	Yes
	35	Non-polarity	Present	Yes
Part of thallus	Apical	Polarity	Absent	Yes
	Sub-apical	Non-polarity	Absent	Yes
	Basal	Non-polarity	Absent	Yes
Density ($g\ L^{-1}$)	1	Polarity	Absent	Yes
	2	Polarity	Absent	Yes
	4	Polarity	Absent	No
	6	Polarity	Absent	No
	8	Polarity	Absent	No

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3.2.5 *G. fisheri* tissue under different colors in laboratory condition

Tissue under different shading color showed differential growth rate (Figure 3.10). Under red light, tissue got highest biomass with 1.6±0.1 $g{\cdot}L^{-1}$ significantly higher ($p<0.05$) than those under white light with 1.5±0.1 $g{\cdot}L^{-1}$ at 40 days (Figure 3.11A), (Table 3.13). The biomass increase of tissues under red light got maximum with 57.3% (Figure 3.11B). Similarly, the RGR of tissues under the red light also got highest with 1.43±0.21 $\%.day^{-1}$ and followed by the tissue in white color 1.19±0.37 $\%.day^{-1}$ (Figure 3.11C). At 40 days, the average length increase of tissues in the white, green, blue and red were 4.5±2.7, 1.88±0.5, 6.1±7.9 and 5.3±1.7 %, respectively (Figure 3.12A), (Table 3.13). Tissues in the red color also produced the highest number of new branches with 2 branches per cm (Figure 3.12B). There was no significant different ($p>0.05$) in the length of main thallus among the treatments. The length of new branches produced under red color was significantly higher ($p<0.05$) than those in the other treatments with 3.4±1.1 cm (Figure 3.12C).

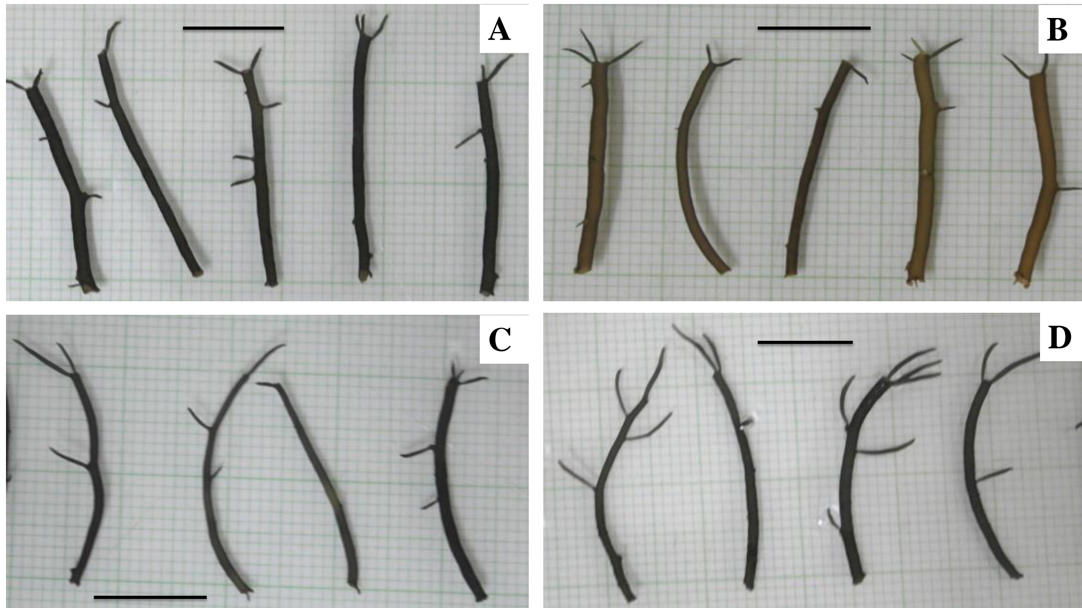

Figure 3.10 *G. fisheri* tissues cultured at 1 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under the different shading colors: (A) White color (B) Green color (C) Blue color (D) Red color.

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Table 3.13 Growth and branch formation of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red

	Shading color	Days 10	20	30	40
Increased biomass (%)	White	27.2 ± 4.7^{b}	39.9 ± 2.9^{b}	49.9 ± 4.6^{c}	54.9 ± 7.9^{ab}
	Green	19.2 ± 8.1^{a}	32.3 ± 4.8^{a}	39.9 ± 5.1^{a}	48.3 ± 5.5^{a}
	Blue	32.6 ± 4.2^{a}	42.5 ± 7.6^{ab}	56.4 ± 8.0^{ab}	65.0 ± 7.7^{a}
	Red	39.8 ± 9.9^{a}	52.3 ± 6.8^{ab}	65.1 ± 5.3^{bc}	78.2 ± 8.1^{b}
RGR (% day^{-1})	White	4.3 ± 0.9^{b}	2.7 ± 0.6^{b}	2.0 ± 0.5^{c}	1.2 ± 0.4^{ab}
	Green	1.9 ± 0.8^{a}	1.7 ± 0.3^{a}	0.8 ± 0.2^{a}	0.7 ± 0.3^{a}
	Blue	2.7 ± 0.4^{a}	1.9 ± 0.4^{ab}	1.2 ± 0.2^{ab}	0.8 ± 0.1^{a}
	Red	2.7 ± 0.5^{a}	2.1 ± 0.3^{ab}	1.9 ± 0.5^{bc}	1.4 ± 0.2^{b}
Number of new branch per cm	White	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{a}
	Green	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{ab}
	Blue	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{ab}
	Red	0.0 ± 0.0^{a}	1.0 ± 0.0^{ab}	1.0 ± 0.0^{b}	2.0 ± 0.0^{b}
Branch length (cm)	White	0.2 ± 0.1^{b}	1.1 ± 0.0^{b}	2.0 ± 0.6^{b}	2.3 ± 0.6^{ab}
	Green	0.1 ± 0.0^{a}	0.5 ± 0.4^{a}	1.1 ± 0.2^{a}	2.0 ± 0.3^{a}
	Blue	0.2 ± 0.1^{a}	1.0 ± 0.2^{ab}	2.0 ± 0.5^{ab}	2.3 ± 0.1^{ab}
	Red	0.2 ± 0.1^{a}	1.0 ± 0.4^{ab}	2.1 ± 0.4^{b}	3.4 ± 0.3^{b}
Biomass (g L^{-1})	White	1.4 ± 0.1^{b}	1.5 ± 0.1^{b}	1.6 ± 0.1^{b}	1.5 ± 0.1^{b}
	Green	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}	1.2 ± 0.1^{a}	1.3 ± 0.1^{a}
	Blue	1.3 ± 0.0^{a}	1.4 ± 0.1^{b}	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}
	Red	1.3 ± 0.1^{a}	1.4 ± 0.1^{ab}	1.6 ± 0.2^{ab}	1.6 ± 0.1^{b}
Incresed length (%)	White	8.1 ± 2.4^{b}	7.4 ± 2.7^{b}	9.6 ± 4.2^{a}	4.5 ± 2.7^{a}
	Green	3.8 ± 1.5^{a}	2.3 ± 1.3^{a}	5.3 ± 2.6^{a}	1.9 ± 0.5^{a}
	Blue	3.1 ± 2.0^{a}	3.3 ± 1.2^{a}	5.7 ± 1.5^{a}	6.1 ± 7.9^{a}
	Red	3.5 ± 1.2^{a}	4.1 ± 1.2^{a}	8.1 ± 3.6^{a}	5.3 ± 1.7^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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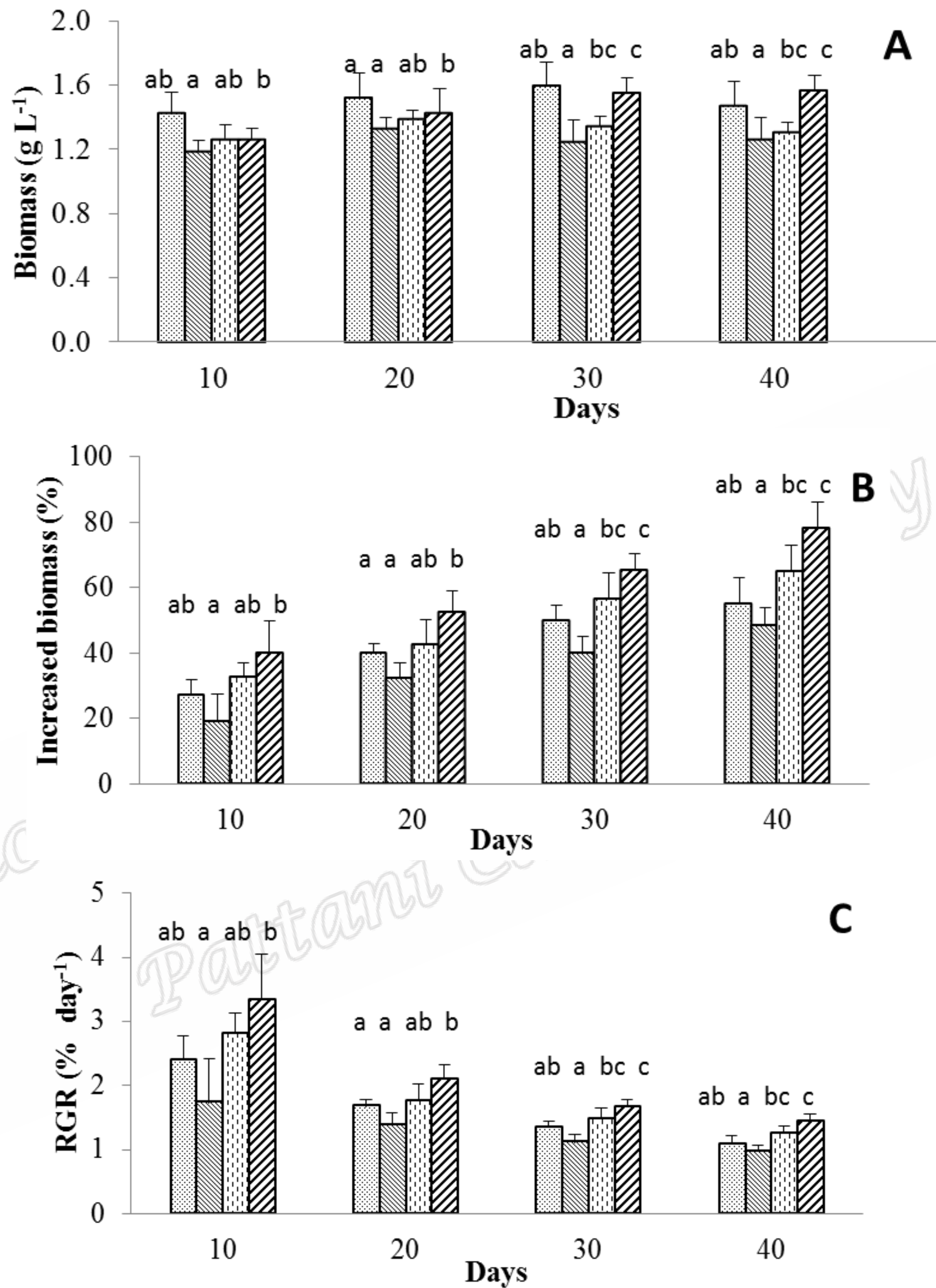

Figure 3.11 *G. fisheri* tissues cultured at 1 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under different shading colors. (A) Increased biomass. (B) RGR. (C) Number of new branches.

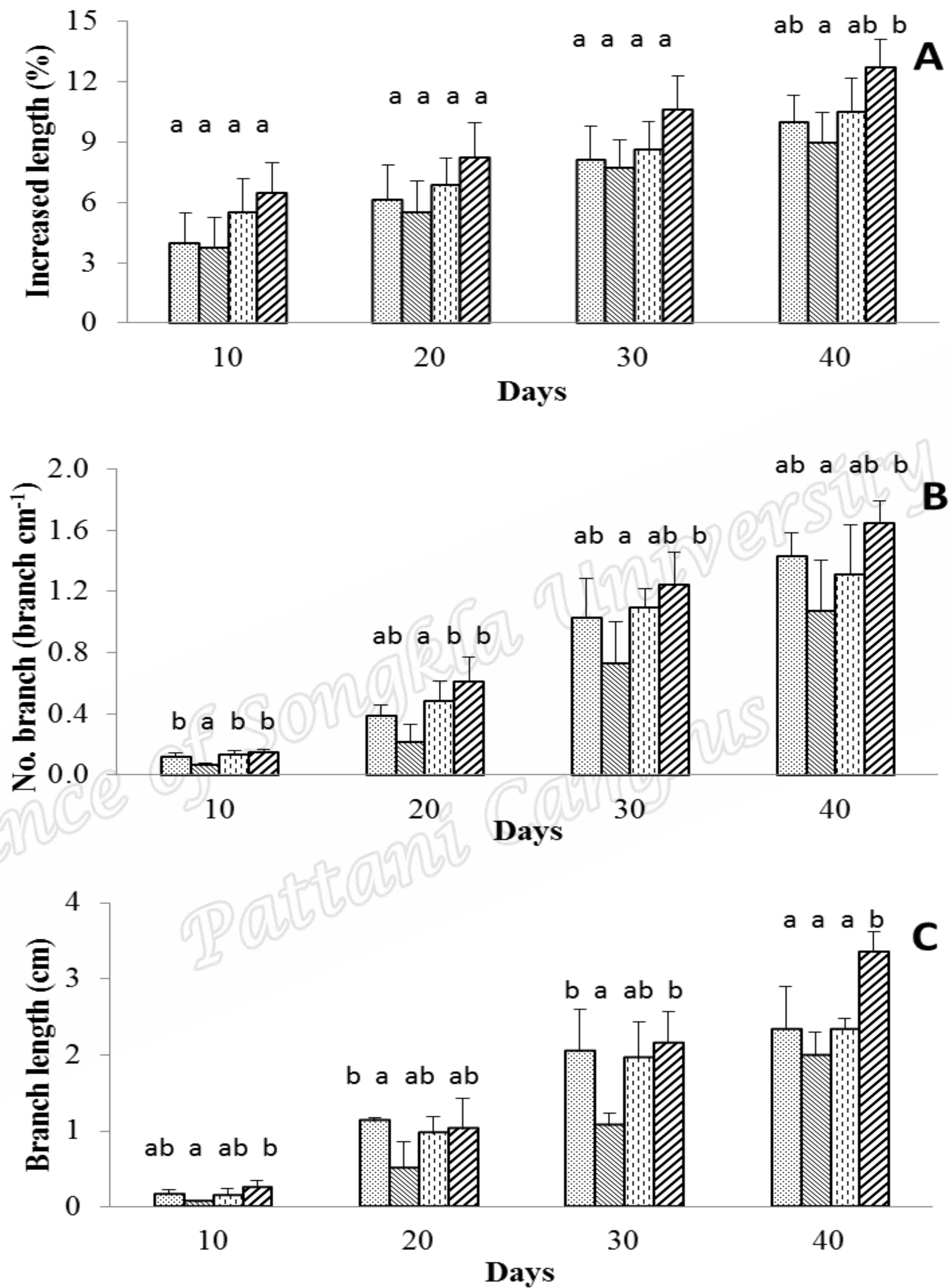

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Figure 3.12 *G. fisheri* tissues cultured at 1 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under the different shading colors. (A) Branch length. (B) Increased length. (C) Biomass.

Pigment analysis showed no significant different (p>0.05) of *G. fisheri* tissues under different light colors (Figure 3.13). Under white light, tissues produced the highest carotenoids and Chl a with 71.4 and 156.7 µg g^{-1} fresh weight (µg g^{-1} FW), respectively whereas under red light tissues got highest R-PE with 214.9 µg g^{-1} FW (Table 3.14).

Table 3.14 Pigment contents of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red

Shading color	Pigment (µg g^{-1} fresh weight)				
	Carotenoids	Chl. a	Chl. b	Chl. c	R-PE
White	71.4±2.3[a]	156.7±13.1[a]	10.6±4.2[a]	16.5±2.2[a]	169.2±48.9[a]
Green	61.0±13.1[a]	110.9±40.3[a]	13.5±3.5[a]	13.1±0.8[a]	132.9±9.9[a]
Blue	62.7±12.1[a]	107.5±34.3[a]	13.8±3.0[a]	13.5±1.0[a]	180.7±33.1[a]
Red	62.2±6.8[a]	110.2±18.4[a]	13.7±1.7[a]	14.1±0.5[a]	214.9±16.3[a]

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at p<0.05.

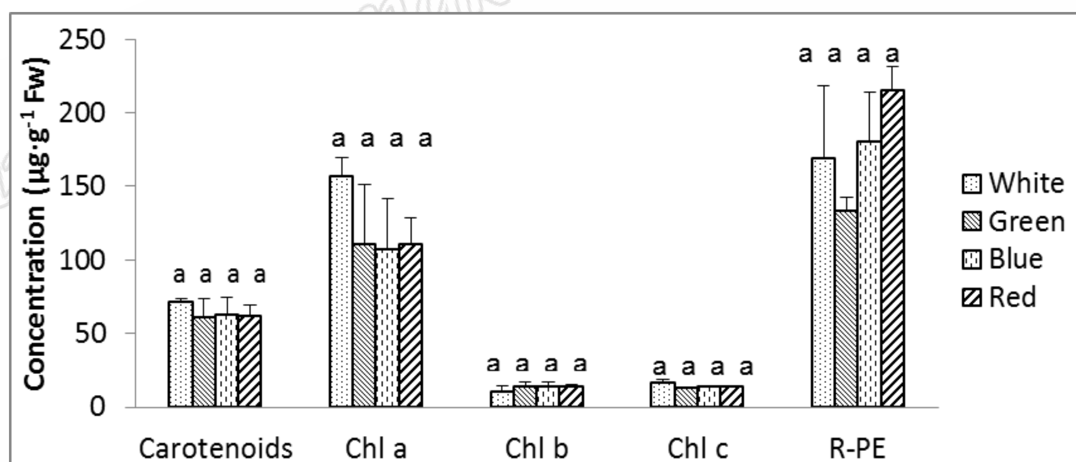

Figure 3.13 Pigment content of *G. fisheri* tissues cultured at 1 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under different shading colors indoor condition.

3.2.6 Growth of *G. fisheri* sporelings on laboratory

Spores showed different growth rate under different shading color (Figure 3.14). At 40 days, the length increase of the sporelings in red light got significantly higher (p<0.05) than those under other shading lights. The sporelings got maximum biomass under red light with 0.7±0.0 g L^{-1} that significant higher (p<0.05) than those under other shading color (Figure 3.15A), (Table 3.15). The RGR of spore under red light was highest with 2.8 %.day^{-1} (Figure 3.15C). The length increase of the sporelings got highest under red light with 45.2±3.5 % and lowest under blue light

with 16.0±9.7% (Figure 3.16A), (Table 3.15). However, there was no significant difference ($p>0.05$) on number of new branches produced from the treatments (Figure 3.16B). The highest number of new branches produced under different white light with 1 branch per cm whereas the longest new branch produced under red light with 3.4 cm (Figure 3.16C).

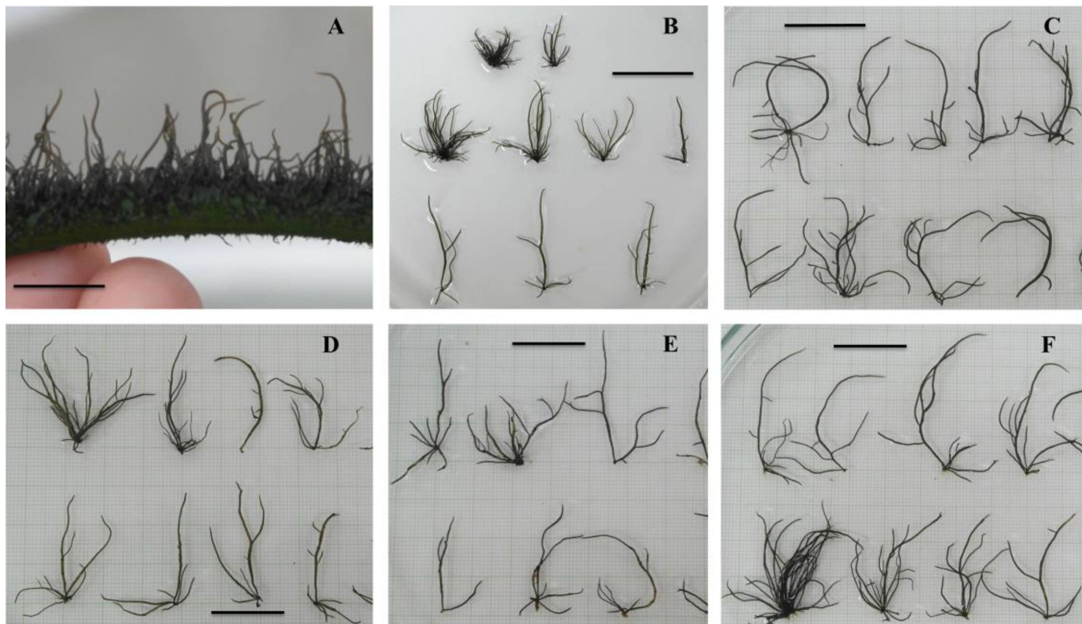

Figure 3.14 *G. fisheri* sporelings cultured at 0.33 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25°C temperature under the different shading color experiment. (A) Sporelings attached in rope. (B) Sporeling materials. (C) Sporelings under white color. (D) Sporeligs under green color. (E) Sporelings under blue color. (F) Sporelings under red color. Scale bar= 1 cm (A, B), 2 cm (C, D, E, F).

Table 3.15 Growth and branch formation of *Gracilaria fisheri* sporelings cultured indoor condition under different shading colors of white, green, blue and red

	Shading color	Days			
		10	20	30	40
Increased biomass (%)	White	10.7±3.1[a]	19.3±5.0[a]	32.0±1.0[a]	40.3±6.7[ab]
	Green	4.7±2.1[a]	16.3±6.7[a]	21.0±2.0[a]	22.7±3.1[a]
	Blue	24.0±8.9[b]	31.7±6.7[b]	59.0±15.9[b]	57.7±20.5[b]
	Red	43.0±5.3[c]	59.3±6.7[c]	85.7±9.5[c]	113.0±13.1[c]
RGR (% day^{-1})	White	1.1±0.3[a]	1.0±0.3[a]	1.1±0.0[a]	1.0±0.2[ab]
	Green	0.5±0.2[a]	0.8±0.3[a]	0.7±0.1[a]	0.6±0.1[a]
	Blue	2.4±0.9[b]	1.6±0.3[b]	2.0±0.5[b]	1.4±0.5[b]
	Red	4.3±0.5[c]	3.0±0.3[c]	2.9±0.3[c]	2.8±0.3[c]
Number of new branch per cm	White	1.0±0.0[a]	1.0±0.0[b]	1.0±0.0[a]	1.0±0.0[a]
	Green	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]
	Blue	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]
	Red	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]	1.0±0.0[a]
Branch length (cm)	White	0.4±0.1[a]	0.5±0.1[bc]	0.5±0.2[a]	0.6±0.1[a]
	Green	0.4±0.2[a]	0.4±0.0[a]	0.4±0.2[a]	0.4±0.1[a]
	Blue	0.4±0.1[a]	0.5±0.1[ab]	0.5±0.1[a]	0.5±0.0[a]
	Red	0.6±0.1[a]	0.6±0.1[c]	0.7±0.1[a]	0.8±0.1[b]
Biomass (g L^{-1})	White	0.4±0.0[a]	0.4±0.0[a]	0.4±0.0[a]	0.5±0.0[a]
	Green	0.3±0.0[a]	0.4±0.0[a]	0.4±0.0[a]	0.4±0.0[a]
	Blue	0.4±0.0[b]	0.4±0.0[b]	0.5±0.1[b]	0.5±0.1[b]
	Red	0.5±0.0[c]	0.5±0.0[c]	0.6±0.0[c]	0.7±0.0[c]
Incresed length (%)	White	9.3±2.6[b]	15.8±6.2[a]	18.8±4.6[a]	18.4±7.6[a]
	Green	14.6±5.9[b]	16.3±10.7[a]	18.0±12.7[a]	17.8±10.1[a]
	Blue	6.0±8.3[a]	8.4±3.7[a]	13.1±8.5[a]	16.0±9.7[a]
	Red	26.2±1.1[b]	26.0±4.8[a]	39.6±5.3[b]	45.2±3.5[b]

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

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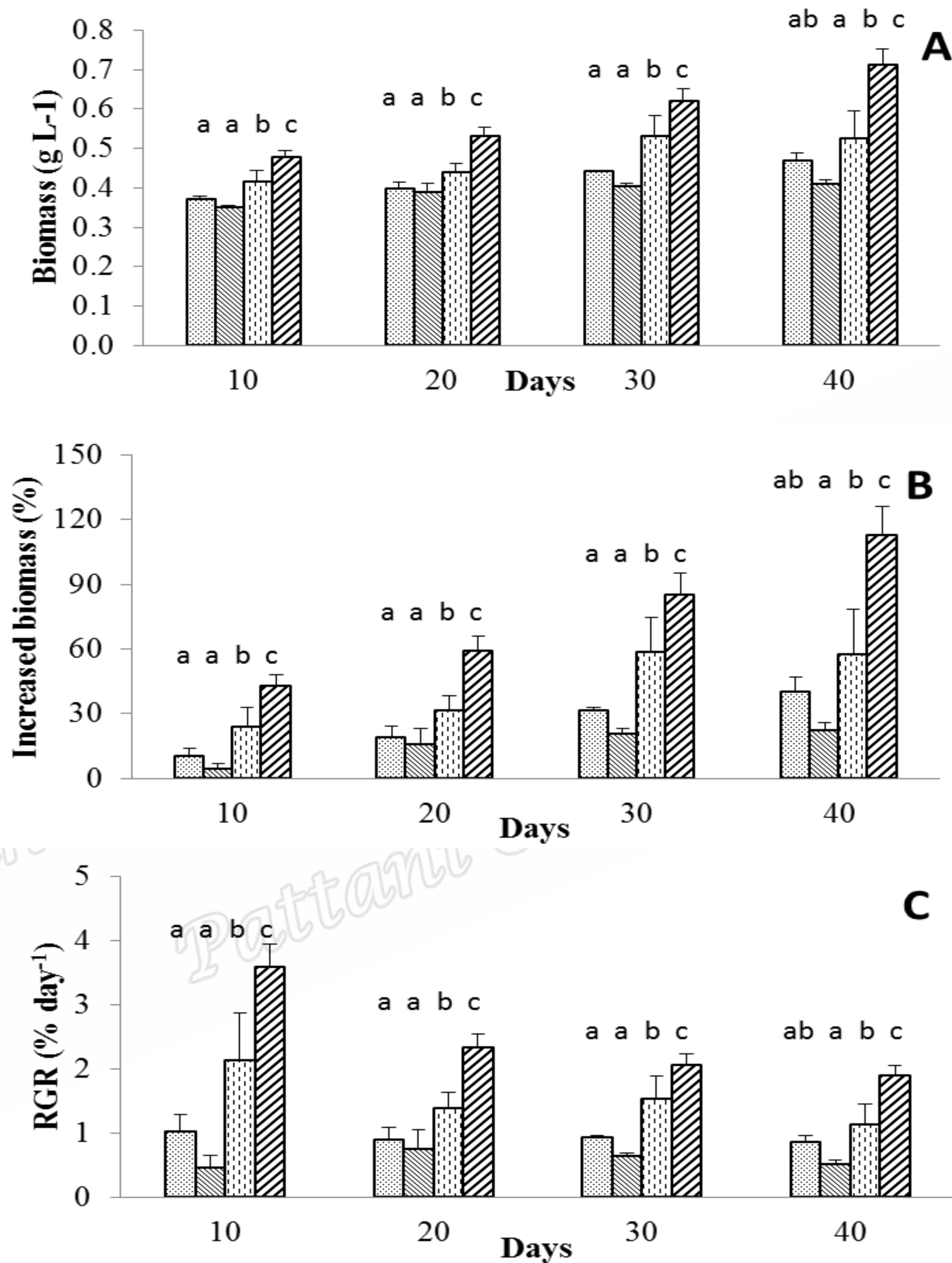

Figure 3.15 *G. fisheri* sporelings cultured at 0.33 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25°C temperature under the different shading color experiment. (A) Increased biomass. (B) RGR. (C) Number of new branches.

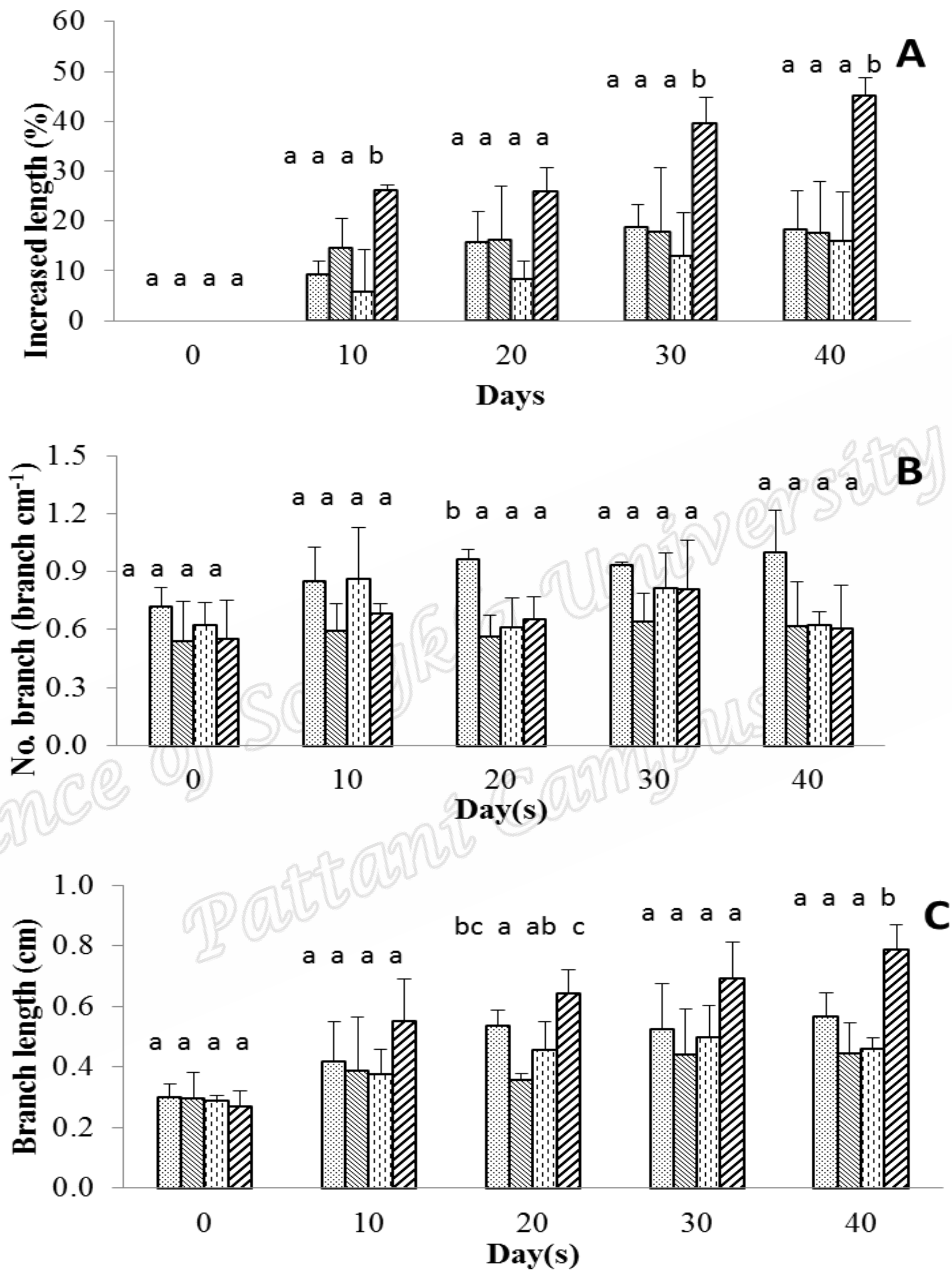

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Figure 3.16 *G. fisheri* sporelings cultured at 0.33 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature under the different shading color experiment. (A) Branch length. (B) Increased length. (C) Biomass.

There was no significant difference ($p>0.05$) on carotenoids and R-PE concentrion in *G. fisheri* spores under different light colors. The highest carotenoids was counted under green light with 154.0 µg g^{-1} FW while the highest R-PE was found under white light with 160.0 µg g^{-1} FW (Figure 3.17, Table 3.16). The amount of Chl a was significantly higher ($p<0.05$) under white and green lights than those in blue light. The highest Chl a was found at tissues under green light with 384.0 µg·g^{-1} FW. With chlorophyll extracts, the absorbance spectra of *G. fisheri* sporelings indoor showed two peaks at 432 nm and 664 nm (Figure 3.18A). These peaks were found same in all treatments whereas, the R-PE extract of *G. fisheri* sporelings got five absorbance peaks at 437, 496, 564, 619 and 678 nm (Figure 3.18B, Table 3.17).

Table 3.16 Pigment contents of *Gracilaria fisheri* tissues cultured indoor condition under different shading colors of white, green, blue and red

Shading color	Pigment (µg g^{-1} fresh weight)				
	Carotenoids	Chl. a	Chl. B	Chl. c	R-PE
White	132.5±4.1^{a}	332.5±6.7^{b}	5.8±3.8^{a}	22.7±2.0^{a}	160.0±3.8^{a}
Green	154.0±23.4^{a}	384.0±61.4^{b}	3.0±2.4^{a}	29.8±6.7^{a}	132.5±8.1^{a}
Blue	90.0±3.6^{a}	174.5±14.3^{a}	17.9±4.0^{b}	21.8±1.8^{a}	148.6±12.5^{a}
Red	127.3±39.7^{a}	293.0±118.7^{ab}	20.5±11.2^{b}	26.2±10.3^{a}	159.5±24.1^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

Table 3.17 Absorbance from chlorophyll and R-PE extract of *Gracilaria fisheri* sporelings cultured indoor condition under different shading colors of white, green, blue and red

Wavelenth	Absorbance from chlorophyll extract				Absorbance from R-PE extract			
	White	Green	Blue	Red	White	Green	Blue	Red
400	1.297	2.045	1.202	1.378	0.755	0.717	0.640	0.729
425	1.836	2.945	1.887	2.141	0.945	0.879	0.775	0.889
450	1.203	1.842	1.170	1.340	0.735	0.701	0.620	0.707
475	0.919	1.419	0.878	0.990	0.530	0.509	0.484	0.534
500	0.552	0.555	0.321	0.332	0.619	0.570	0.574	0.599
525	0.311	0.140	0.084	0.092	0.477	0.422	0.450	0.467
550	0.313	0.112	0.065	0.077	0.502	0.428	0.467	0.486
575	0.322	0.234	0.131	0.154	0.417	0.359	0.399	0.424
600	0.312	0.256	0.140	0.168	0.378	0.324	0.322	0.379
625	0.406	0.379	0.207	0.244	0.454	0.386	0.379	0.444
650	0.465	0.697	0.378	0.471	0.303	0.271	0.246	0.312
675	0.649	0.811	0.448	0.512	0.553	0.503	0.427	0.522
700	0.096	0.018	0.009	0.008	0.158	0.161	0.117	0.168

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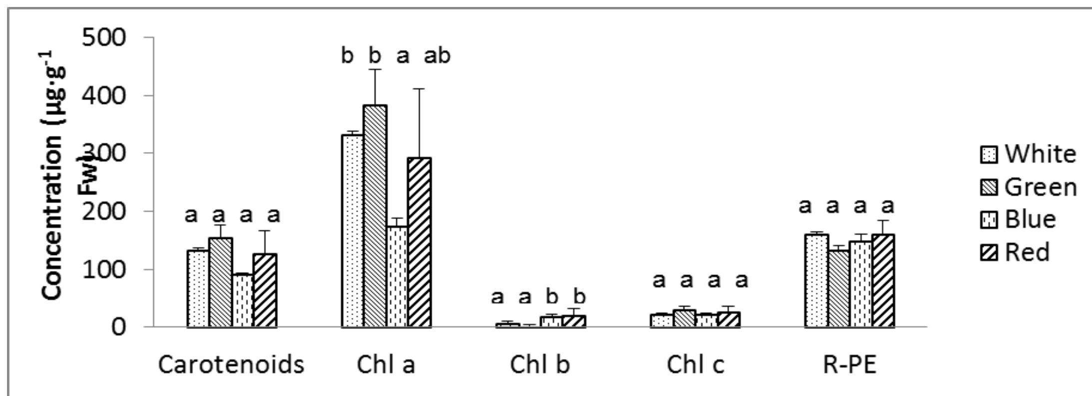

Figure 3.17 Pigment content in *G. fisheri* sporelings cultured at 0.33 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol m^{-2}s^{-1} light intensity, 25ºC temperature under the different shading color experiment.

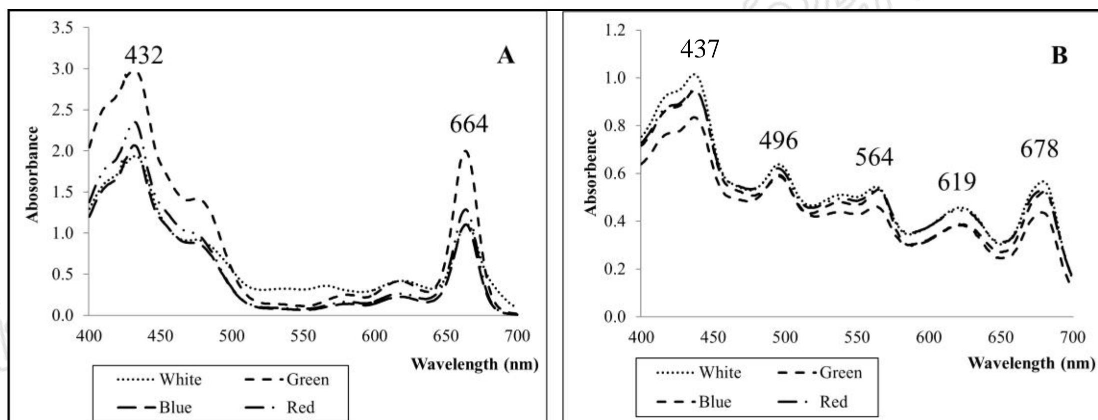

Figure 3.18 Spectrum absorbance of *G. fisheri* sporelings cultured at 0.33 g L^{-1} density, 20 ppt salinity, 12L:12D photopheriod, 20 µmol m^{-2}s^{-1} light intensity, 25ºC temperature under the different shading color experiment. (A) From chlorophyll extract. (B) From R-PE extract.

3.3 Upper scale cultivation of *G. fisheri*

3.3.1 *G. fisheri* tissue upper scale indoor condition

After 40-day cultivation at the optimal conditions, *G. fisheri* tissue got the average biomass of 4.6 g L^{-1} with the average RGR was 8.9 %.day^{-1}. The total length of tissue from this experiment was 2.2 cm with the average number of branch of 3 branch cm^{-1}. The average branch length was 1.2 cm (Table 3.18).

Table 3.18 Growth of biomass, main thallus and new branches of *Gracilaria fisheri* cultured under optimal conditions of 2 cm length apical thallus, at 20 ppt salinity, 1 g L^{-1} density, 12L:12D photopheriod, 20 µmol $m^{-2}s^{-1}$ light intensity, 25ºC temperature at 40 days

Parameter / Day(s)	0	10	20	30	40
Biomass (gL^{-1})	1.0±0.0	1.4±0.1	2.1±0.1	3.2±0.1	4.6±0.3
RGR (%.day^{-1})	0.0±0.0	4.2±0.6	5.4±0.4	7.3±0.5	8.9±0.7
Number of branch	0.0±0.0	2.0±1.0	5.0±0.0	7.0±0.0	6.0±1.0
Length of thallus (cm)	2.0±0.0	2.1±0.0	2.1±0.0	2.1±0.1	2.2±0.1
Length of branch (cm)	0.0±0.0	1.1±0.2	3.7±0.4	8.7±1.1	1.2±0.3

Data are showed as mean±SD, number of replicates n=3.

3.3.2 *G. fisheri* tissue at outdoor condition

At first week of culture, the biomass of tissue in green house is significantly higher ($p<0.05$) than those in other treatments (Figure 3.19A). However, after the first week until the sixth week there was no significant difference ($p>0.05$) on the biomass of tissues among the treatments. At the seventh and 8th week, the highest final biomass was observed in tissues under green house with 873.3±74.7 g·m^{-2} which was significantly higher ($p<0.05$) than those at white and black Saran house (Table 3.19). Similarly, the RGR of tissues at the last two week got maximum under green house with 3.24±0.28 and 2.89±0.40 %·day^{-1} at 7th and 8th weeks, respectively (Figure 3.19B).

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Table 3.19 Growth rate of *Gracilari fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black

	Shading color	Week(s)							
		1	2	3	4	5	6	7	8
Increased biomass (%)	White	19±2^{a}	35±8^{a}	62±9^{a}	78±13^{a}	99±12^{a}	125±13^{a}	125±13^{a}	123±15^{a}
	Green	25±2^{b}	43±5^{a}	74±5^{a}	89±2^{a}	112±3^{a}	151±8^{a}	159±14^{b}	162±22^{b}
	Blue	21±2^{a}	34±4^{a}	64±3^{a}	78±5^{a}	100±8^{a}	132±12^{a}	147±20^{ab}	144±12^{ab}
	Black	21±2^{a}	38±3^{a}	66±9^{a}	78±7^{a}	99±12^{a}	134±16^{a}	140±10^{ab}	133±11^{a}
RGR (% day^{-1})	White	2.8±0.2^{a}	2.5±0.6^{a}	3.0±0.4^{a}	2.8±0.4^{a}	2.8±0.3^{a}	3.0±0.3^{a}	2.6±0.3^{a}	2.2±0.3^{a}
	Green	3.5±0.3^{b}	3.1±0.3^{a}	3.5±0.2^{a}	3.2±0.1^{a}	3.2±0.1^{a}	3.6±0.2^{a}	3.2±0.3^{b}	2.9±0.4^{b}
	Blue	3.1±0.3^{a}	2.4±0.3^{a}	3.1±0.2^{a}	2.8±0.2^{a}	2.9±0.2^{a}	3.1±0.3^{a}	3.0±0.4^{ab}	2.6±0.2^{ab}
	Black	2.9±0.3^{a}	2.7±0.2^{a}	3.1±0.4^{a}	2.8±0.3^{a}	2.8±0.3^{a}	3.2±0.4^{a}	2.8±0.2^{ab}	2.4±0.2^{a}
Biomass (g m^{-2})	White	398±5^{a}	451±27^{a}	542±31^{a}	594±42^{a}	664±41^{a}	751±44^{a}	752±44^{a}	742±49^{a}
	Green	416±8^{b}	478±16^{a}	581±17^{a}	629±8^{a}	706±11^{a}	838±27^{a}	863±46^{b}	873±75^{b}
	Blue	405±6^{a}	447±14^{a}	548±11^{a}	594±15^{a}	667±26^{a}	772±39^{a}	823±68^{ab}	814±40^{ab}
	Black	402±8^{a}	461±11^{a}	554±31^{a}	594±24^{a}	664±39^{a}	779±53^{a}	799±32^{ab}	778±37^{b}
Epiphytes (%)	White	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	12±1^{b}	20±4^{b}
	Green	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	11±2^{b}	20±3^{b}
	Blue	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	8±1^{b}	18±3^{ab}
	Black	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	4±1^{a}	15±4^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

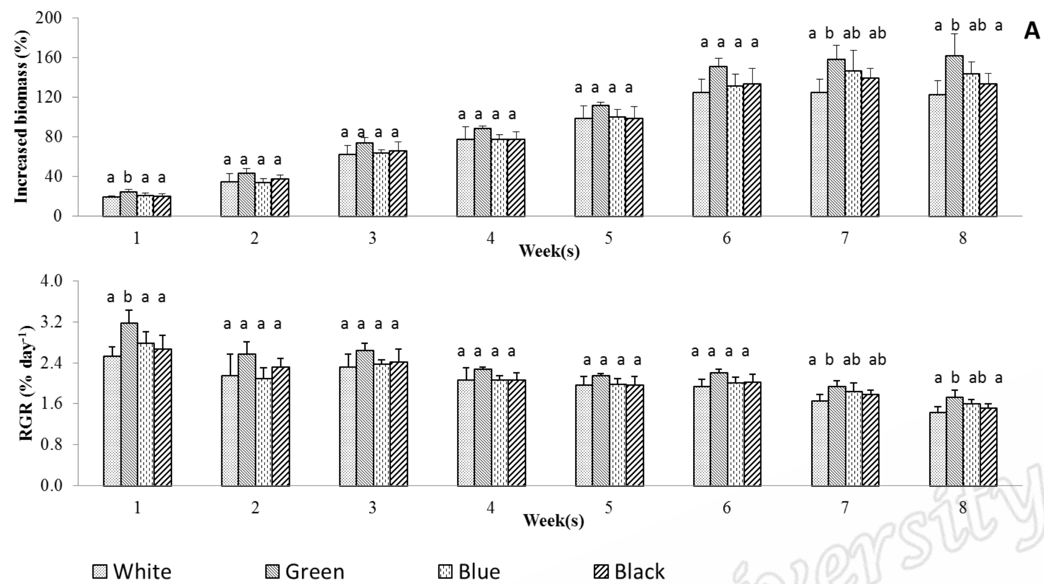

Figure 3.19 Growth of *G. fisheri* tissues cultured at 300 g m^{-2} density, 20 ppt salinity, 113 µmol m^{-2} s^{-1} light intensity under the different shading color outdoor condition. (A) Increased biomass. (B) RGR

After sixth week, the culture was affected by epiphytes (*Rhizoclonium tortuosum* and *Uva intestinalis*). It was found that the tissues were less affected by epiphytes under black Saran house. However, tissues under white were most affected by epiphytes. At seventh week, the percentage of contaminant epiphytes in white, green, blue and black houses were in the range of 4.1-12.2%, respectively; this number was gradually increasing until the eighth week up to 15.5-20.5 % (Figure 3.20).

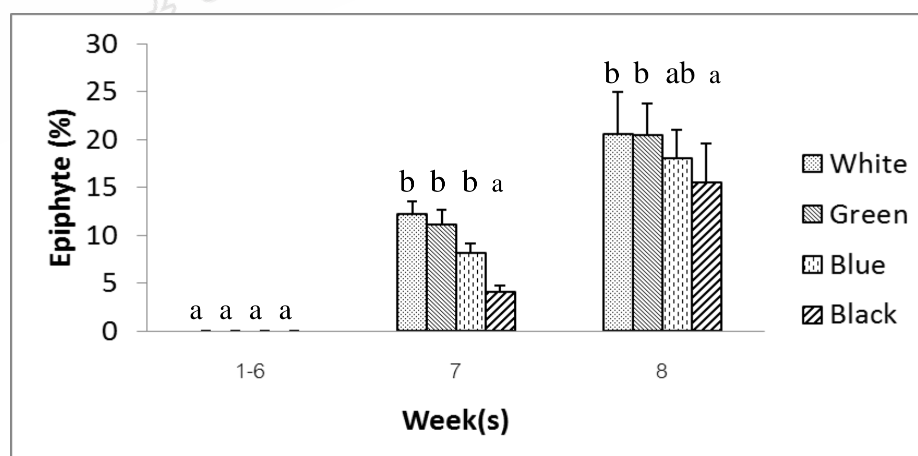

Figure 3.20 Percentage of epiphytes in *G. fisheri* tissues cultured at 300 g m^{-2} density, 20 ppt salinity, 113 µmol m^{-2} s^{-1} light intensity under the different shading color outdoor condition

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G. fisheri tissues showed the low pigment contituents of tissues under black Saran house. Tissues had significantly lower ($p<0.05$) carotenoids and R-PE concentration under black Saran house than those in others. The lowest carotenoids and Chl a of tissues in black Saran house were 13.3 and 23.9 $\mu g \cdot g^{-1}$ FW, respectively (Figure 3.21, Table 3.20). The highest Chl a was found at tissues under green Saran house with 32.6 $\mu g \cdot g^{-1}$ FW whereas tissues under white Saran house produced the highest R-PE with 84.1 $\mu g \cdot g^{-1}$ FW. With acetone extracts, the absorbance spectra of *G. fisheri* tissues outdoor showed two peaks at 432 nm and 664 nm (Figure 3.22A) while the absorbance spectra extracted without acetone got five peaks at 467, 495, 563, 618 and 680 nm (Figure 3.22B, Table 3.21).

Table 3.20 Pigment contents of *Gracilaria fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black

Shading color	Pigment ($\mu g\ g^{-1}$ fresh weight)				
	Carotenoids	Chl. a	Chl. b	Chl. c	R-PE
White	24.2 ± 4.5^{b}	27.0 ± 7.8^{a}	4.3 ± 2.7^{a}	4.9 ± 1.0^{a}	84.1 ± 14.6^{c}
Green	25.4 ± 4.8^{b}	32.6 ± 1.1^{a}	0.8 ± 0.6^{a}	4.7 ± 1.4^{a}	57.5 ± 12.9^{b}
Blue	22.2 ± 2.7^{b}	29.2 ± 2.5^{a}	2.5 ± 1.2^{a}	3.9 ± 1.2^{a}	34.5 ± 8.5^{a}
Black	13.3 ± 2.0^{a}	23.9 ± 8.9^{a}	3.3 ± 2.0^{a}	3.6 ± 1.9^{a}	38.1 ± 1.0^{a}

Data are showed as mean±SD, number of replicates n=3. Mean values in each row followed by the different superscript letters are significantly different at $p<0.05$.

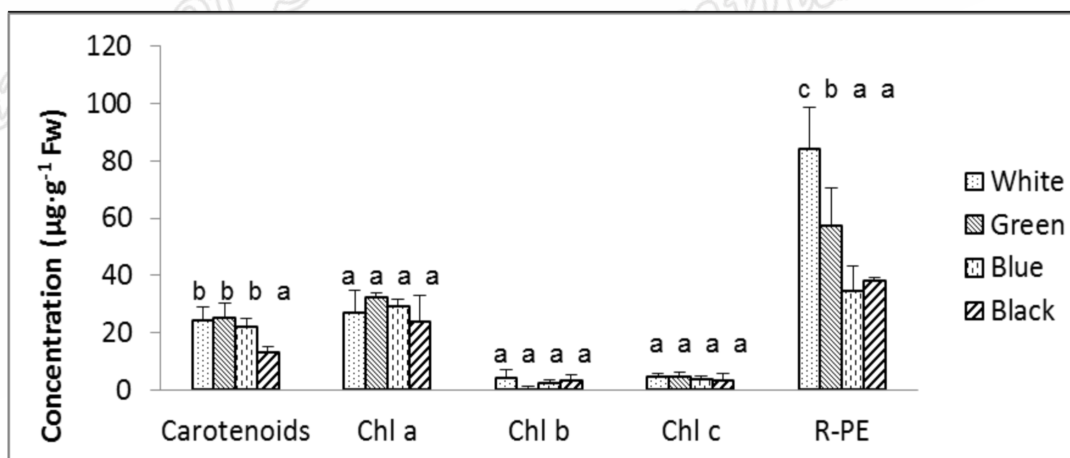

Figure 3.21 Pigment *G. fisheri* tissues cultured at 300 g m^{-2} density, 20 ppt salinity, 113 $\mu mol\ m^{-2}\ s^{-1}$ light intensity under the different shading color outdoor condition

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Table 3.21 Absorbance from chlorophyll and R-PE extract of *Gracilaria fisheri* tissues cultured outdoor condition under different shading colors of white, green, blue and black

	Absorbance from chlorophyll extract				Absorbance from R-PE extract			
Wave length	White	Green	Blue	Black	White	Green	Blue	Black
400	0.284	0.155	0.180	0.170	0.461	0.382	0.232	0.322
425	0.444	0.220	0.276	0.251	0.489	0.390	0.204	0.301
450	0.320	0.136	0.193	0.149	0.411	0.335	0.175	0.262
475	0.256	0.100	0.150	0.113	0.331	0.271	0.151	0.216
500	0.105	0.037	0.054	0.044	0.345	0.271	0.149	0.201
525	0.025	0.008	0.010	0.007	0.277	0.205	0.123	0.149
550	0.017	0.005	0.006	0.005	0.270	0.190	0.114	0.130
575	0.029	0.012	0.014	0.013	0.238	0.170	0.102	0.114
600	0.032	0.014	0.016	0.016	0.210	0.149	0.082	0.095
625	0.047	0.022	0.026	0.026	0.225	0.153	0.079	0.094
650	0.086	0.046	0.052	0.050	0.168	0.121	0.067	0.087
675	0.093	0.047	0.055	0.057	0.242	0.179	0.081	0.124
700	0.004	0.000	0.002	0.002	0.121	0.091	0.050	0.068

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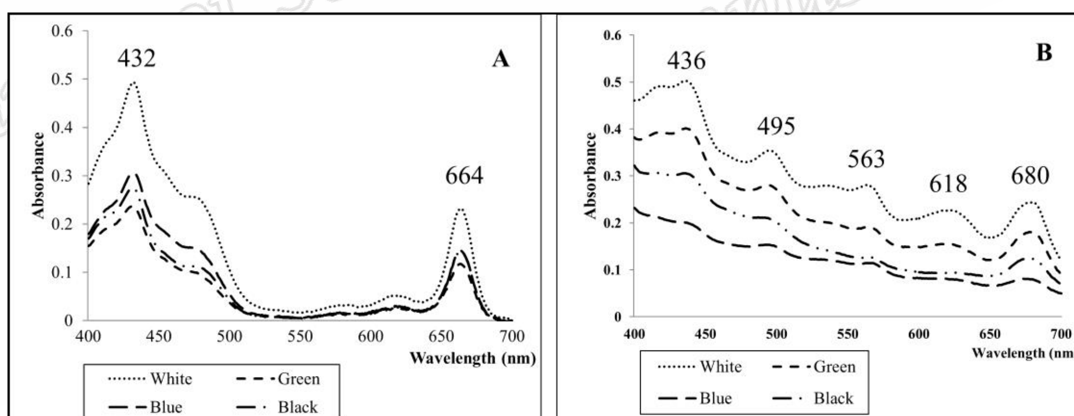

Figure 3.22 Spectrum absorbance of *G. fisheri* tissues cultured at 300 g m^{-2} density, 20 ppt salinity, 113 µmol m^{-2} s^{-1} light intensity under the different shading color outdoor condition. (A) From chlorophyll extract. (B) From R-PE extract.

CHAPTER 4

DISCUSSIONS

4.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment.

The result found that *G. fisheri* yield related inversely with the concentration of nitrate-nitrogen and phosphate-phosphorus in water. It possibily was that the both nutrients in water was absorbed by the seaweed to grow and then only few concentration in water. The amount of chlorophyll in seaweed showed positive relation with Mg in water. This finding was supported by Lobban and Harrison (1994) that Mg is an important component of chlorophyll and forming a metalloporphyrin.

The highest production was observed in ponds with lower salinity of 15 ppt at Songkhla province because the samples were collected in the raining season. However, the optimal salinity for *G. fisheri* was found 20-25 ppt (Prud'homme van Reine and Trono, 2001). Environmental features such as pH, salinity, temperature might affect metal accumulation (Astorga-Espana *et al*., 2015; Rodenas de la Rocha *et al*., 2009). Besides, the element composition also was dependent on the amount and composition of polysaccharides in the seaweed cell walls (Astorga-Espana *et al*., 2015).

The major elements (Ca, Mg, K and Na) in water showed much higher than that in sediment and seaweed whereas trace elements and heavy metals in sediment and seaweed were higher than that in water. In seaweed, the accumulation of major and trace elements showed in the pattern of Mg>K>Ca>Na and Mn>Fe>Zn>Cu. The mount of major elements Ca, Mg, K and Na in this study were 9.52, 12.13, 9.64 and 2.96 mg g^{-1}DW in *G. fisheri* whereas *G. confervoids* contained 16.2, 6.57, 11.2 and 27.8; *G. corticata* maintained 11.72, 4.58, 114.75, 26.29 mg g^{-1} DW, respectively (Moreda-Pineiro *et al*., 2012). The variation of trace elements Fe, Zn and Cu showed the same pattern of Fe>Zn>Cu in seaweed and water. This result was similar to trace element pattern in *Gracilaria verrucosa* (Khaled *et al*., 2014).

In this study, the ratio of Na/K in *G. fisheri* was low in the range of 0.21-1.29. Therefore, this might avoid the incidence of hypertension (Astorga-Espana *et al*., 2015; Benjama and Masniyom, 2012; Lobban and Harrison, 1994) and this ratio is normally consider as lower than 1 (Astorga-Espana *et al*., 2015). Lobban and Harrison (1994) mentioned that K and Na are important for osmotic regulation, pH control and protein conformation and stability in seaweed. It showed that the amount of K and Na in *G. fisheri* at Pattani were significantly higher ($p<0.05$) than those of the other two provinces. This might be caused by the higher salinity in Pattani than Songkhla and Surat Thani provinces. The earlier study reported that major elements, trace elements and heavy metals in *G. fisheri* in Pattani varied from seasons. The amounts of K in seaweed at Pattani was lower than those was previously reported (Benjama and Masniyom, 2012). In this study, it showed the higher amount of Ca, Mg and Na in *G.fisheri* at Pattani than that were reported earlier (Benjama and Masniyom, 2012). The amount of Cu were relative high in the seaweed (2.1 µg g^{-1}

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DW), water (60.1 μg L^{-1}) and in sediment (130.1 μg g^{-1} DW). The amount of macroalgae was reported as lower than 10 μg g^{-1} DW while Cu in marine sediment was reported in the range of 10-50 μg g^{-1} DW (Neff, 2002).

The sequence of Cr, Pb and Cd showed the same pattern of Pb>Cr>Cd in the seaweed, water and sediment. The earlier study also mentioned that the sequence of heavy metals Cr, Pb and Cd in *G. verrucosa* was same (Khaled *et al*., 2014). Heavy metal in seaweed and sediment showed the similar sequence of Ni>Pb>Cr>Cd whereas it was Pb>Ni>Cr>Cd in water. Besides, the amount of Pb in *G. fisheri* was found as high as 11.53 mg g^{-1} DW, however, it fluctuated among the provinces and ponds of each province. The concentration of heavy metals in *G. fisheri* at Pattani province, Pb was higher whereas Cd was lower than those were reported in the earlier study (Benjama and Masniyom, 2012). For all marine organisms, Cr, Cd and Pb are considered as toxic for organism health that might cause to chronic disease (Neff, 2002). In this study, it was found that the amount of Cd in the seaweed relates inversely to salinity in the water. It was reported that Cd in water decreases when salinity increases because of the competition of Cd and Ca or Mg in marine water (Neff, 2002). The study also found that the amounts of Pb in the seaweed, water and sediment in all ponds were much higher than Cd in the seaweed, water and sediment. The average ratio of Pb/Cd in the seaweed, water and sediment were 57, 14 and 28, repectively. In this study, Cd concentration in sediment ranged from 1.0 to 1.2 μg L^{-1} DW that was relatively low while Cd range in marine water was 0.0005-490 ug L^{-1} (Neff, 2012).

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4.2 Tissue culture under different conditions

The different segment lengths did not strongly affect to *G. fisheri* tissue growth. The use of fragments in the range of 1-5 cm for initial length was possible in the tissue growth. However, this study presented that the optimal initial length of *G. fisheri* was observed at 2 cm fragments because of its highest RGR with 11.0 %.day^{-1}. The earlier studies on algal tissue culture were done at selected lengths *i.e.,* 1.0 cm length for *G. verrucosa* and *G. chorda* tissue (Choi *et al*., 2006), 0.5-1.0 cm length for *G. vermiculophylla* tissue (Yokoya *et al*., 1999), 2.0-3.0 cm length for *G. edulis* and *G. tenuistipitata* tissue (Yu *et al*., 2013).

Salinity is one of the most important factors that regulate the growth, reproduction and distribution of seaweed (Choi *et al*., 2006; He *et al*., 2002; Yokoya *et al*., 1999; Yu *et al*., 2013). Several species of *Gracilaria* have been reported with wide salinity tolerance such as *G. verrucosa* and *G. chorda* of 5-35 ppt (Choi *et al*., 2006), *G. vermiculophylla* of 5-60 ppt (Yokoya *et al*., 1999), *G. edulis* and *G. tenuistipitata* of 5-40 ppt (Yu *et al*., 2013). *G. fisheri* tolerated in the salinity range of 10-28 ppt (Ruangchuay *et al*., 2010). In this study, *G. fisheri* tissues expressed as a stenohaline species that salinity is considered as the main affective factor which could cause bleaching area on the tissue and the lethal tissue. At low salinity of 15 ppt and high salinity of 30 and 35 ppt, the fragment started bleaching after 10 days of cultivation which caused a decrease in biomass.

This was agreed with the earlier studies on *Gracilaria* tissues that either too high or too low salinity might limit *Gracilaria* growth (Wilson *et al*., 2013; Yu *et al*., 2013); for instance the inhibited growth and bleaching of tissues occurred in *G.*

tenuistipitata at 21 ppt (He *et al*., 2002), *Gracilaria corticata* at 15 ppt (Kumar *et al*., 2010), *G. verrucosa* at less than 25 ppt (Mensi *et al*., 2011), *G. edulis* at 5 and 40 ppt (Yu *et al*., 2013). However, there is no previous report about relation between bleaching symptom in tissue and ice-ice phenomenon in thallus which occurred in low light intensity and low level of nutrition (Santelices and Doty, 1989). The result showed the similarity on optimal salinity for *G. fisheri* tissue and thallus since this species was reported with the optimal salinity range of 20-25 ppt (Prud'homme van Reine and Trono, 2001). For *Gracilaria* tissues culture, the optimal salinity also varies among species, *e.g*., 42 ppt for *G. verrucosa* (Cirik *et al*., 2010), 30 ppt for *Gracilaria tikvahiae* (Kim and Yarish, 2014), and 30-32 ppt for *G. vermiculophylla* (Yokoya *et al*., 1999). At the optimal salinity, the RGR of *G. fisheri* tissues in present study provided 12.6 % day^{-1} while other species such as *G. verrucosa* with 4.9 % day^{-1} and *G. chorda* 4.5 % day^{-1} at 25 ppt (Choi *et al*., 2006), *G. edulis* with 13.6 % day^{-1} at 25 ppt and *G. tenuistipitata* with 19.7 % day^{-1} at 15 ppt (Yu *et al*., 2013). According to Lobban and Harrison (1994), RGR of seaweed depend on species and culture conditions *i.e.,* temperature, light, nutrition level.

Part of tissue is considered as the next important factor on *G. fisheri* tissue culture. In this study, the apical fragment was chosen due to giving the highest RGR with 12.7 % day^{-1}. Hence, it has been more supported to choose apical zone for *Gracilaria* tissue culture since the apical part was reported as the growing point and produced the highest RGR (Martinfar *et al*., 2013). Different parts of thallus determine the growth polarity *e. g*., apical tissues grew with apicobasal polarity whereas sub-apical and basal parts grew with non-polarity. That led to the average number of new branches at apical part being significantly lower ($p<0.05$) than those of other zones. Lobban and Harrison (1994) mentioned that algal apical part composes a lot of apical meristematic cells that can repeatedly divide and grow fast rather than other cells. The apical fragment was chosen for the previous studied *Gracilaria i.e*., *G. verrucosa* and *G. chorda* (Choi *et al*., 2006), *Gracilariopsis persica* (Martinfar *et al*., 2013), *G. vermiculophylla* (Yokoya *et al*., 1999).

Generally, the higher stocking density of tissue caused the negative impact to *G. fisheri* tissue growth and branch formation especially branchlet yield. The branchlets were only produced in the lower densities, 1 and 2 g L^{-1}. Therefore, fragments at lowest stocking density of 1 g L^{-1} showed highest RGR with 31.0 % day^{-1}. This survey was also reported in several species of *Gracilaria;* for instance, at lower density (0.5 g L^{-1}) *G. tikvahiae* grew with the higher growth rate of 13.2 % day^{-1} in comparison to those cultured at higher density (10 g L^{-1}) of 4.5 % day^{-1} (Kim and Yarish, 2014).

G. fisheri tissues and sporelings showed the significantly higher ($p<0.05$) growth rate under red light than those in the other treatments. It was mentioned that the better growth rate in red than blue light of *Porphyra umbilicalis* (Figueroa *et al*., 1995) and some *Gracilaria* spp. e.g., *Gracilaria edulis*, *Gracilaria crassa* and *Gracilaria corticata* (Jayasankar and Kulandaivelu, 2001). It was also reported that red light regulates algal rhizoid formation (Kim *et al.,* 2015) and promotes thallus expansion and cell division (Figueroa *et al*., 1995). Besides, Talarico and Maranzana (2000) said that *Porphyra* and *Palmaria* significantly increased cell wall thickness and growth under red light than in blue light. Likewise, the number and length of new banch in *G. fisheri* was also favoured under red light in this study. The similar

finding on *G. edulis* and *G. corticata* showed the growth of thallus more prominent with high elongated and branched under red light due to the ability to maintain photosynthesis activity (Jayasankar and Kulandaivelu, 2001).

4.3 Tissue culture under different shading color

There is no effect of white and monochromatic lights on pigment content of *G. fisheri* tissues and has long been reported that red macroalgae is only "light intensity adapter" since white and monochromatic lights have same influence on pigment content of the algae (Talarico and Maranzana, 2000). However, the Chl a concentration in *G. fisheri* spore showed significantly higher ($p<0.05$) in green light than in other lights. This finding was similar as *G. edulis* tissues that chlorophyll increased in green light higher than under white, red and blue light (Jayasankar and Kulandaivelu, 2001). Green light affects the spore germination (Kim *et al.,* 2015).

Tissues at outdoor experiment showed the significantly higher ($p<0.05$) biomass in green Saran house than those in other treatments. Figueroa *et al*. (1995) mentioned that at very low intensity of green light, several red algae increase growth higher than in red or blue light. The studied showed that carotenoid content in *G. fisheri* tissue was significant lower ($p<0.05$) in black Saran house than in other treatments. Carotenoids associated with the photosynthetic membranes of all photosynthetic organisms (Naguit and Tisera, 2009). It was mentioned in the previous study that carotenoid and chlorophyll content rather influence by strain than by light source (Kim *et al*., 2015). R-phycoerythrin of tissues showed the significantly higher ($p<0.05$) under white Saran house than in other treatments. Unlikely, it was previously reported that green light promoted maximum increase of R-PE. In red algae, R-PE might play a role in adaptation to sudden irradiance and light spectral changes (Talarico and Maranzana, 2000).

Tissue culture outdoor was affected by the epiphytes (*Rhizoclonium tortuosum* and *Ulva intestinalis*) at the last two weeks. The epiphytes occurred may come from higher light intensity since the rainy season stop. This epiphytic contaminant is a common problem for *G. fisheri* cultivation. Therefore, the result of this study might give the suggestion of black Saran house to limit the epiphytic impact for *G. fisheri* maintenance and cultivation.

The absorbance spectra analysis from chlorophyll extracts of *G. fisheri* tissues outdoor and spore indoor showed the similar result with two peaks at 432 nm and 664 nm. This result showed the slightly difference on absorbance spectra of *G. edulis* which peaked at 441, 443, 476 and 666 nm from chlorophyll extract (Eswasan *et al*., 2002). However, from R-PE extract, the peaks were slightly different from *G. fisheri* sporelings (at 436, 495, 563, 618 and 680 nm) and tissues (at 437, 496, 564, 619 and 678 nm). This result showed slightly different absorbance spectra of *G. fisheri* and with *G. edulis*, *Gracilaria crassa* and *G. corticata* which had absorbance peaks at 433, 495, 565, 621 and 676 nm from R-PE extract (Jayasankar and Kulandaivelu, 2001). Costa and Plastino (2011) reported that the absorbance spectra of red, greenish-brown and green strains of *Gracilaria birdiae* from Chl a extracts peaked at 494 and 564 nm and from phycobiliprotein extracts got three peaks at 494, 564 and 614 nm. The earlier studies reported that the region for R-PE extract is 550-

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630 nm (Kawsar *et al*., 2011) and maximum spectra at 665 nm for chlorophyll extract (Jodlowska and Latala, 2011).

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CHAPETER 5

CONCLUSION

5.1 Relation between biological characteristics of seaweed and physical, chemical parameters of environment.

The yield of *G. fisheri* seemed conversed relation with the nutrient in water. The agar and pigment related positively with the elements which played an important role in photosynthesis. The r-phycoerythrine quite closed to sedimentation and some heavy metals. Element concentration and metal accumulation in seaweed were also related to the water and sediment characteristics. Trace elements in seaweed were mainly dependent on some parameters in sediment and few parameters in water. The concentration of Cu was relation to sand and silt in sediment, the water depth and transparency of water but Cu and Cr in sediment and water hardness related positively to the amount of Mn in the seaweed. Heavy metal accumulation in seaweed mainly depends on water characteristics. The major elements in water were higher than those in sediment and seaweed. The major and trace elements in seaweed were in the order of Mg>K>Ca>Na and Mn>Fe>Zn>Cu, respectively.

5.2 Tissue culture under different conditions

Tissues of *Gracilaria fisheri* in all experiments showed the same characteristics of growth that tissue grew slow at the beginning time (the first 10 days) and started branching, grew faster in weight and new branches after 10 days. Salinity level, part of thallus and propagule density have specific effect on growth and branch formation of *G. fisheri* tissue. Pigment loss of the tissue was only occurred in extreme salinity. Different parts of thallus determine the different growth polarity whereas propagule density regulates branchlet yield of new branch. Tissue length has less effect on *G. fisheri* tissue comparing to the three other factors. In this study, the biomass and RGR did not relate to the number and length of branch of tissues. The biomass and relative growth rate are criteria to evaluate the *G. fisheri* tissue growth. The optimal conditions for *G. fisheri* tissues was recommended to choose the apical fragment, cut into small pieces of 1-2 cm length, culture in the range of 20-25 ppt salinity and use low propagule density of 1 g L^{-1}. The maximum RGR at the optimal conditions, 2 cm length, 20 ppt salinity, apical fragment and 1 g L^{-1} was 31.0 % day^{-1}; the final biomass and length of tissues enhanced 12.4 times and 2.2 times, respectively after 40 days. Therefore, *G. fisheri* tissue culture may be an advantageous method for fast growth and continuous cultivation that is applicable for further cultivation in tanks or other culture systems.

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5.3 Tissue culture under different shading color

Shading from plastic colors caused different spectrum occurrences. *G. fisheri* could adapt to grow under those conditions. Light quality strongly influenced on growth of *G. fisheri* sporelings and tissues but little affected on pigment constituents. Red light is more effective light for the growth of *G. fisheri* tissues and spores in laboratory condition. However, under green Saran plastic house, tissues showed the highest growth rate with the relative high pigment content. Besides, the study found that *G. fisheri* cultivation was less impacted by epiphytes under back Saran house. The study showed the feasible and successful to maintain the strain cultivation for *G. fisheri* sporelings and tissues; hence, it might protect contamination of outdoor cultivation due to high light intensity.

5.4 Suggestions

5.4.1 The further study on the element concentrations in the seaweed, water and sediment should be conducted in the different season to give more information about the seasonal effects on the element concentrations.

5.4.2 More study on tissue culture of *G. fisheri* in different conditions should be conducted with other factors such as temperature, ligh intensity and others in the future.

5.4.3 The indoor study about effect of different shading color condition is recommended to study more about the biological responses of *G. fisheri* tissues and sporelings to know more about spectral effect on *G. fisheri* growth and metabolism.

5.4.4 Outdoor culture of *G. fisheri* tissue should be investigated in red Saran house as similar as the indoor study to see the effect of red color shading on the tissue growth. Besides, the study is suggested to be conducted in the same season to reduce the environmental effects.

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Pattani Campus

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APPENDICES

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APPENDIX 1
METHODOLOGY FOR ELEMENT ANALYSIS

1.1. Element analysis in seaweed and sediment (AOAC, 2000)

1.1.1 Preparation of test sample

Dry ashing: Accurately weigh 1 g test portion, dried and ground, into glazed, high-form porcelain and carefully and 3-4 mL HNO_3 (1+1). Evaporate excess HNO_3 on hot plateset at 100-120^oC (Figure 3.23). Return crucible to furnace and ash additional 1 h at 500^oC. Cool crucible, dissolve ash in 10 mL HCl (1+1), and transfer quantitatively to 50 mL volumetric flask.

Wet ashing: - Accurately weigh 1 g test portioin, dried and ground, into 150 mL Pyrex beaker. Add 10 mL HNO_3 and let soak thoroughy. Add 3 mL 60% $HClO_4$ and heat on hot plate, slowly at first, until frothing ceases. Heat until HNO_3 is almost evaporated. If charring occurs, cool, and 10 mL HNO_3, and continue heating. Heat to white fumes of $HClO_4$. Cool, add 10 mL HCl (1+1), and trasfer quantitatively to 50 mL volumetric flask.

1.1.2 Determination

To solution in 50 mL volumetric flask, add 10 mL 5% Lanthanum (La) solution. Add dilute to volume Let silica settle, decant supernate, and proceed as in 965.09 D (see 2.6.01)

Make necessary dilutions with 10% HCl to obtain solutions with range of instrument.

1.1.3 Calculation

Element, ppm (µg/g) = (µg/mL) x F/g sample

Element, % = ppm (µg/g) x 10^{-4}

Where F= (mL original dilution x mL final dilution)/mL aliquot if original 50 mL is diluted).

1.2. Element analysis in water

1.2.1 Apparatus

Use pyrex, quartz, or Teflon labware exclusively; lean thoroughly with detergent and H_2O; soak in HNO_3 (1+1) for 1 week; deionese with H_2O, dilute HNO_3, and H_2O, in that order. Use deionized, distilled H_2O, whenever H_2O is specified.

Atomic absorption spectrophotometer. Spectrophotometer cabable of operating. Operator become familiar with settings and operations of his apparatus, using table only as guide. Using Boiling burner for aqueous solutions, and premix burner with solvent.

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1.2.2 Reagents

Deionized distilled water.

Nitric acid: Diluted 500 mL redistiled HNO_3 to 1 L with H_2O. (Caution: perform distillation in hood with protective ash in place.)

Hydrochloric acid: Dilute 500 mL HCl to 1L with H_2O and distil in all-Pyrex apparatus.

Metal standard solutions: 1 Stock solution. Accurately weigh amount of metal specified in table into beaker and add dissolving medium. When metal in completely dissolved, transfer quantitatively to 1L volumetric flask and dilute to volume with H_2O. (2) Working solutions. Prepare daily. Dailute aliqouts of stock solutions with H_2O to make ≥4 standard solutions of each element within range of determination. Add 1.5 mL HNO_3/L to all working standard solutions before diluting to volume. Add 1 mL $LaCl_3$/10 mL Mg working standard solution.

Lanthanum stock solution. 50 g La/L ca 50% HCl. Slowly and 250 mL HCl to 50.65 g La_2O_3, dissolve, and dilute to 1 L.

Amonium pyrrolidine dithiocarbamate (APDC) solution. Dissolve 1 g APDC in 100 mL H_2O. Prepare fresh daily.

Preparation of specimen

Dissolved metals. As soon as practicable after collection, filter known volume specimen through 0.45µm membrane. Use first 50-100 mL to rinse flask and discard. Collect filtrate and preserve solution by adding 3 mL HNO_3 (1+1)/L

Suspended metals. Transfer residue and membrane to 250 mL beaker and add 3mL HNO_3. Cover with watch glass to dryness. Cool, and add 3 mL HNO_3, and heat until digestion is complete, generally indicated by light colored residue. Add 2mL HCL (1+1), and heat gently to dissolve residue. Wash watch glass and beaker with H_2O and filter. Wash filter and discard. Dilute filtrate with H_2O to concentration within range of instrument.

Total metal. Transfer aliquot of well mixed sample to beaker and add 3 mL HNO_3. Heat, and evaporate to dryness. (Do not boil). Continue as in beginning "Cool, and add 3 mL HNO_3."

1.2.3 Determination

Ca and Mg determination is eliminated by adding La stock solution to specimen and working standard solutions so that final dilutions contain 1% La.

General method. Set up instrument (Figure 3.24), or previously established optimum settings. Secondary or less sensitive lines may be used to reduce necessary dilution, if desired. Read 4 standard solutions within range before amd after each group of 6-12 specimens, and re-establish 0 A each time. Prepared calibration curve from average f each standard before and after specimen group. Read specimen concentration from plot of *A* against mg/L

Special extration method. When Pb or Cd concentration is too low for direct determinations, transfer specimen aliquot to 250 mL beaker and dilute to 100 mL with H_2O. Prepare blank and standards in sam manner. Adjust pH of specimen and standard solution to 2.5 with HCl, using pH meter. Transfer quantitatively to 200 mL volumetric flask, add 2.5 mL APDC solution, and mix. Add 10 mL methyl isobutyl ketone and shake vigorously 1 min. Let layers seperate; then add H_2O until ketone layer is in neck of flask. (Centrifuation maybe necessary). Aspirate ketone layer and

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record reading of standards and specimens against blank. (Fuel-to-air ratio should be adjusted to as blue a flame as possible, since organic solvent adds to fuel suply). Prepare clibration curve from average of each standard and read specimen concentration from plot (mg/L).

Figure 1. Heating the solution of seaweed and HNO_3

Figure 2. Analysis metal and mineral contents by using ASS 100

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1.2.4 Caculation

General method: mg Metal/L = (mg metal in aliquot/L)x F
where F = final dilution/mL aliqout
Special extraction method: mg Metal/L = mg metal in aliqout/L

1.3. Sediment particle size analysis (Pipette method; Sheldrick and Wang, 1993)

13.1 Removal of Carbonates

1) Weigh 10 g of 2 mm air dried sediment into 300 mL fleaker
2) Add 50 mL of water and mix
3) Add 1mL HCl slowly until the pH fall between 3.5-4 and remains for 10 minutes.

1.3.2 Removal of organic matter

1) Add 10 mL of hydrogen peroxide to the fleakers until no more frothing occurs.
2) Put the fleakers on hot plot at 90°C and continue adding H_2O_2 and heating until most of organic matter destroy
3) Continue to heat the sample for 45 minutes to remove excess hydrogen peroxide

1.3.4 Removal of soluble salts

1) Place the fleakers in a rack and filter the remaining peroxide
2) Add 150 mL water in a jet strong enough to stir the sample
3) Remove sediment adhearing to the filter candle
4) Place the sample in an oven vernight at 105°C

1.3.5 Dispersion of sample

1) Add 10 mL of sodium metaphosphate dispersing
2) Stopper tightly and shake end-over-end overnight

1.3.6 Separation of sand fractions

1) Pour the suspensions through a 300 mesh sieve up to 1L.
2) Wash the sand retained on the sieve
3) Transfer the sand to 100 mL beaker and oven dry at 105°C
4) Transfer dried sand to a set of sieves
5) Weigh each sand fraction and record the weight to determine percent of sand

1.3.7 Determination of clay

1) Stir the material in the sedimentation cylinders for 4 minutes
2) Stir the suspension for 30 second with a hand stirrer (up and down motion)
3) After a predetermined settling time due to the sample's temperature
4) Take 20 mL of the suspension from the fleakers at 5 cm into 100 mL beakers
5) Evaporate the water and dry I the oven at 105°C for at least 24 hours
6) Record the weight to determine the percent of clay

The percentage of silt is determined by:

% silt = 100- (% sand + % clay)

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1.4. Sediment organic matter analysis (Walkley-Black Procedure; Nelson and Sommers, 1982)

1.4.1 Sediment (0.5-2 g) put into the flask

1.4.2 Add 10 mL $K_2Cr_2O_7$ and shake

1.4.3 Add 20 mL of solution H_2SO_4 concentrated and shake slowly for about 1-2 min, then wait for 20-30 min

1.4.4 Add 100 mL water

1.4.5 Add 4-5 drops *o*-Phenanthroline indicator

The blank is prepared in the same way but without sediment. The samples were titrated by $(NH_2)_2Fe(SO_4)_2.6H_2O$ 0.5N until the color changing from green to brown color.

The organic matter of sediment was determined by:

Organic matter = Organic Carbon × 1.724

$$\text{Total C, \%} = \frac{ml(blank) - ml(sample)}{g\ soil} \times N_{HCl} \times 0.6$$

(Nelson and Sommers, 1982)

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Appendix 2

CULTURE MEDIUM

Table 1. The nutrient component of Modified Grund Medium

Component	Stock Solution ($g.L^{-1}dH_2O$)	Quantity Used (mL)	Concentration in final medium (M)
Na_2 β-glycerophosphate	5.36	10	2.48×10^{-4}
$NaNO_3$	42.52	10	5.00×10^{-3}
$FeSO_4.7H_2O$	0.28	10	1.00×10^{-5}
$MnCl_2.4H_2O$	1.96	10	1.00×10^{-4}
$Na_2EDTA.2H_2O$	3.72	10	1.00×10^{-4}
Vitamin stock solution (Table 3)		10	-

Source: Mensi *et al*. (2011)

Table 2. The component of vitamins stock solution

Component	Stock solution	Used Quantity	Concentration in final medium (M)
Thiamine.HCl (vitamin B_1)	-	200 mg	5.93×10^{-6}
Biotin (vitamin H)	0.1	1 mL	4.09×10^{-9}
Cyanocobalamin (vitamin B_{12})	0.2	1mL	1.48×10^{-9}

Source: Mensi *et al*. (2011)

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Appendix 3

ANALYSIS OF VARIANCE (ANOVA)

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Table 1. ANOVA of elements in *Gracilaria fisheri* from the cultivated ponds

		Sum of Squares	df	Mean Square	F	Sig.
Ca	Between Groups	1.414	2	.707	.354	.711
	Within Groups	17.960	9	1.996		
	Total	19.373	11			
Mg	Between Groups	9.710	2	4.855	3.881	.061
	Within Groups	11.259	9	1.251		
	Total	20.968	11			
K	Between Groups	1135.645	2	567.823	113.212	.000
	Within Groups	45.140	9	5.016		
	Total	1180.785	11			
Na	Between Groups	25.001	2	12.500	8.588	.008
	Within Groups	13.100	9	1.456		
	Total	38.100	11			
Cu	Between Groups	.000	2	.000	1.000	.405
	Within Groups	.000	9	.000		
	Total	.000	11			
Mn	Between Groups	.093	2	.046	1.079	.380
	Within Groups	.386	9	.043		
	Total	.479	11			
Zn	Between Groups	.000	2	.000	.913	.435
	Within Groups	.001	9	.000		
	Total	.001	11			
Fe	Between Groups	.949	2	.474	2.032	.187
	Within Groups	2.101	9	.233		
	Total	3.049	11			
Ni	Between Groups	10.411	2	5.205	1.609	.253
	Within Groups	29.119	9	3.235		
	Total	39.530	11			
Cr	Between Groups	4.058	2	2.029	.613	.563
	Within Groups	29.803	9	3.311		
	Total	33.860	11			
Cd	Between Groups	.005	2	.002	9.663	.006
	Within Groups	.002	9	.000		
	Total	.007	11			
Pb	Between Groups	6.882	2	3.441	.423	.667
	Within Groups	73.151	9	8.128		
	Total	80.033	11			

Table 2. ANOVA of content and other characteristics of *Gracilaria fisheri*

		Sum of Squares	df	Mean Square	F	Sig.
Yield	Between Groups	2853.500	2	1426.750	59.794	.000
	Within Groups	214.750	9	23.861		
	Total	3068.250	11			
Carotenoids	Between Groups	658.652	2	329.326	.477	.636
	Within Groups	6214.845	9	690.538		
	Total	6873.497	11			
Chla	Between Groups	4910.826	2	2455.413	.588	.575
	Within Groups	37583.401	9	4175.933		
	Total	42494.227	11			
Chlb	Between Groups	13.775	2	6.887	.158	.856
	Within Groups	392.692	9	43.632		
	Total	406.467	11			
Chlc	Between Groups	35.684	2	17.842	.354	.711
	Within Groups	453.733	9	50.415		
	Total	489.417	11			
Phycoerytrine	Between Groups	1155.796	2	577.898	2.130	.175
	Within Groups	2441.397	9	271.266		
	Total	3597.193	11			
Agar	Between Groups	154.854	2	77.427	4.193	.052
	Within Groups	166.190	9	18.466		
	Total	321.043	11			
Moisture	Between Groups	24.634	2	12.317	19.364	.001
	Within Groups	5.725	9	.636		
	Total	30.359	11			
Contaminants	Between Groups	109.500	2	54.750	9.386	.006
	Within Groups	52.500	9	5.833		
	Total	162.000	11			

Table 3. ANOVA of color of *G. fisheri*

		Sum of Squares	df	Mean Square	F	Sig.
dL	Between Groups	14.745	2	7.372	.825	.469
	Within Groups	80.467	9	8.941		
	Total	95.212	11			
da	Between Groups	.160	2	.080	.613	.563
	Within Groups	1.174	9	.130		
	Total	1.334	11			
db	Between Groups	.822	2	.411	.246	.787
	Within Groups	15.031	9	1.670		
	Total	15.853	11			
dE	Between Groups	14.540	2	7.270	.833	.466
	Within Groups	78.523	9	8.725		
	Total	93.063	11			

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Table 4. ANOVA of elements and other characteristics in water of cultivated ponds

		Sum of Squares	df	Mean Square	F	Sig.
Ca	Between Groups	1147756.167	2	573878.083	.398	.683
	Within Groups	1.296E7	9	1440504.833		
	Total	1.411E7	11			
Mg	Between Groups	95455.167	2	47727.583	8.471	.009
	Within Groups	50710.500	9	5634.500		
	Total	146165.667	11			
K	Between Groups	130991.167	2	65495.583	1.029	.396
	Within Groups	572885.750	9	63653.972		
	Total	703876.917	11			
Na	Between Groups	478443.500	2	239221.750	.574	.583
	Within Groups	3752535.500	9	416948.389		
	Total	4230979.000	11			
Cu	Between Groups	.000	2	.000	.233	.797
	Within Groups	.002	9	.000		
	Total	.002	11			
Mn	Between Groups	.104	2	.052	3.320	.083
	Within Groups	.141	9	.016		
	Total	.245	11			
Zn	Between Groups	.001	2	.001	3.627	.070
	Within Groups	.002	9	.000		
	Total	.003	11			
Fe	Between Groups	.764	2	.382	7.154	.014
	Within Groups	.481	9	.053		
	Total	1.245	11			
Ni	Between Groups	.023	2	.011	.477	.635
	Within Groups	.215	9	.024		
	Total	.238	11			
Cr	Between Groups	.001	2	.001	.796	.480
	Within Groups	.007	9	.001		
	Total	.008	11			
Cd	Between Groups	.000	2	.000	.362	.706
	Within Groups	.001	9	.000		
	Total	.002	11			
Pb	Between Groups	.043	2	.021	.495	.625
	Within Groups	.389	9	.043		
	Total	.432	11			

		Sum of Squares	df	Mean Square	F	Sig.
Phosphate-phosphorus	Between Groups	.007	2	.003	5.193	.032
	Within Groups	.006	9	.001		
	Total	.013	11			
Nitrate-nitrogen	Between Groups	.191	2	.096	74.562	.000
	Within Groups	.012	9	.001		
	Total	.203	11			
Alkalinity	Between Groups	4016.167	2	2008.083	2.342	.152
	Within Groups	7716.500	9	857.389		
	Total	11732.667	11			
Hardness	Between Groups	4266387.167	2	2133193.583	1.430	.289
	Within Groups	1.343E7	9	1492204.611		
	Total	1.770E7	11			
pH	Between Groups	.087	2	.043	.414	.673
	Within Groups	.943	9	.105		
	Total	1.029	11			
Salinity	Between Groups	195.167	2	97.583	12.591	.002
	Within Groups	69.750	9	7.750		
	Total	264.917	11			
Temperature	Between Groups	14.042	2	7.021	2.368	.149
	Within Groups	26.688	9	2.965		
	Total	40.729	11			
Depth	Between Groups	4038.000	2	2019.000	3.063	.097
	Within Groups	5932.250	9	659.139		
	Total	9970.250	11			
Transparency	Between Groups	1567.167	2	783.583	3.125	.093
	Within Groups	2256.750	9	250.750		
	Total	3823.917	11			
Light	Between Groups	38850.500	2	19425.250	.195	.826
	Within Groups	897289.750	9	99698.861		
	Total	936140.250	11			

Table 5. ANOVA of elements in sediment of the cultivated ponds

		Sum of Squares	df	Mean Square	F	Sig.
Ca	Between Groups	83044.319	2	41522.160	7.963	.010
	Within Groups	46927.875	9	5214.208		
	Total	129972.195	11			
Mg	Between Groups	1700.418	2	850.209	1.141	.362
	Within Groups	6704.578	9	744.953		
	Total	8404.996	11			
K	Between Groups	347.478	2	173.739	.110	.897
	Within Groups	14170.498	9	1574.500		
	Total	14517.976	11			
Na	Between Groups	412.134	2	206.067	.584	.577
	Within Groups	3173.087	9	352.565		
	Total	3585.221	11			
Cu	Between Groups	.044	2	.022	4.569	.043
	Within Groups	.044	9	.005		
	Total	.088	11			
Mn	Between Groups	49.705	2	24.852	5.707	.025
	Within Groups	39.194	9	4.355		
	Total	88.899	11			
Zn	Between Groups	.046	2	.023	.524	.609
	Within Groups	.396	9	.044		
	Total	.442	11			
Fe	Between Groups	4.110	2	2.055	3.378	.080
	Within Groups	5.475	9	.608		
	Total	9.585	11			
Ni	Between Groups	17440.543	2	8720.271	3.602	.071
	Within Groups	21789.395	9	2421.044		
	Total	39229.937	11			
Cr	Between Groups	26.525	2	13.263	2.712	.120
	Within Groups	44.011	9	4.890		
	Total	70.537	11			
Cd	Between Groups	.123	2	.061	.388	.689
	Within Groups	1.426	9	.158		
	Total	1.549	11			
Pb	Between Groups	193.453	2	96.726	.653	.544
	Within Groups	1333.263	9	148.140		
	Total	1526.716	11			

Table 6. ANOVA of sediment charateristics

Percentage (%)		Sum of Squares	df	Mean Square	F	Sig.
Organic carbon	Between Groups	5.844	2	2.922	7.597	.012
	Within Groups	3.461	9	.385		
	Total	9.305	11			
Organic matter	Between Groups	17.347	2	8.674	7.627	.012
	Within Groups	10.235	9	1.137		
	Total	27.582	11			
Sand	Between Groups	3534.773	2	1767.386	2.386	.147
	Within Groups	6667.089	9	740.788		
	Total	10201.861	11			
Clay	Between Groups	2.187	2	1.094	1.536	.267
	Within Groups	6.410	9	.712		
	Total	8.597	11			
Silt	Between Groups	3712.996	2	1856.498	2.599	.129
	Within Groups	6428.055	9	714.228		
	Total	10141.051	11			

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Table 7. ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	285.509	4	71.377	.707	.605
	Within Groups	1010.000	10	101.000		
	Total	1295.509	14			
Day20	Between Groups	827.520	4	206.880	.843	.529
	Within Groups	2453.440	10	245.344		
	Total	3280.960	14			
Day30	Between Groups	3358.933	4	839.733	.518	.725
	Within Groups	16224.000	10	1622.400		
	Total	19582.933	14			
Day40	Between Groups	61374.933	4	15343.733	3.703	.042
	Within Groups	41440.000	10	4144.000		
	Total	102814.933	14			

Table 8. ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	2.855	4	.714	.707	.605
	Within Groups	10.100	10	1.010		
	Total	12.955	14			
Day20	Between Groups	2.069	4	.517	.843	.529
	Within Groups	6.134	10	.613		
	Total	8.202	14			
Day30	Between Groups	3.729	4	.932	.517	.725
	Within Groups	18.019	10	1.802		
	Total	21.749	14			
Day40	Between Groups	38.359	4	9.590	3.703	.042
	Within Groups	25.900	10	2.590		
	Total	64.259	14			

Table 9. ANOVA of increased length (%) of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	149.337	4	37.334	33.601	.000
	Within Groups	11.111	10	1.111		
	Total	160.448	14			
Day20	Between Groups	550.840	4	137.710	11.747	.001
	Within Groups	117.234	10	11.723		
	Total	668.075	14			
Day30	Between Groups	311.653	4	77.913	10.570	.001
	Within Groups	73.712	10	7.371		
	Total	385.364	14			
Day40	Between Groups	572.229	4	143.057	12.180	.001
	Within Groups	117.449	10	11.745		
	Total	689.679	14			

Table 10. ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.118	4	.029	.723	.596
	Within Groups	.406	10	.041		
	Total	.524	14			
Day20	Between Groups	.334	4	.084	.851	.525
	Within Groups	.981	10	.098		
	Total	1.315	14			
Day30	Between Groups	1.344	4	.336	.518	.725
	Within Groups	6.490	10	.649		
	Total	7.833	14			
Day40	Between Groups	24.550	4	6.137	3.703	.042
	Within Groups	16.576	10	1.658		
	Total	41.126	14			

Table 11. ANOVA of number of new branch per cm of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	1.553	4	.388	3.588	.046
	Within Groups	1.082	10	.108		
	Total	2.635	14			
Day20	Between Groups	2.790	4	.698	6.626	.007
	Within Groups	1.053	10	.105		
	Total	3.843	14			
Day30	Between Groups	4.587	4	1.147	7.485	.005
	Within Groups	1.532	10	.153		
	Total	6.119	14			
Day40	Between Groups	5.541	4	1.385	14.254	.000
	Within Groups	.972	10	.097		
	Total	6.512	14			

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Table 12. ANOVA of branch length (cm) of *G. fisheri* tissue culture under different initial lengths

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.003	4	.001	3.136	.065
	Within Groups	.002	10	.000		
	Total	.005	14			
Day20	Between Groups	.023	4	.006	4.632	.022
	Within Groups	.012	10	.001		
	Total	.035	14			
Day30	Between Groups	.133	4	.033	8.768	.003
	Within Groups	.038	10	.004		
	Total	.170	14			
Day40	Between Groups	1.227	4	.307	5.298	.015
	Within Groups	.579	10	.058		
	Total	1.806	14			

Table 13. ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	5693.211	4	1423.303	1.419	.297
	Within Groups	10032.347	10	1003.235		
	Total	15725.557	14			
Day20	Between Groups	22272.997	4	5568.249	5.568	.013
	Within Groups	10001.200	10	1000.120		
	Total	32274.197	14			
Day30	Between Groups	77795.936	4	19448.984	8.580	.003
	Within Groups	22668.773	10	2266.877		
	Total	100464.709	14			
Day40	Between Groups	237267.291	4	59316.823	16.096	.000
	Within Groups	36852.053	10	3685.205		
	Total	274119.344	14			

Prince of Songkla University Pattani Campus

Table 14. ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	56.932	4	14.233	1.419	.297
	Within Groups	100.323	10	10.032		
	Total	157.256	14			
Day20	Between Groups	55.682	4	13.921	5.568	.013
	Within Groups	25.003	10	2.500		
	Total	80.685	14			
Day30	Between Groups	86.410	4	21.602	8.579	.003
	Within Groups	25.179	10	2.518		
	Total	111.589	14			
Day40	Between Groups	148.365	4	37.091	16.072	.000
	Within Groups	23.078	10	2.308		
	Total	171.443	14			

Table 15. ANOVA of increased length (%) of *G. fisheri* tissue culture under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	290.094	4	72.524	33.111	.000
	Within Groups	21.903	10	2.190		
	Total	311.997	14			
Day20	Between Groups	394.208	4	98.552	6.683	.007
	Within Groups	147.474	10	14.747		
	Total	541.682	14			
Day30	Between Groups	716.298	4	179.075	10.197	.001
	Within Groups	175.622	10	17.562		
	Total	891.920	14			
Day40	Between Groups	904.020	4	226.005	8.789	.003
	Within Groups	257.153	10	25.715		
	Total	1161.173	14			

Table 16. ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	2.236	4	.559	1.384	.307
	Within Groups	4.040	10	.404		
	Total	6.276	14			
Day20	Between Groups	8.844	4	2.211	5.349	.014
	Within Groups	4.133	10	.413		
	Total	12.977	14			
Day30	Between Groups	31.263	4	7.816	8.526	.003
	Within Groups	9.167	10	.917		
	Total	40.429	14			
Day40	Between Groups	94.773	4	23.693	16.295	.000
	Within Groups	14.540	10	1.454		
	Total	109.313	14			

Prince of Songkla University Pattani Campus

Table 17. ANOVA of number of new branch per cm of *G. fisheri* tissue under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.908	4	.227	9.781	.002
	Within Groups	.232	10	.023		
	Total	1.140	14			
Day20	Between Groups	4.065	4	1.016	5.294	.015
	Within Groups	1.920	10	.192		
	Total	5.985	14			
Day30	Between Groups	2.344	4	.586	6.713	.007
	Within Groups	.873	10	.087		
	Total	3.217	14			
Day40	Between Groups	2.969	4	.742	6.157	.009
	Within Groups	1.205	10	.121		
	Total	4.174	14			

Table 18. ANOVA of branch length (cm) of *G. fisheri* tissue under different salinity levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.010	4	.003	.634	.650
	Within Groups	.041	10	.004		
	Total	.051	14			
Day20	Between Groups	.126	4	.032	5.787	.011
	Within Groups	.055	10	.005		
	Total	.181	14			
Day30	Between Groups	.475	4	.119	16.376	.000
	Within Groups	.073	10	.007		
	Total	.548	14			
Day40	Between Groups	1.032	4	.258	11.297	.001
	Within Groups	.228	10	.023		
	Total	1.260	14			

Prince of Songkla University Pattani Campus

Table 19. ANOVA of increased biomass (%) of *G. fisheri* tissue culture from different parts of thallus

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	1264.062	2	632.031	3.959	.080
	Within Groups	957.787	6	159.631		
	Total	2221.849	8			
Day20	Between Groups	7521.170	2	3760.585	5.470	.044
	Within Groups	4125.099	6	687.516		
	Total	11646.269	8			
Day30	Between Groups	9490.667	2	4745.333	1.639	.270
	Within Groups	17373.333	6	2895.556		
	Total	26864.000	8			
Day40	Between Groups	25755.556	2	12877.778	6.653	.030
	Within Groups	11613.333	6	1935.556		
	Total	37368.889	8			

Table 20. ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture from different parts of thallus

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	12.641	2	6.320	3.959	.080
	Within Groups	9.578	6	1.596		
	Total	22.218	8			
Day20	Between Groups	18.803	2	9.402	5.470	.044
	Within Groups	10.312	6	1.719		
	Total	29.115	8			
Day30	Between Groups	10.537	2	5.269	1.635	.271
	Within Groups	19.332	6	3.222		
	Total	29.870	8			
Day40	Between Groups	16.097	2	8.049	6.653	.030
	Within Groups	7.258	6	1.210		
	Total	23.356	8			

Table 21. ANOVA of increased length (%) of *G. fisheri* tissue culture from different parts of thallus

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	1024.722	2	512.361	54.689	.000
	Within Groups	56.212	6	9.369		
	Total	1080.933	8			
Day20	Between Groups	7140.500	2	3570.250	27.202	.001
	Within Groups	787.500	6	131.250		
	Total	7928.000	8			
Day30	Between Groups	21195.722	2	10597.861	179.709	.000
	Within Groups	353.833	6	58.972		
	Total	21549.556	8			
Day40	Between Groups	24827.722	2	12413.861	38.652	.000
	Within Groups	1927.000	6	321.167		
	Total	26754.722	8			

Tale 22. ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture from different parts of thallus

Biomass		Sum of Squares	df	Mean Square	F	Sig.
Day	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
Day10	Between Groups	.506	2	.253	3.963	.080
	Within Groups	.383	6	.064		
	Total	.888	8			
Day20	Between Groups	3.013	2	1.506	5.457	.045
	Within Groups	1.656	6	.276		
	Total	4.669	8			
Day30	Between Groups	3.796	2	1.898	1.639	.270
	Within Groups	6.949	6	1.158		
	Total	10.746	8			
Day40	Between Groups	10.302	2	5.151	6.653	.030
	Within Groups	4.645	6	.774		
	Total	14.948	8			

Prince of Songkla University Pattani Campus

Table 23. ANOVA of number of new branch per cm of *G. fisheri* tissue from different parts of thallus

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
Day10	Between Groups	.624	2	.312	4.111	.075
	Within Groups	.455	6	.076		
	Total	1.079	8			
Day20	Between Groups	7.536	2	3.768	35.605	.000
	Within Groups	.635	6	.106		
	Total	8.171	8			
Day30	Between Groups	5.841	2	2.920	12.568	.007
	Within Groups	1.394	6	.232		
	Total	7.235	8			
Day40	Between Groups	6.822	2	3.411	11.488	.009
	Within Groups	1.782	6	.297		
	Total	8.604	8			

Table 24. ANOVA of branch length (cm) of *G. fisheri* tissue from different parts of thallus

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	2	.000	.	.
	Within Groups	.000	6	.000		
	Total	.000	8			
Day10	Between Groups	.001	2	.000	.795	.494
	Within Groups	.003	6	.000		
	Total	.003	8			
Day20	Between Groups	.040	2	.020	20.360	.002
	Within Groups	.006	6	.001		
	Total	.046	8			
Day30	Between Groups	.817	2	.408	24.107	.001
	Within Groups	.102	6	.017		
	Total	.919	8			
Day40	Between Groups	1.225	2	.612	14.935	.005
	Within Groups	.246	6	.041		
	Total	1.471	8			

Prince of Songkla University Pattani Campus

Table 25. ANOVA of increased biomass (%) of *G. fisheri* under different density levels

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	25274.883	4	6318.721	10.407	.001
	Within Groups	6071.346	10	607.135		
	Total	31346.229	14			
Day20	Between Groups	248492.226	4	62123.057	34.576	.000
	Within Groups	17967.167	10	1796.717		
	Total	266459.393	14			
Day30	Between Groups	1017627.644	4	254406.911	61.704	.000
	Within Groups	41229.993	10	4122.999		
	Total	1058857.637	14			
Day40	Between Groups	1970420.267	4	492605.067	44.486	.000
	Within Groups	110732.944	10	11073.294		
	Total	2081153.211	14			

Table 26. ANOVA of RGR (% day^{-1}) of *G. fisheri* under different density levels

RGR		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	252.798	4	63.199	10.410	.001
	Within Groups	60.713	10	6.071		
	Total	313.511	14			
Day20	Between Groups	621.288	4	155.322	34.575	.000
	Within Groups	44.924	10	4.492		
	Total	666.211	14			
Day30	Between Groups	1130.510	4	282.628	61.725	.000
	Within Groups	45.788	10	4.579		
	Total	1176.298	14			
Day40	Between Groups	1231.513	4	307.878	44.480	.000
	Within Groups	69.218	10	6.922		
	Total	1300.730	14			

Table 27. ANOVA of increased length (%) of *G. fisheri* under different density levels

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	2735.000	4	683.750	9.052	.002
	Within Groups	755.333	10	75.533		
	Total	3490.333	14			
Day20	Between Groups	10486.667	4	2621.667	22.794	.000
	Within Groups	1150.167	10	115.017		
	Total	11636.833	14			
Day30	Between Groups	26677.767	4	6669.442	23.120	.000
	Within Groups	2884.667	10	288.467		
	Total	29562.433	14			
Day40	Between Groups	26019.567	4	6504.892	16.067	.000
	Within Groups	4048.667	10	404.867		
	Total	30068.233	14			

Table 28. ANOVA of biomass (g L^{-1}) of *G. fisheri* under different density levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	98.400	4	24.600	.	.
	Within Groups	.000	10	.000		
	Total	98.400	14			
Day10	Between Groups	222.310	4	55.578	142.633	.000
	Within Groups	3.897	10	.390		
	Total	226.207	14			
Day20	Between Groups	238.555	4	59.639	113.411	.000
	Within Groups	5.259	10	.526		
	Total	243.814	14			
Day30	Between Groups	240.096	4	60.024	52.172	.000
	Within Groups	11.505	10	1.151		
	Total	251.601	14			
Day40	Between Groups	424.145	4	106.036	41.225	.000
	Within Groups	25.722	10	2.572		
	Total	449.867	14			

Prince of Songkla University Pattani Campus

Table 29. ANOVA of number of new branch per cm of *G. fisheri* under different density levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.097	4	.024	.380	.818
	Within Groups	.640	10	.064		
	Total	.737	14			
Day20	Between Groups	5.897	4	1.474	6.621	.007
	Within Groups	2.227	10	.223		
	Total	8.124	14			
Day30	Between Groups	37.011	4	9.253	23.093	.000
	Within Groups	4.007	10	.401		
	Total	41.017	14			
Day40	Between Groups	70.320	4	17.580	41.725	.000
	Within Groups	4.213	10	.421		
	Total	74.533	14			

Table 30. ANOVA of branch length (cm) of *G. fisheri* under different density levels

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	4	.000	.	.
	Within Groups	.000	10	.000		
	Total	.000	14			
Day10	Between Groups	.001	4	.000	.123	.971
	Within Groups	.020	10	.002		
	Total	.021	14			
Day20	Between Groups	.127	4	.032	2.249	.136
	Within Groups	.141	10	.014		
	Total	.268	14			
Day30	Between Groups	.366	4	.092	12.211	.001
	Within Groups	.075	10	.007		
	Total	.441	14			
Day40	Between Groups	.005	4	.001	8.167	.003
	Within Groups	.001	10	.000		
	Total	.006	14			

Prince of Songkla University Pattani Campus

Table 31. ANOVA of pigment content (µg g^{-1} FW) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Carotenoids	Between Groups	206.163	3	68.721	.748	.554
	Within Groups	735.412	8	91.927		
	Total	941.575	11			
Chla	Between Groups	5018.199	3	1672.733	2.024	.189
	Within Groups	6612.687	8	826.586		
	Total	11630.886	11			
Chlb	Between Groups	20.534	3	6.845	.652	.604
	Within Groups	83.995	8	10.499		
	Total	104.530	11			
Chlc	Between Groups	20.202	3	6.734	3.976	.053
	Within Groups	13.549	8	1.694		
	Total	33.752	11			
R-PE	Between Groups	10275.926	3	3425.309	3.555	.067
	Within Groups	7707.576	8	963.447		
	Total	17983.503	11			

Table 32. ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	1187.606	3	395.869	8.133	.003
	Within Groups	584.091	12	48.674		
	Total	1771.696	15			
Day20	Between Groups	850.208	3	283.403	3.705	.043
	Within Groups	917.947	12	76.496		
	Total	1768.155	15			
Day30	Between Groups	3379.903	3	1126.634	8.828	.002
	Within Groups	1531.433	12	127.619		
	Total	4911.337	15			
Day40	Between Groups	2464.149	3	821.383	6.532	.007
	Within Groups	1508.997	12	125.750		
	Total	3973.147	15			

Table 33. ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	11.878	3	3.959	8.116	.003
	Within Groups	5.854	12	.488		
	Total	17.732	15			
Day20	Between Groups	2.124	3	.708	3.705	.043
	Within Groups	2.293	12	.191		
	Total	4.418	15			
Day30	Between Groups	3.740	3	1.247	8.803	.002
	Within Groups	1.700	12	.142		
	Total	5.440	15			
Day40	Between Groups	1.544	3	.515	6.534	.007
	Within Groups	.945	12	.079		
	Total	2.490	15			

Prince of Songkla University Pattani Campus

Table 34 ANOVA of increased length (%) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	66.125	3	22.042	6.432	.008
	Within Groups	41.125	12	3.427		
	Total	107.250	15			
Day20	Between Groups	59.125	3	19.708	6.781	.006
	Within Groups	34.875	12	2.906		
	Total	94.000	15			
Day30	Between Groups	50.562	3	16.854	1.687	.223
	Within Groups	119.875	12	9.990		
	Total	170.438	15			
Day40	Between Groups	40.312	3	13.438	.729	.554
	Within Groups	221.125	12	18.427		
	Total	261.438	15			

Table 35. ANOVA of biomass (g L^{-1}) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	3	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	15			
Day10	Between Groups	.119	3	.040	7.945	.003
	Within Groups	.060	12	.005		
	Total	.179	15			
Day20	Between Groups	.085	3	.028	3.699	.043
	Within Groups	.092	12	.008		
	Total	.177	15			
Day30	Between Groups	.336	3	.112	8.536	.003
	Within Groups	.158	12	.013		
	Total	.494	15			
Day40	Between Groups	.248	3	.083	6.518	.007
	Within Groups	.152	12	.013		
	Total	.401	15			

Table 36. ANOVA of number of new branch per cm of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	3	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	15			
Day10	Between Groups	.675	3	.225	8.133	.003
	Within Groups	.332	12	.028		
	Total	1.007	15			
Day20	Between Groups	1.184	3	.395	7.002	.006
	Within Groups	.676	12	.056		
	Total	1.860	15			
Day30	Between Groups	.548	3	.183	3.711	.043
	Within Groups	.591	12	.049		
	Total	1.139	15			
Day40	Between Groups	.689	3	.230	3.598	.046
	Within Groups	.766	12	.064		
	Total	1.455	15			

Prince of Songkla University Pattani Campus

Table 37. ANOVA of branch length (cm) of *G. fisheri* tissue culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	3	.000	.	.
	Within Groups	.000	12	.000		
	Total	.000	15			
Day10	Between Groups	.010	3	.003	14.246	.000
	Within Groups	.003	12	.000		
	Total	.013	15			
Day20	Between Groups	.018	3	.006	3.525	.049
	Within Groups	.021	12	.002		
	Total	.039	15			
Day30	Between Groups	.029	3	.010	5.289	.015
	Within Groups	.022	12	.002		
	Total	.051	15			
Day40	Between Groups	.044	3	.015	3.622	.045
	Within Groups	.049	12	.004		
	Total	.093	15			

Table 38. ANOVA of increased biomass (%) of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	2597.583	3	865.861	28.703	.000
	Within Groups	241.333	8	30.167		
	Total	2838.917	11			
Day20	Between Groups	3458.000	3	1152.667	29.120	.000
	Within Groups	316.667	8	39.583		
	Total	3774.667	11			
Day30	Between Groups	7550.250	3	2516.750	28.984	.000
	Within Groups	694.667	8	86.833		
	Total	8244.917	11			
Day40	Between Groups	13754.917	3	4584.972	28.390	.000
	Within Groups	1292.000	8	161.500		
	Total	15046.917	11			

Table 39. ANOVA of RGR (% day^{-1}) of *G. fisheri* spore culture under different shading colors indoor experiment

RGR		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	25.976	3	8.659	28.703	.000
	Within Groups	2.413	8	.302		
	Total	28.389	11			
Day20	Between Groups	8.645	3	2.882	29.120	.000
	Within Groups	.792	8	.099		
	Total	9.437	11			
Day30	Between Groups	8.404	3	2.801	28.829	.000
	Within Groups	.777	8	.097		
	Total	9.181	11			
Day40	Between Groups	8.633	3	2.878	28.722	.000
	Within Groups	.802	8	.100		
	Total	9.435	11			

Table 40. ANOVA of increased length (%) of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day10	Between Groups	707.687	3	235.896	8.504	.007
	Within Groups	221.905	8	27.738		
	Total	929.591	11			
Day20	Between Groups	469.078	3	156.359	3.285	.079
	Within Groups	380.736	8	47.592		
	Total	849.814	11			
Day30	Between Groups	1238.651	3	412.884	5.851	.020
	Within Groups	564.514	8	70.564		
	Total	1803.165	11			
Day40	Between Groups	1748.128	3	582.709	8.726	.007
	Within Groups	534.207	8	66.776		
	Total	2282.335	11			

Table 41. ANOVA of biomass (g L^{-1}) of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.000	3	.000	.	.
	Within Groups	.000	8	.000		
	Total	.000	11			
Day10	Between Groups	.028	3	.009	27.602	.000
	Within Groups	.003	8	.000		
	Total	.031	11			
Day20	Between Groups	.040	3	.013	29.309	.000
	Within Groups	.004	8	.000		
	Total	.044	11			
Day30	Between Groups	.085	3	.028	30.250	.000
	Within Groups	.007	8	.001		
	Total	.092	11			
Day40	Between Groups	.152	3	.051	29.599	.000
	Within Groups	.014	8	.002		
	Total	.166	11			

Table 42. ANOVA of number of new branch per cm of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.061	3	.020	.776	.539
	Within Groups	.211	8	.026		
	Total	.273	11			
Day10	Between Groups	.153	3	.051	1.609	.262
	Within Groups	.254	8	.032		
	Total	.407	11			
Day20	Between Groups	.304	3	.101	7.667	.010
	Within Groups	.106	8	.013		
	Total	.409	11			
Day30	Between Groups	.125	3	.042	1.382	.317
	Within Groups	.241	8	.030		
	Total	.366	11			
Day40	Between Groups	.339	3	.113	2.938	.099
	Within Groups	.307	8	.038		
	Total	.646	11			

Table 43. ANOVA of branch length (cm) of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Day0	Between Groups	.002	3	.001	.163	.918
	Within Groups	.024	8	.003		
	Total	.026	11			
Day10	Between Groups	.062	3	.021	1.069	.415
	Within Groups	.154	8	.019		
	Total	.216	11			
Day20	Between Groups	.133	3	.044	9.134	.006
	Within Groups	.039	8	.005		
	Total	.172	11			
Day30	Between Groups	.108	3	.036	1.995	.193
	Within Groups	.145	8	.018		
	Total	.253	11			
Day40	Between Groups	.220	3	.073	11.436	.003
	Within Groups	.051	8	.006		
	Total	.272	11			

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Table 44. ANOVA of pigment content (µg g^{-1} FW) of *G. fisheri* spore culture under different shading colors indoor experiment

		Sum of Squares	df	Mean Square	F	Sig.
Carotenoid	Between Groups	6370.582	3	2123.527	3.945	.054
	Within Groups	4306.672	8	538.334		
	Total	10677.254	11			
Chla	Between Groups	71574.871	3	23858.290	5.272	.027
	Within Groups	36205.856	8	4525.732		
	Total	107780.727	11			
Chlb	Between Groups	678.288	3	226.096	5.589	.023
	Within Groups	323.648	8	40.456		
	Total	1001.936	11			
Chlc	Between Groups	119.871	3	39.957	1.010	.437
	Within Groups	316.425	8	39.553		
	Total	436.296	11			
RPE	Between Groups	1497.174	3	499.058	2.439	.139
	Within Groups	1636.649	8	204.581		
	Total	3133.823	11			

Prince of Pattani Campus

Table 45. ANOVA of RGR (% day^{-1}) of *G. fisheri* tissue culture under different shading colors outdoor condition

		Sum of Squares	df	Mean Square	F	Sig.
1	Between Groups	1.369	3	.456	5.280	.015
	Within Groups	1.037	12	.086		
	Total	2.406	15			
2	Between Groups	1.061	3	.354	2.364	.122
	Within Groups	1.795	12	.150		
	Total	2.856	15			
3	Between Groups	.738	3	.246	2.126	.150
	Within Groups	1.388	12	.116		
	Total	2.125	15			
4	Between Groups	.430	3	.143	1.886	.186
	Within Groups	.911	12	.076		
	Total	1.340	15			
5	Between Groups	.366	3	.122	1.658	.229
	Within Groups	.883	12	.074		
	Total	1.249	15			
6	Between Groups	.839	3	.280	3.104	.067
	Within Groups	1.081	12	.090		
	Total	1.921	15			
7	Between Groups	.976	3	.325	3.556	.048
	Within Groups	1.098	12	.091		
	Total	2.074	15			
8	Between Groups	1.092	3	.364	4.602	.023
	Within Groups	.949	12	.079		
	Total	2.041	15			

Table 46. ANOVA of epiphytes (%) and pigment content (µg g^{-1} FW) of *G. fisheri* tissue culture under different shading colors outdoor condition

		Sum of Squares	df	Mean Square	F	Sig.
Epiphyte at 7th week	Between Groups	157.076	3	52.359	39.360	.000
	Within Groups	15.963	12	1.330		
	Total	173.039	15			
Epiphyte at 8th week	Between Groups	68.455	3	22.818	4.575	.023
	Within Groups	59.854	12	4.988		
	Total	128.310	15			
Carotenoids	Between Groups	361.337	3	120.446	8.716	.002
	Within Groups	165.822	12	13.819		
	Total	527.159	15			
Chla	Between Groups	160.474	3	53.491	1.441	.280
	Within Groups	445.419	12	37.118		
	Total	605.893	15			
Chlb	Between Groups	26.183	3	8.728	2.647	.097
	Within Groups	39.563	12	3.297		
	Total	65.746	15			
Chlc	Between Groups	5.048	3	1.683	.834	.501
	Within Groups	24.221	12	2.018		
	Total	29.268	15			
R-PE	Between Groups	6204.639	3	2068.213	18.230	.000
	Within Groups	1361.376	12	113.448		
	Total	7566.015	15			

Table 47. ANOVA of increased biomass (%) of *G. fisheri* tissue culture under different shading colors outdoor condition

		Sum of Squares	df	Mean Square	F	Sig.
1	Between Groups	67.203	3	22.401	5.338	.014
	Within Groups	50.358	12	4.196		
	Total	117.560	15			
2	Between Groups	208.069	3	69.356	2.365	.122
	Within Groups	351.881	12	29.323		
	Total	559.950	15			
3	Between Groups	323.845	3	107.948	2.105	.153
	Within Groups	615.386	12	51.282		
	Total	939.232	15			
4	Between Groups	339.205	3	113.068	1.897	.184
	Within Groups	715.110	12	59.593		
	Total	1054.316	15			
5	Between Groups	453.599	3	151.200	1.687	.222
	Within Groups	1075.335	12	89.611		
	Total	1528.935	15			
6	Between Groups	1492.466	3	497.489	3.138	.065
	Within Groups	1902.623	12	158.552		
	Total	3395.089	15			
7	Between Groups	2338.199	3	779.400	3.549	.048
	Within Groups	2635.399	12	219.617		
	Total	4973.598	15			
8	Between Groups	3384.011	3	1128.004	4.598	.023
	Within Groups	2943.977	12	245.331		
	Total	6327.988	15			

Table 48. ANOVA of biomass (g m^{-2}) of *G. fisheri* tissue culture under different shading colors outdoor condition

Week		Sum of Squares	df	Mean Square	F	Sig.
1	Between Groups	747.182	3	249.061	5.354	.014
	Within Groups	558.223	12	46.519		
	Total	1305.404	15			
2	Between Groups	2312.207	3	770.736	2.369	.122
	Within Groups	3904.552	12	325.379		
	Total	6216.759	15			
3	Between Groups	3603.942	3	1201.314	2.110	.152
	Within Groups	6833.192	12	569.433		
	Total	10437.134	15			
4	Between Groups	3770.143	3	1256.714	1.899	.184
	Within Groups	7940.295	12	661.691		
	Total	11710.437	15			
5	Between Groups	5034.147	3	1678.049	1.686	.223
	Within Groups	11945.750	12	995.479		
	Total	16979.897	15			
6	Between Groups	16575.512	3	5525.171	3.135	.066
	Within Groups	21152.172	12	1762.681		
	Total	37727.684	15			
7	Between Groups	25978.577	3	8659.526	3.549	.048
	Within Groups	29279.138	12	2439.928		
	Total	55257.714	15			
8	Between Groups	37597.027	3	12532.342	4.598	.023
	Within Groups	32704.610	12	2725.384		
	Total	70301.637	15			

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List of Publication and Conference

Nguyen, T.P., Ruangchuay, R. and Lueangthuvapranit, C. 2013. Comparison on Cultivation Characteristics in Two Seasons of Agarophytic Seaweed, *Gracilaria fisheri* (Rhodophyta) at Pattani Province, Southern Thailand. In the 3rd International Fiseries Symposium, Pattaya, Thailand, 28-30 November, 2013.

Nguyen, T.P., Ruangchuay, R. and Lueangthuvapranit, C. 2014. Tissue Culture of Agarophytic Seaweed *Gracilaria fisheri* (Rhodophyta) in Different Conditions. In the 4th International Fiseries Symposium, Surabaya, Indonesia, 30-31 October, 2014.

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