THE CULTURE STUDIES ON THE FORMATION AND GROWTH OF THE SECONDARY BRANCHES AND REPRODUCTIVE STRUCTURES OF *HYPNEA SPINELLA* (C. AGARDH) KÜTZING (GIGARTINALES, RHODOPHYTA) FROM SETSE COASTAL AREA

Sein Moh Moh Khaing[1], Myo Min Tun[2], Khin Khin Gyi[3] & Wint Thuzar Nwe[4]

Abstract

The plants of *Hypnea spinella* (C. Agardh) Kützing collected from the tidal pools in the upper intertidal zone of Setse coastal area (Lat. 15° 52' N, Long. 97° 35' E) had been culture under the laboratory conditions. It was carried out to investigate the formation and growth of *H. spinella* (C. Agardh) Kützing based on the early stages of secondary branches and reproductive structures in culture. In this study, the maximum growth was found in the salinity 25‰. After five days, a new filamentous sprout grew and seven days later, a single apical cell which develops at terminate of the branches and branchlets. After ten days, laterals and new branch filaments arised and after fifteen days initiating, short branchlets which gradually grow up on the branches like spiny outgrowth. Moreover, tetrasporangial form observed at the basal, middle and upper portion of the branchlets encircling the entire surface were described.

Keywords: branches, culture, *Hypnea spinella*, laboratory, salinity, Setse.

Introduction

Red algae are found from the intertidal to the deep limits of the photic zone, displaying a wide variety of morphologies including unicells, filaments, crusts, sacs, blades, and finely branched forms. They are most abundant in tropical waters and most of them are benthic and some are epiphytic on other seaweeds and seagrasses. Red algae are the largest in numbers of species among the marine algae. Many are microscopic but most are macroscopic in multicellular forms. Moreover, some members of the red algae are calcareous. The red algae contain photosynthetic pigments, chlorophyll a and c, carotenes, xanthophylls and phycobilins especially r-phycoerythrin which causes the red coloration. Their cell walls are composed of cellulose and pectic compounds such as agar, carrageenan, and furcellaran (Guiry and Guiry 2017).

In red algae, *Hypnea* species are found as economically and medically important seaweeds and also can be recorded as a part of livelihood resource for many countries. The morphotaxonomy and utilization of *H. spinella* (C. Agardh) Kützing of the Asian, American, British, African and Austalian coasts has been studied by several researchers (Tanaka 1941, Taylor 1950, Krishnamurthy and Joshi 1970, Lewmanomont and Ogawa 1995, Jha *et al.* 2009, Coppejans *et al.* 2009, 2010, Pham *et al.* 2011, Guiry and Guiry 2017). Similar studies have received from three coastal regions of Myanmar by Aung Myint 1975, Soe-Htun *et al*. 2009 a, Soe-Htun *et al*. 2009 b, Hlaing Hlaing Htoon 2009 and Sein Moh Moh Khaing 2017.

In the present study, *H. spinella* (C. Agardh) Kützing collected from the Setse coastal area had been cultured under laboratory conditions. The stages of development of branches, branchlets, and reproductive structures of this species were presented in the present research. The main objectives of this study are: 1) to investigate the formation of secondary branches of *H. spinella* (C. Agardh) Kützing, and 2) to know the early stages of reproductive structures of *H. spinella* (C. Agardh) Kützing under the laboratory conditions.

[1] Dr, Lecturer, Department of Marine Science, Mawlamyine University
[2] Dr, Lecturer, Department of Marine Science, Mawlamyine University
[3] Dr, Lecturer, Department of Marine Science, Mawlamyine University
[4] Assistant Lecturer, Department of Marine Science, Mawlamyine University

Materials and Methods

The plants of *H. spinella* (C. Agardh) Kützing were collected from the tidal pools in the upper intertidal zone of Setse coastal area (Lat. 15° 52' N, Long. 97° 35' E) in Thanpyuzayat Township, Mon State, from May 2016 to February 2017. The collected plants were kept in ice-box and brought to the laboratory immediately after collection. Morphological studies were mainly based on the fresh and the herbarium specimens deposited in the herbarium of Marine Science Department.

In the laboratory, the fresh and healthy specimens were thoroughly washed with painting brushes in the sterile seawater to remove epiphytes and some contaminants. The culture apparatus (glass bottles, cover slices, Petri dishes, forceps and brushes) were cleaned with tap water, and then they were sterilized with boiling water. Natural sea water was collected from Setse coast, and stored it in plastic drums placed in laboratory. For the culture experiments, seawater was filtered with the Whatman No. 1 filter papers. Natural seawater was heated for hypersalinities and diluted with distilled water to reduce salinity. Sterile seawater was adjusted to salinities (20‰, 25‰, and 30‰) using a refractometer.

For the experiments on the vegetative growth of secondary branches, fresh and healthy plants were selected and used as seed materials. The plants were cut into pieces of 5 mm in length by hand using double-edged razor blades. The five pieces of healthy plants were inoculated onto cover slices in the Petri dishes, 25 ml of seawater of Provasoli's Enriched Seawater (PES) medium (Provasoli 1968) (Table. 1) in 20‰, 25‰ and 30‰ salinities. The medium was replenished every 3 days intervals. Cultures were maintained on the culture shelf at room temperature (28°C±1) under continuous light for 30 days. The formation and growth of the secondary branches were examined every day under compound microscope (Olympus CO11 Japan) throughout the experimental period. Microscopic measurements were recorded by micrometer (µm) using ocular meter. The important characteristics of this study were photographed under the light microscope with a Sony DSC-WX80 digital camera and the results assembled from digital photographs are processed by Adobe Photoshop CS4. This study followed the classification system of Matinfar *et al*. (2013).

Table 1 Provasoli's Enriched Seawater Medium (PES).

Stock solutions (Each in 100 ml water)		Millimeters of stock solutions to be added
$NaNO_3$	35 g	10
Na_2 glycerophosphate	5 mg	10
Vitamin B_{12}	1 mg	10
Thiamine	50 mg	10
Biotin	0.5 mg	10
Tri buffer		-
Fe (as EDTA 1:1 molar)		250
Fe $(NH_4)_2$ $(SO_4).6H_2O$, + Na_2 EDTA,	351 mg 300 mg/ 500 ml	
P II trace metals		250

Add 20 ml of the above stock solution mixture to 1000 ml of filtered seawater to prepare full-strength medium

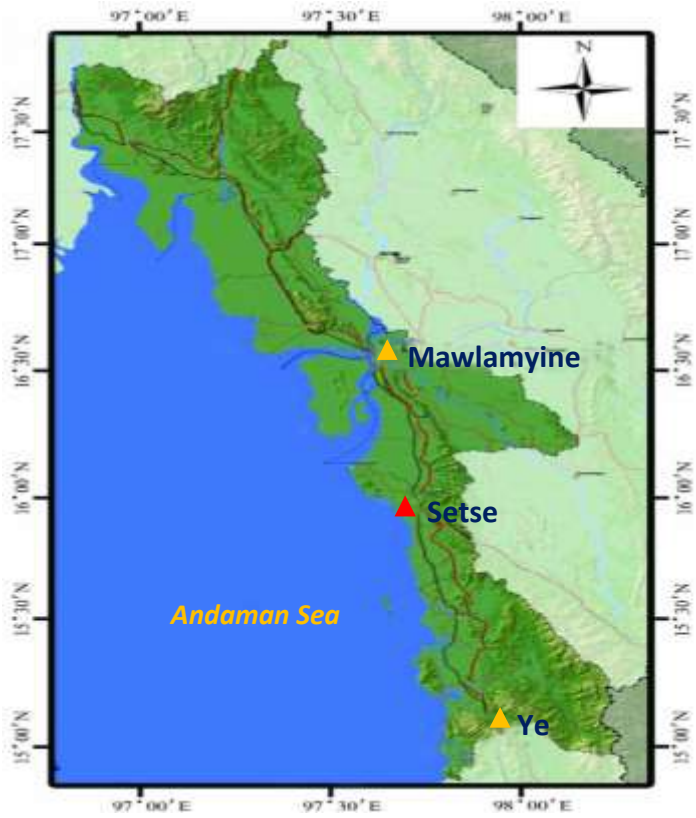

Figure 1 Map showing the collection site of the *Hypnea spinella* (C. Agardh) Kützing from Setse coastal area.

Results

A classification system of the *Hypnea spinella* (C. Agardh) Kützing

Phylum : Rhodophyta

Class : Florideophyceae

Order : Gigartinales

Family : Cystocloniaceae Kützing 1843

Genus : *Hypnea* J. V. Lamouroux 1813

Species : *Hypnea spinella* (C. Agardh) Kützing

General morphology of *Hypnea spinella* (C. Agardh) Kützing

References.- Taylor 1950:135; Krishnamurthy and Joshi 1970: 22; Aung Myint 1975: 52-57, figs. 7-10, 44; Coppejans *et al.* 2009: 182, fig. 150; Hlaing Hlaing Htoon 2009: 45-46, figs. 86-88; Soe-Htun *et al*. 2009a: 148-149, fig. 5; Soe-Htun *et al*. 2009b: 296; Coppejans *et al* . 2010: 198; fig. 123; Myo Min Tun 2013:101, figs. 113-115.

Plant purplish green to red in color, 2-3 cm in height, occuring as intricate cushion, pulviniformis thallus, terete, texture firm fleshy, alternately and dichotomously branches are freely erect, 5-7 mm long and 0.5 mm broad branches form prostrate with tip rising upwards by many accessory holdfasts. Thallus decorated with small spines or branchlets about 1-3 mm long, acutes originated from the primary branches, more frequent in the middle and basal parts of the plants. Spherical tetrasporangial sori decorated out from the surface of the frond, especially at the upper and middle regions of the branchlets.

Development of secondary branches and reproductive structures of *Hypnea spinella* (C. Agardh) Kützing

In this study, the maximum growth was found in the salinity 25‰. The secondary branches of *H. spinella* grew well at room temperature and this salinity regime. The color of secondary branches was purplish red under fluorescent light tested.

After five days, a new filamentous sprout grew about 50-60 µm long (Fig. 5). In the young filamentous sprout pericentral and central cells were formed. Seven days later, a single apical cell which develops at the terminate of the branches and branchlets, cuts off a daughter cell (sub-apical cell) which later give rise to an axial filament which inturn cut off lateral pseudoparenchymatous tissue, consisting of large and elongate periaxial cells (Fig. 6).

H. spinella have a distinct apical cell which is generally ovoid to obovoid, sometimes oblong to round or tholus-shaped (Fig. 3). It can be seen prominent and rarely submerged at the tip of the thallus under microscope. Two dividing surfaces are apparent and usually a sub-apical cell present under it (Fig. 19). After that, the young filamentous sprouts become polysiphonous. The elongation of sprout continues, measuring about 150 µm long and 30 µm broad. Ten days later, laterals and new branch filaments arised (Fig. 7). Branch was up to 1.3-1.8 mm in length with acute pieces and dichotomized branched. After that, thallus with successive branches, cell divisions and branching pattern with alternate branching. Prostrate branches on the cover slice in the present study were terete and like a rod; straight, long and thin. Terminal portion of branches are gradually tapering towards the apex or the tips of the branches may be acute and straight, but some of them from the small accessory discs or modified hooks for secondary attachment.

In this stage, accessory holdfasts emerge from the upper, middle and basal portions of secondary branches (Fig. 8). Occurrence of numerous accessory disc-like holdfasts is a common feature in this study. Holdfasts are generally hemisphere in shape, 40-50 µm in diameter and these are modified by further division of cortical cells (Figs. 9, 10). And then, the formation of accessory discs were tested by pull out small branches gently from cover slice using forcep.

Fifteen days later, short branchlets which gradually grow up on the branches like spiny outgrowth seemingly determinate are called branchlets measuring about 180 µm long and 40 µm broad (Figs. 14-16). Frequently, these can be mistaken with short undeterminate branches, especially at the upper portion of the branches. Branchlets are important parts for tetrasporangial sori that occur on them.

Twenty days later, tetrasporangia form at the basal and upper portion of the branchlets encircling the entire surface, 60-80 µm in diameter (Figs. 30, 35). Nevertheless, some tetrasporangial sori occur in the middle region of the branchlets (Figs. 31, 32). Tetrasporangia occur on the swollen surfaces of fertile branchlets. External appearance of the tetrasporangial sori can be distinguished by their darkly colored spots which occur abundantly and visible under the microscope.

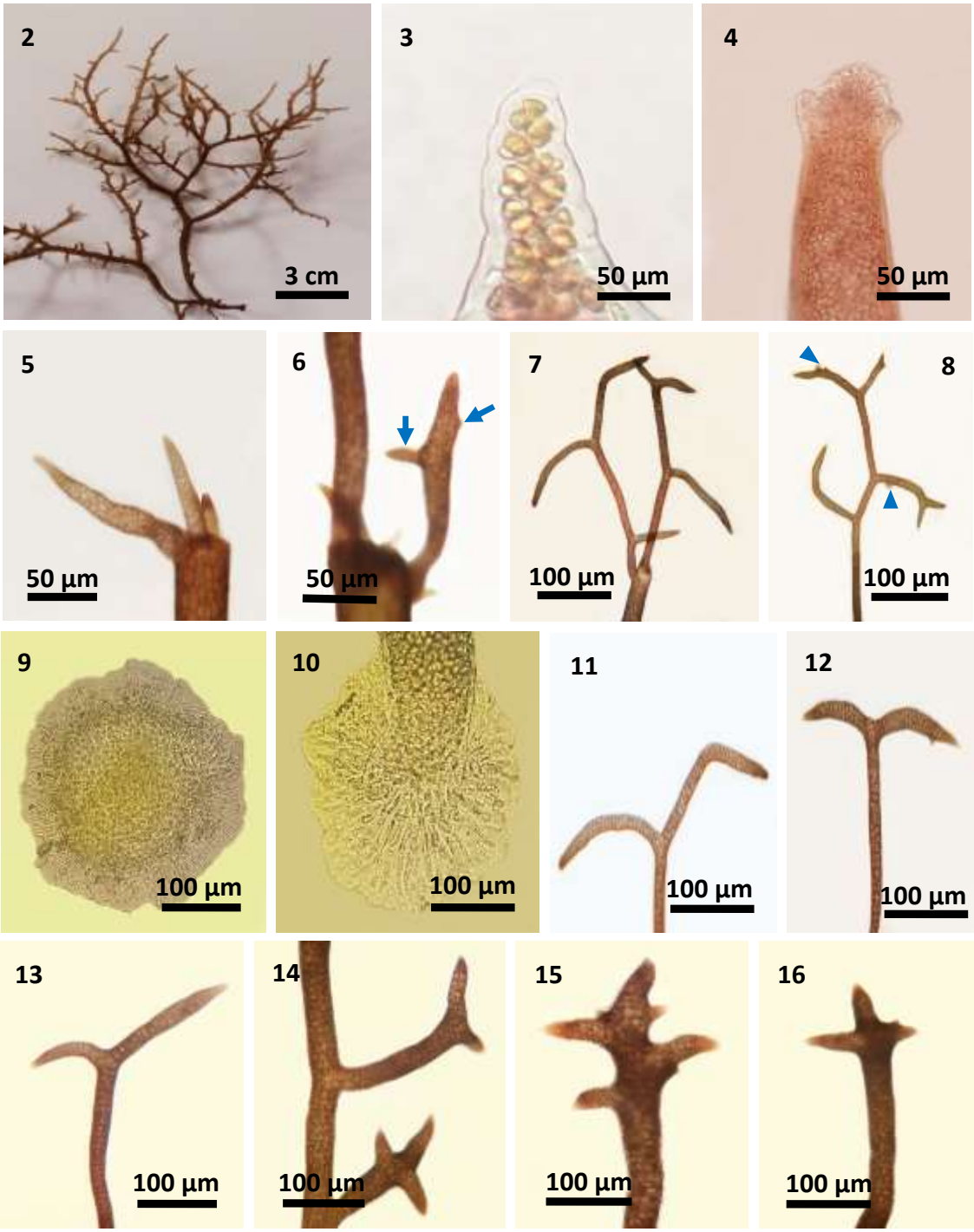

Figure 2-16. The formation and growth of the secondary branches of *H. spinella* (C. Agardh) Kützing: 2) Habit of *H. spinella* (C. Agardh) Kützing ; 3) Ovoid or oboviod apical cell; 4) Apical portion of new filament; 5) New filamentous sprouts on main branch; 6) Small branchlet and new shoot on new branch (arrows); 7) Laterals and new branch filaments; 8) Accessory holdfasts at the upper and basal portions of secondary branches (arrowheads); 9) Accessory holdfast on the apical portion of branch and composed with divisions of cortical cells; 10) Circular-shaped accessory holdfast; 11-12) Different secondary branches; 13-16) Two to seven times spinulose branchlets on the upper portion of the small branches.

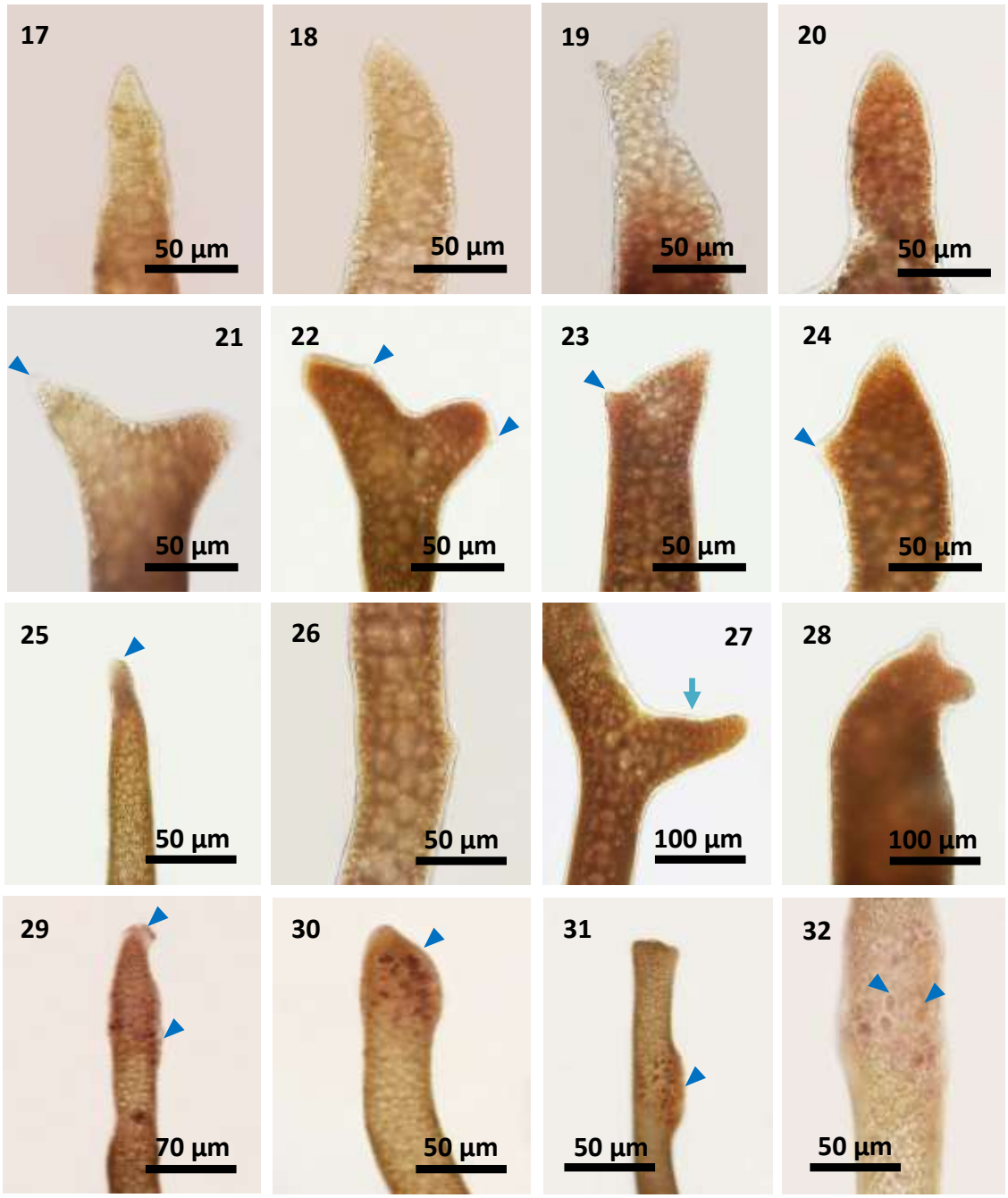

Figure 17-32. The formation and growth of branchlets and reproductive structures of *H. spinella* (C. Agardh) Kützing: 17-18) Apical portions of new branchlets; 19) Cell division at the apical portion of branchlet; 20) Apical portion of branchlet; 21-24) Different new shoots on the apical portion of branchlets (arrowheads); 25) New accessory disc at the apical portion of branchlet (arrowhead); 26) Small shoot at the margin portion of branch; 27) Branchlet at the middle portion of branch (arrow); 28) Two new shoots at the apical portion of branchlet; 29) Tetrasporangial and accessory disc at the upper portion of branchlets encircling the entire surface (arrowheads); 30) Tetrasporangial at the apical portion of branchlets (arrowhead); 31-32) Tetrasporangial at the middle portion of branchlet (arrowheads).

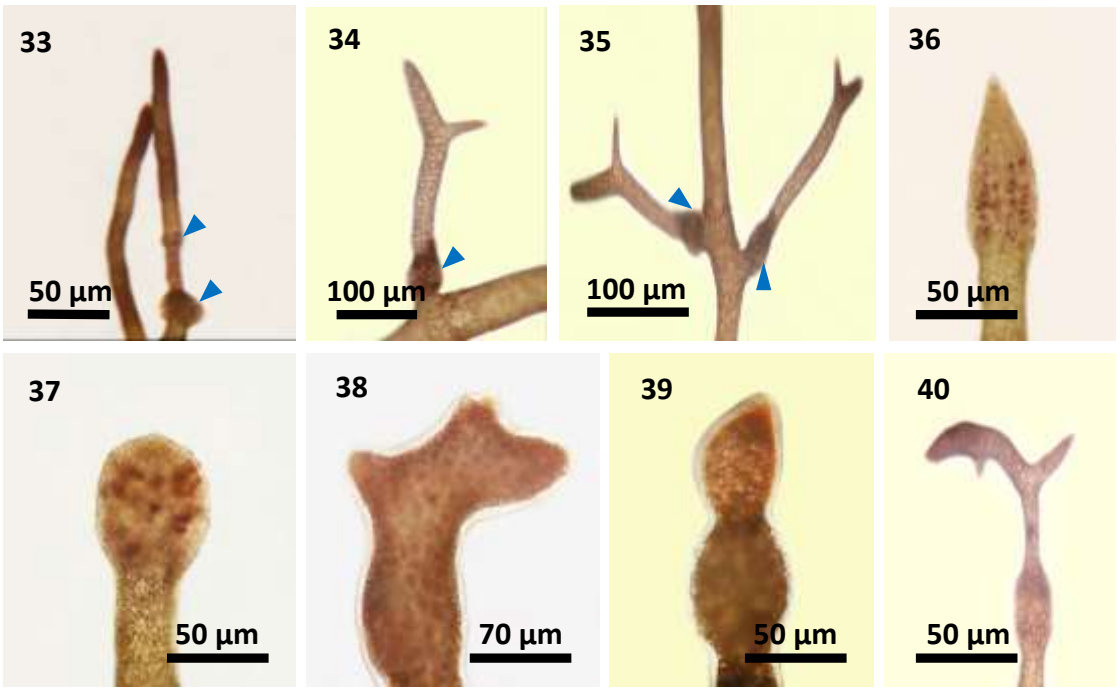

Figure 33-40 The formation and growth of branchlets and reproductive structures of *H. spinella* (C. Agardh) Kützing: 33) Tetrasporangial on the middle and basal portions of branchlets (arrowheads); 34-35) Tetrasporangial on the basal portion of branchlets (arrowheads); 36-37) Tetrasporangial at the apical portions of branchlets; 38-40) Different formation of tetrasporangial sori.

Discussion

In culture study, the maximum growth was found in the salinity 25‰. However, the growth of secondary branches was slow in the salinity 20‰ and 30‰. The secondary branches of *H. spinella* (C. Agardh) Kützing grew well at room temperature and this salinity regime. The light intensity was one of the important factors, which controlled the growth of the *H. spinella* (C. Agardh) Kützing, rather than light quality. The result of this study coincides with the growth of *H. spinella* (C. Agardh) Kützing which commonly occur in the tidal pools at the upper intertidal zone.

Teoh *et al*. (2010) studied temperature tolerance and temperature optimum of algae isolated from different habitats. They stated that temperature optimum of *H. cenomyce* J. Agardh and *H. spinella* (C. Agardh) Kützing was 30° C and, temperature tolerance of these species was 15-30° C respectively. Ding *et al*. (2013) observed the growth rates and changes of several photosynthetic pigments in *H. cervicornis* by setting up different ranges of salinity. The growth rate first increase then decrease as the temperature increases, while growth tends to decline as salinity increases. The optimum salinity and temperature conditions for growth are 25 ‰ and 25° C, respectively. And then, salinity and temperature have significant effects on photosynthetic pigments in this species.

Yokoya *et al*. (2007) studied growth responses and photosynthetic characteristics of wild and phycoerythrin-deficient strains of *H. musciformis* (Wulfen) J. V. Lamouroux. They described that the growth responses to irradiance, photoperiod and temperature variations, pigment contents, and photosynthetic characteristics of the brown and green strains of *H. musciformis* (Wulfen) J. V. Lamouroux. The result showed that growth rates increased as a function of irradiance, but with further increase in irradiance, become light saturated and remained almost unchanged. The highest

growth rates of the brown and green strains were observed in temperature of 20-25° C under long and short photoperiods.

Ribeiro *et al*. (2013) studied effects of nitrogen and phosphorous availabilities on growth, pigment, and protein contents in *H. cervicornis*. They advised the selection of seaweed species for use as biofilters should be based on the knowledge of their nutrient requirements and tolerance to wide variations of nutrients concentrations.

Treatments were composed of sterilized seawater enrich with 25 % von Stosch solution (without nitrogen and phosphorous), and nitrate or ammonium and phosphate were added in a combination of 100:1 and 10:1 nitrogen/phosphorous (N/P). Growth rates of *H. cervicornis* increased linearly with addition of ammonium, but with nitrate addition, growth varied following saturation kinetic, and the highest growth rate (14.45% d^{-1}) was observed in N/P ratio of 10:1. An excess of nutrients was accumulated as proteins and phycobiliproteins (mainly as allophycocyanin and phycoerythrin) at higher phosphate availability (N/P ratio of 10:1), and *H. cervicornis* tolerated the highest ammonium and nitrate concentrations. These physiological responses suggest that this species could be used as biofilter for nutrient removal in eutrophicated seawater and could be cultivated in integrated multitrophic aquaculture system.

In the present study, nitrogen, phosphate, and ammonium were contained in Provasoli's Enriched Seawater (PES) medium, but not adjusted increase and decrease ratio of these concentrations. However, the effects of these elements for the growth of branches of *H. spinella* (C. Agardh) Kützing were studied in culture period. Phosphorous is vital to seaweed growth and it involved in many functions, including photosynthesis, nutrient movement within this plant, important in cell division and development of new cells.

The growth rate of all branches gradually decreased after 25 days. Results of this study indicate that replenishment of medium and changes of Petri dishes were important for the growth of this species. If Petri dishes were should be change in time-lag of culture period, unseen contamination can occur in these dishes.

In this study, partial view of habitat and variation of characters in thallus and branches formation were recorded monthly in the field. Detailed studies indicate that *H. spinella* (C. Agardh) Kützing mainly grows in the tidal pools at the upper intertidal zone of Setse coastal area. Maximum development of this species generally occurs during rainy season in this area. *Gracilaria canaliculata* are the important substrata for some *H. spinella.* Moreover, seasonal variations of these marine algae and associated seaweeds seemed to be quantitatively rare in numbers due to the turbidity by the heavy rate of sedimentation in May and developing of the crop attains its peak from June to October.

In this research, a gradual degradation of this species commences in November and most of this species in this area are disappear from December to April. Nevertheless, seasonal variations of the genus *Hypnea* in Rakhine coastal region demonstrate that regeneration commences in December and developing of the crop attains its peak from January to March. From this study, commencement and declination periods are not entirely uniform for three coastal regions and each year. Similar observation was given by Aung Myint (1975) who had studied on the genus *Hypnea.*

Conclusion

Cultural studies on the secondary branches and reproductive structures of *H. spinella* (C. Agardh) Kützing were carried out in order to know the formation and growth stages under the laboratory conditions. In this study, the maximum growth was found in the salinity 25‰. The secondary branches of were grew well at room temperature and this salinity regime. The color of secondary branches was purplish red under fluorescent light intensity tested. In this study, twenty

days later, tetrasporangia formed at the basal and upper portion of the branchlets encircling the entire swollen surfaces of fertile branchlets.

Acknowledgements

I am indebted to Dr. Aung Myat Kyaw Sein, Rector of Mawlamyine University and Dr. San San Aye, Pro-Rector of Mawlamyine University, for their encouragement and supports in preparing this work. I am very grateful to Dr. San Tha Tun, Professor and Head of the Department of Marine Science, Mawlamyine University, for his valuable suggestions and constructive criticisms on this study. I would like to thanks all staffs of Aquaculture Research Centre, Setse for their support which facilitated smooth execution of the Setse field. I would like to thank my beloved parents, U Myo Chit and Daw Mya San Yi, for their physical, moral and financial supports throughout this study.

References

Aung Myint. (1975). *Studies on the Hypneaceae (Gigartinales, Rhodophyta) of Burma*. MSc. Thesis, Department of Marine Biology, Moulmein College (Unpublished).

Coppejans, E., Leliaert, F., Dargent, O., Gunasekara, R. and Cleck, O. D. (2009). *Sri Lankan Seaweeds*. Abc Taxa, Belgian Development Cooperation. **6**: 265pp.

Coppejans, E., Prathep, A., Leliaert, F., Lewmanomont, K. and De Clerck, O. (2010). *Seaweeds of Mu Ko Tha Lae Tai* (*SE Thailand*)*: Methodologies and Field guide to the dominant species.* Biodiversity research and training program. Thailand. 274 pp.

Ding, L., Ma, Y., Haung, B. and Chen, S. (2013). Effects of seawater salinity and temperature on growth and pigment contents in *Hypnea cervicornis* J. Agardh (Gigartinales, Rhodophyta). *Biomed Research International*. 10 pp.

Guiry, M. D. and Guiry, G. M. (2017). "*AlgaeBase*.World-wide electronic publication", National University of Ireland, Galway [http://www.algaebase.org.]

Hlaing Hlaing Htoon. (2009). *Studies on the marine algae of Setse coastal area*. MRes Thesis, Department of Marine Science, Mawlamyine University (Unpublished).

Jha, B., Reddy, C.R.K, Thakur, M.C. and Rao, M.U. (2009). *Seaweeds of India.* The diversity and distribution of seaweeds of Gujarat Coast. Springer Dordrecht Heidelberg London New York. 215 pp.

Krishnamurthy, V. and Joshi, H.V. (1970). "A check-list of Indian Marine Algae". *Central Salt and Marine Chemicals Research Institute* (*CSMCRI*) *Bhavnagar.* 36 pp.

Matinfar, M., Rafiee, F., Nejatkhah, M. P., Joon L., Hong. Y.K. (2013). "Optimal conditions for tissue growth and branch induction of *Gracilariopsis persica*". *Iranian Journal of Fisheries Sciences*. **12**(1):24-33.

Myo Min Tun. (2013). *The Flora and ecology and marine algae in Kampani coastal areas*. MRes Thesis, Department of Marine Science, Mawlamyine University (Unpublished).

Pham, M. N., Tan. H. T.W., Mitrovic, S. and Yeo, H. H. T. (2011). *A checklist of the algae of Singapore*. National University of Singapore. 104 pp.

Provasoli, L. (1968). "Media and prospects for the cultivation of marine algae". *In*: Waternable. A. and Hattoria, A. (Eds). Cultures and Collection of Algae, Proc. U.S. Japan Conf. Hakone, 1966, *Jap. Soc. Pl Physiol*. 63-75

Ribeiro, A. L. N. L., Tesima, K. E., Souza, J. M. C. and Yokoya, N. S. (2013). "Effects of nitrogen and phosphorous availabilities on growth, pigment, and protein contents in *Hypnea cervicornis* J. Agardh (Gigartinales, Rhodophyta)". *J. Appl. Phycol*. **25**(4): 1151-1157.

Sein Moh Moh Khaing. (2017). *Morphotaxonomy of the carrageenophyte species of the family Cystocloniaceace (Gigartinales, Rhodophyta) of Myanmar*. PhD Thesis, Department of Marine Science, Mawlamyine University (Unpublished).

Soe-Htun, Mya Kyawt Wai, Thida Nyunt, Soe Pa Pa Kyaw and Mu Mu Aye. (2009a). "Notes on some marine benthic red algae of Gwa Coastal Areas II. Rhodophyta (Cryptonemiales, Gigartinales, Gracilariales, Rhodymeniales and Ceramiales)". *Jour. Mya. Acad. Arts & Sc.* **7**(5): 143-181.

Soe-Htun, Mya Kyawt Wai, Thida Nyunt, Soe Pa Pa Kyaw, Yin Yin Htay and Mu Mu Aye. (2009b). "Checklist, distribution and potential utilization of marine algae of Myanmar II. Rhodophyta (Red algae)". *Jour. Myan. Acad. Arts & Sci*. **7**(5): 279-305.

Tanaka, T. (1941). *The genus Hypnea from Japan*. Hokkido University. 250 pp.

Taylor, W.R. (1950). *Plants of Bikini and other northern Marshall Island.* The university of Michigan press. 227 pp.

Trono, G. C. and Gronier, P. (2002). "*Hypnea* J. V. Lamour". *In*: Trono, G. C. and Reine, W. F. P. (Eds), Plant resources of South East Asia. *Prosea Foundation, Bogor, Indonesia*: pp. 208-212.

Teoh, M.T. Chu, W.L. and Phang, S.M. (2010). "Effect of temperature change on physiology and biochemistry of algae: A review". *Malaysian Journal of Science*. **29**(2): 82-97.

Yokoya, N. S., Necchi, Jr. O., Martins, A. P., Gonalez, S. F. and Plastino, E. M. (2007). "Growth responses and photosynthetic characteristics of wild and phycoerythrin-deficient strains of *Hypnea musciformis* (Wulfen) J. V. Lamour. (Gigartinales, Rhodophyta)". *J. Appl. Phycol*. **19**(3): 192-205.