



Original Article

Gametangial characteristics in the sexual reproduction of *Halimeda macroloba* Decaisne (Chlorophyta: Halimedaceae)

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Received 10 November 2011; Accepted 22 March 2012

Abstract

Halimeda macroloba Decaisne is a reef builder that provides habitats for many marine organisms and is important for the production of calcium carbonate sediments. This species is one of the most common and abundant algae in tropical regions of the Indo-Pacific Ocean. However, the trigger for reproduction of *H. macroloba* is not well understood. We have examined some environmental factors that might trigger reproduction and determined the gametangial characteristics of *H. macroloba*. The peak seasonal reproduction of *H. macroloba* occurred within only a 3-month period (May-July). Nutrient and salinity were found to have triggered reproduction. Gametangial clusters were produced along the outer margin of segments and the gametophores had a long stipe and were dichotomously branched at their distal end. Divided and sessile gametangia were observed. This description of the variable gametangial morphology of *H. macroloba* is reported for the first time.

Keywords: gametangial clusters, nutrient, reproduction, salinity

1. Introduction

Halimeda is a group of siphonous, calcified green macroalgae that are abundant and widely distributed in tropical waters because they are successful competitors, integral components of many reefs, and have a high growth rate (Vroom and Smith, 2003a; van Tussenbroek and van Dijk, 2007; Sinutok *et al.*, 2008). It is of great importance in a broad range of ecological systems including reef construction, provision of habitat for marine organisms, stabilization of sedi-

ments, and production of calcium carbonate sand (Hillis-Colinvaux, 1980; Drew and Abel, 1988, Beach *et al.*, 2003, Vroom and Smith, 2003a; van Tussenbroek and van Dijk, 2007). In recent years, macroalgae especially *Halimeda* has become a dominant component of many reefs in the tropical regions of the Indo-Pacific oceans including Thai waters, through increasing their cover, biomass and abundance. The increasing cover of *Halimeda* has been documented from many reefs such as those in Jamaica (Lapointe and Thacker, 2002) and the Florida Keys (Beach *et al.*, 2003; Vroom *et al.*, 2003). In addition, *Halimeda* may be used as an indicator of coral reef degradation (Beach *et al.*, 2003; Vroom *et al.*, 2003).

Halimeda exhibits both asexual and sexual reproductive processes. Asexual reproduction involves the develop-

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ment of new thalli from either the segments, filaments of the holdfast or small fragments detached by wave motion and damaged by herbivores, redband parrotfishes and surgeon fishes (Drew and Abel, 1988; Walter and Smith, 1994). On the other hand, sexual reproduction has been observed in the field by Clifton and Clifton (1999). They reported that *Halimeda* developed gametangial clusters during the night, with gametangia maturing during the first day and second night, and gametes are released around dawn of the third day. Then, the thallus breaks down and dies after releasing the gametes.

The trigger for reproduction is not well understood and remains disputed (Drew and Abel, 1988; Clifton, 1997). Some workers have suggested that water temperature, nutrient and environmental stresses are the triggers for reproduction (Drew and Abel, 1988; van Tussenbroek *et al.*, 2006). Some species of *Halimeda* do show seasonal and lunar periodicity (Drew and Abel, 1988; Clifton, 1997; Clifton and Clifton, 1999) and reproductive occurrences within the same population are synchronized (Vroom *et al.*, 2003; Clifton, 1997). van Tussenbroek *et al.* (2006) noted that a sudden decrease in temperature, as a result of hurricane Wilma, may have been the trigger for the unusual synchronous spawning of green algae. However, the effects of interacting environmental factors on algal sexual reproduction remain poorly understood and merit further investigation. Field studies of sexual reproduction by natural populations of *Halimeda* and the triggers that stimulate them remain unexplored.

Gametangial clusters of *Halimeda* vary in size, shape, and position on the segment. Gametangia are produced on the surface or the margins of segments in *H. incrassata* (Ellis) Lamouroux, *H. tuna* (Ellis et Solander) Lamouroux and *H. velasquezii* Taylor (Vroom and Smith, 2003b). In addition, the length and diameter of gametophores and the number of gametangia also vary. Vroom and Smith (2003a) produced a review that defined the evolutionary lineages of 19 *Halimeda* species using their reproductive characteristics and assessed the phylogenetic importance of the reproductive structures within the genus. The gametangial structure of *H. macroloba* Decaisne was described in 1940 (Kanda, 1940 in Vroom and Smith, 2003b) but no other researches on their reproductive structures have since been reported.

In this study, we described in detail the reproductive structures of *H. macroloba* that were observed both in the field and in a water tank and examined the effect of environmental factors on the reproduction of *H. macroloba*.

2. Materials and Methods

This study was carried out in the shallow (2-3 m in depth) coastal waters at Ko Rab, Mu Ko Thale Tai National Park, the Gulf of Thailand in Southern Thailand (9° 19' 20" N, 99° 46' 80" E). The site, which was a sand plain with some dead coral fragments, was visited biweekly during January-December 2008.

2.1 Field experiment

In the field, the natural density of *H. macroloba* was measured to assess population changes using a 50 cm x 50 cm quadrat and three line transects of 50 m long. *Halimeda* were counted in 3 quadrats at 10 m intervals along the transect line. There were 90 quadrats in total. For the reproduction study, fifteen ambient and 15 nutrient enriched plots (50 cm x 50 cm) were permanently marked using thread and labelled with plastic tags. To examine the role of nutrients, Osmocote[®], a slow-release fertilizer, was enclosed in three mesh bags made from nylon hosiery with each containing 60 g of Osmocote fertilizer (14-14-14 [N-P-K]). The mesh bags were placed in the center and at two diagonally opposite corners in each plot in the field. Ambient and enriched plots were placed a maximum of 1 or 2 m apart. *H. macroloba* was observed weekly for their reproductive occurrences.

2.2 Water tank experiment

Three hundred and sixty young thalli (length was 9.4 ± 0.87 cm (mean \pm SE)) collected in the field were cleaned of all visible epibionts and transported to the laboratory in seawater. Twenty algae were transplanted into each of the 18 outdoor water tanks (50 L each) and attached to the sandy substrate. To make the water move, one filter pump was placed in each water tank. The water tanks were situated outdoors under natural light conditions. Total light intensity was $1,711 \mu\text{mol m}^{-2}\text{s}^{-1}$. Water temperature and salinity varied between 26-31°C and 31-33 psu, respectively. Growth and reproduction of plants were observed daily in May-July, 2008. For the nutrient study, nutrients levels were manipulated into 2 levels: enriched (enriched nitrogen and phosphorus) and ambient levels. In the 6 enriched water tanks, Osmocote[®] slow-release fertilizer (14-14-14 [N-K-P]) was enclosed in mesh bag constructed from nylon hosiery and containing 60 g of Osmocote fertilizer and another 6 tanks were left ambient. To examine the effect of salinity on the reproduction of *Halimeda*, the salinity of 6 tanks was varied from 31-33 psu up to 40 psu in June 2008. For preparing higher salinities, sea water was partially evaporated.

In both the field and laboratory, fertile plants of *Halimeda* were collected and preserved in 4% formaldehyde in seawater for later examination. Reproductive occurrences were recorded and gametangial characteristics were observed. The structure and size of the reproductive organs were measured. The width and length of the gametangia and gametophore were measured (Figure 1). The gametophores were cut off with a blade and measured and recorded using a color digital camera attached to a compound microscope (Model: BX 51TF Olympus, No. 1H20773, Japan). The density of gametes was determined using light microscopy and a haemocytometer.

Tissue nutrients were measured to check for significant nutrient enrichment. The C:N:P ratios were analysed at

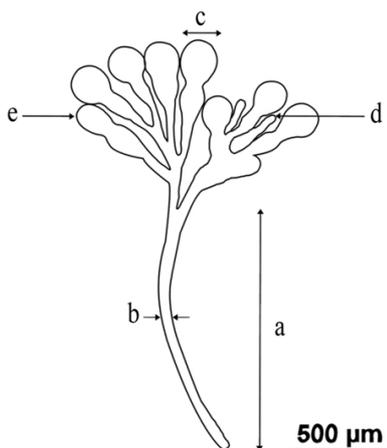


Figure 1. Diagram of *Halimeda macroloba* from gametophore measurements. a. Length of gametophore. b. Width of gametophore. c. Width of gametangia. d. Discharge papilla. e. Gametangium.

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3. Results

The mean density of *H. macroloba* at the study site was 104 ± 5 thalli m^{-2} ($n = 90$ quadrats) during January-December 2008. The peak seasonal reproduction was between May and July when reproductive occurrences were observed both in the field and laboratory (Figures 2 a,b). Twelve reproductive thalli of *H. macroloba* were seen and collected from the ambient plots in the field on May 25, Jun 8, 17, and 22, 2008. The bearing thalli, matured gametangia, were seen in July. These algae experienced temperatures of 26-31°C and a salinity of 31-33 psu, similar to those in the water tanks. Five reproductive thalli of *H. macroloba* reproduced rapidly within 1 week after nutrient enrichment. In the water tanks, 5 individual reproductive thalli also were found in the first week of May after enrichment.

After two weeks in nutrient-enriched quadrats, the C:P and N:P ratios from *H. macroloba* increased from 674 ± 83 and 30.7 ± 1.0 to 807 ± 9.3 and 47.2 ± 2.0 , respectively, whereas the C:N ratios decreased from 21.9 ± 2.00 to 17.2 ± 1.95 . (mean \pm SE). There were significant decreases in C:N ratios ($p = 0.048$, *t*-test) and significant increase in N:P ratios ($p = 0.018$,

t-test) observed (Table 1). For the salinity study, reproductive occurrences were observed when the salinity in the water tanks increased from 31-33 psu to 40 psu.

H. macroloba developed gametophores and stalks on the first night after the beginning of maturation, gametangia became mature during the second day, and gametes were released from the discharge papillae around dawn of the third day.

Reproductive occurrences were not synchronized. The frequency of the reproductive thalli was 2.67%, 3.33% and 0.83% of the mature individuals in the field, in the nutrient enriched quadrats and in the laboratory, respectively.

Gametangial clusters were formed along the outer rim of the upper segments (Figure 2). The gametophore was quite long and usually dichotomously branched between 0 and 3 times (Figures 3 a,b). Gametangia were formed at the distal end of the gametophores. The diameters of the gametangia ranged from 157 to 613 µm and the gametophores ranged

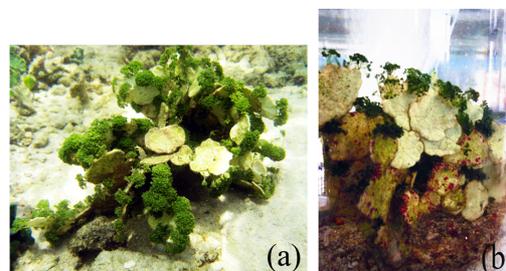


Figure 2. The reproductive thallus observed in the field (a) and the aquarium (b), gametophores are produced along the outer rim of the segments.

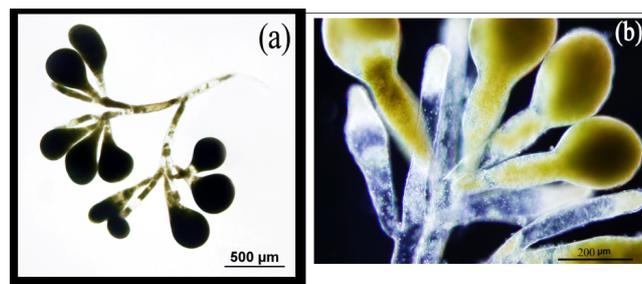


Figure 3. (a). A gametophore *H. macroloba* showing dichotomous branching, 3 times. (b). A gametangium and papillae.

Table 1. Contents (% dry wt.) and the ratios of carbon (C), nitrogen (N), phosphorus (P), and C:N, C:P, and N:P of the tissue *Halimeda macroloba* in nutrient enriched quadrats. (Values in parentheses = SE, $n = 15$)

	%C	%N	%P	C:N	C:P	N:P
Initial (ambient)	22.1(3.88)	1.17(0.10)	0.08(0.01)	21.9(2.00)	674(82.7)	30.7(0.97)
Week 1	19.1(1.11)	1.16(0.10)	0.07(0.00)	19.5(1.10)	734(31.9)	38.1(3.29)
Week 2	20.2(1.83)	1.37(0.10)	0.06(0.01)	17.22(0.85)	807(9.35)	47.2(1.95)

from 2212 to 2276 μm in length, 118 to 132 μm in width and contained 1-9 discharge papillae (Figures 4 a,b). Gametangia varied in size and shape within the thallus. The gametangial form varied from globose, tear-shaped to pear-shaped. Divided and sessile gametangia were also observed in both the field and laboratory (Figure 4a). They became mixed with normal morphologies (globose shape with a short stipe) (Figure 4b). There were discharge papillae through which gametes could be released (Figures 3b and 4a). Gametes released from the reproductive thalli were macrogametes (female gametes). They were generally large and brown to dark green. The number of gametes released was around $2.6\text{-}3 \times 10^6$ cells/mL. Gametes were biflagellate and the size ranged from 4 to 28 μm (Figure 5).

4. Discussion

Our results showed that the peak seasonal reproduction of *H. macroloba* occurred within only 3 months of the year (May-July) during the dry season. The observed peak of reproduction is similar to the time of fertility of *H. macroloba* from the Philippines and Indonesia (Hillis-Colinvaux, 1972) that was also in May and July, respectively but it is contrary to the study of *H. macroloba* reproduction in Guam where it was fertile throughout the year (Merten, 1971). Our reproductive events indicated that there was no correlation with a 28-day cycle. The events were restricted to only 3 months of the year, thereby showing seasonality. However, the number of reproductive events may be too small to be certain of this and extensive surveys and observations should be conducted

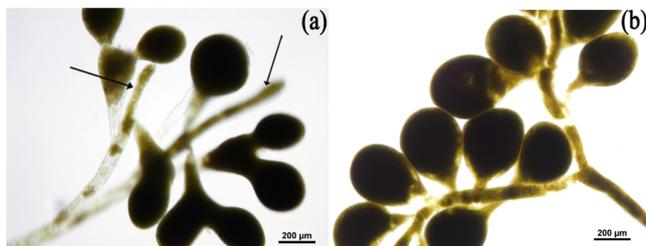


Figure 4. Various shapes of the gametangium of *H. macroloba*. (a). Divided and sessile gametangia (b). globose morphology. Arrows in Fig 4 (a) show the discharge papillae.

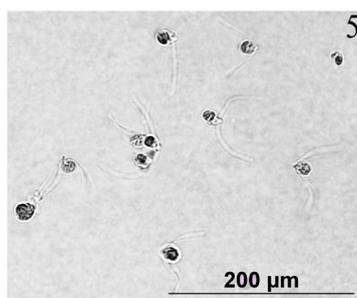


Figure 5. Biflagellated female gametes of *Halimeda macroloba*.

daily. The reproductive occurrences were not synchronized and not all mature individuals reproduced at the same time. In contrast to Clifton and Clifton (1999), the reproduction of 17 other green algae species were synchronized and spawned on the same morning. Non-synchronized sexual reproduction of *H. macroloba* in Thai waters is an unusual occurrence compared to the reproduction of other green algae. Reproduction by *Caulerpa racemosa* was likely to be synchronized when a large observed population of *C. racemosa* disappeared after undergoing sexual reproduction (Prathep *et al.*, 2006).

In this study, we found that the occurrence of the reproductive events in *H. macroloba* was rare. Our results were similar to a study of *H. macroloba* at Tankhen Bay, Phuket, from the Andaman Sea, that also reported no fertile plants during July 2006 - July 2007, although high densities of 24.0 thalli m^{-2} were observed (Sinutok, 2008). It was suggested that the life span of *H. macroloba* was 8-12 months, including around 1 month to develop from a newly recruited plant to a partially calcified plant and another 1-4 months to become a mature plant (Sinutok, 2008). We also found dense populations of *H. macroloba* at >200 thalli m^{-2} at Lidee Island in the Andaman Sea, with an infrequent reproductive occurrence (Unpublished data, J. Mayakun). In these Thai waters it seems to be an unusual reproductive phenomenon. Vroom *et al.* (2003) also reported that only one or two reproductive events of *Halimeda tuna* were found each month during the summer and early autumn periods.

Halimeda reached a high density of 500 thalli m^{-2} at the Glory Be reef (Hillis-Colinvaux, 1980; Littler *et al.*, 1988). This high density of *H. macroloba* might be due to asexual reproduction by vegetative fragmentation or development of new thalli at the ends of segments or from filaments of the holdfast. Thalli can be fragmented by herbivorous/carnivorous fish bites and physical disturbance such as wave action (Walter and Smith, 1994; Walter *et al.*, 2002; Vroom *et al.*, 2003). These asexual fragments can settle and quickly develop into new thalli. In addition, *Halimeda* quickly produced new segments of around 1-2 segments per day (Unpublished data, J. Mayakun). Asexual reproduction can increase *Halimeda* numbers by clonal propagation. Thus, asexual reproduction by rhizoid extension or thallus fragmentation probably plays a more important role in maintaining the population than does sexual reproduction.

There are many environmental and biological factors that might trigger the reproduction of *Halimeda* such as water temperature, lunar cycle, and stresses. Abel and Drew (1985) suggested that increased water temperature may have been the trigger for *Halimeda* reproduction and there might be a correlation between fertility and seawater temperature. In contrast, van Tussenbroek *et al.* (2006) noted that the sudden decrease in temperature, as a result of hurricane Wilma, may have been the trigger for the unusual synchronous spawning of green algae. However, the effects of interacting environmental factors on algal sexual reproduction remain poorly understood.

In this study, we attempted to demonstrate that nutrient enrichment and increased salinity might be the triggers for the reproduction of *H. macroloba* as it rapidly reproduced within 1-2 weeks after enrichment. Our tissue nutrient study indicated that the reproduction was affected by both nitrogen and phosphorus. C:N:P ratios in the tissue showed that the C:N, C:P, and N:P (21.9, 674, and 30.7) ratios were similar to the values of other benthic macroalgae and seagrass (22, 700, and 35, respectively) as reported by Atkinson and Smith (1983). Decreasing C:N ratios indicate that the growth and reproduction of *H. macroloba* was limited by nitrogen and this is supported by the studies on the productivity of *H. opuntia* (Linnaeus) Lamouroux on Caribbean barrier reef (Lapointe *et al.*, 1987), *Penicillus capitatus* Lamarck, *P. dumetosus* (J.V. Lamouroux) Blainville and *H. opuntia* in the shallow nearshore waters of southwestern Florida Bay in the Florida Keys (Delgado and Lapointe, 1994). In addition, nutrient availability has been shown to affect plant growth rate and influence the time for reproduction in plants (Reekie, 1997). Our study has demonstrated a nutrient limitation for *H. macroloba*. Algae in our study are unlikely to be exposed to elevated nutrients associated with sewage input and terrestrial runoff because our study site is located around 12 km away from Ko Samui, the third largest island of Thailand that is a tourist destination. So, nutrient enrichment might have influenced the growth rate and the cycle of reproduction.

In the ambient tanks, reproduction occurred when the salinity increased from 31-33 psu to 40 psu. Observations on 4 species of Floridian *Halimeda* namely, *Halimeda discoidea* Decaisne, *Halimeda goreauii* W.R. Taylor, *Halimeda incrasata*, and *Halimeda opuntia*, showed that fertile thalli of the *Halimeda discoidea* collected from northern Palm Beach County, Florida were only observed when the salinity increased to 40 psu (Unpublished data, J. Mayakun). There is some evidence to show that sexual reproduction of *Enteromorpha* was suppressed at lower salinities (<23 ppt) (Fong *et al.*, 1996; Pringle, 1986). Thus, salinity is probably one of the environmental factors involved in triggering reproduction. However, the reproductive triggers might include synergistic effects of interacting environmental factors. So, the relative importance of nutrient enrichment and increased salinity on algal reproduction should be experimentally tested in a factorial design experiment both in the field and in outdoor water tanks. Further experiments are still needed to better understand the reproductive triggers of *H. macroloba* in the tropical regions of the Indo-Pacific oceans including Thai waters.

Comparison of gametangial clusters among *Halimeda* species showed variations in size, shape, and positions on the segment (Drew and Abel, 1988; Vroom and Smith, 2003b). Gametangial clusters of *H. macroloba* usually occur on the distal edges of segments whereas several species have gametangial clusters on one side of the segments. Compared to the size of other species, *H. macroloba* in the present study has the largest gametangial diameters larger than those of *H. macroloba* collected and studied by Dong and Tseng (1980), which ranged from 135.7-200.0 μm (Vroom and Smith, 2003b).

We found divided and sessile gametangia in both the field and laboratory. The gametangial morphology of *H. macroloba* is reported herein for the first time.

Acknowledgements

This study is a part of the doctoral thesis research of the first author at the Prince of Songkla University (PSU), Thailand. The first author would like to thank the Seaweed & Seagrass Research Unit team, PSU, that assisted with the field work. We are also indebted to Dr. Peter Vroom for ideas and comments. Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0063/2550) to Jaruwan Mayakun and Anchana Prathep is acknowledged. This work was also partly funded by the Prince of Songkla University Graduate Studies Grant and the Graduate School, PSU. Thanks also to Dr Brian Hodgson for assistance with the English.

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