Taiwania 62(2): 168–174, 2017
DOI: 10.6165/tai.2017.62.168

Reproductive organ characteristics and phenology of a seagrass *Thalassia hemprichii* (Ehrenberg) Ascherson in the Andaman Sea, Thailand

Pattama TONGKOK[1], Chatcharee KAEWSURALIKHIT[2,4] and Prasart KERMANEE[1,3,*]

1. Department of Botany, Faculty of Science, Kasetsart University, Bangkok, 10900, Thailand.
2. Department of Fishery Biology, Faculty of Fisheries, Kasetsart University, Bangkok, 10900, Thailand.
3. Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok, 10900, Thailand.
4. Center for Advanced Studies for Agriculture and Food, Kasetsart University, Bangkok, 10900, Thailand.
[*] Corresponding author's email: fscipsk@ku.ac.th

(Manuscript received 13 January 2017; accepted 14 April 2017; online published 3 May 2017)

ABSTRACT: Characteristics of the reproductive organs and phenology of *Thalassia hemprichii* using samples collected from the Andaman Sea, West coat of Thailand, are described. Permanent slides of specimens were prepared by the paraffin method. Preparations were observed under a stereo microscope and a light microscope. The occurrences of reproductive organs were recorded monthly for one year. Staminate and carpellate flowers are actinomorphic. They have three tepals and are unscented. Secretory cells were found in tepals, connective parenchyma of the anther and ovary wall. The pollen grains are spherical and 30-50 µm in diameter. Numerous long papillae were found on the stigmas. One to nine viviparous seeds were observed per fruit. The reproductive organs occurred in the greatest number during the lowest tides during the night time and when temperatures were 24 to 26 ^{o}C (December to March). The reproductive organs tend to change with water depth. They decrease when the water depth increases. The highest number of staminate flowers (168.67±47.05 m^{-2}), carpellate flower (205.67±41.23 m^{-2}) and fruit (161.17±31.16 m^{-2}) were found at 1.5 m above lowest low water (LLW).

KEY WORDS: Anatomy, the Andaman Sea, Morphology, Reproductive organs, *Thalassia hemprichii.*

INTRODUCTION

Thalassia hemprichii (Ehrenberg) Ascherson is a seagrass in the family Hydrocharitaceae. It is widely distributed (Meñez *et al*., 1983) and is the dominant seagrass in mixed seagrass beds and is the climax seagrass species in the Indo-Pacific region (Lacap *et al*., 2002; den Hartog, 1970; Short *et al*., 2010). It is found from southern Japan to Taiwan, extending to Hainan, China, the Philippines, and throughout Southeast Asia (Short *et al*., 2010). *Thalassia hemprichii* is an obligate hydrophil. Its pollen grains are distributed by sea water (hydrophilous pollination) (Cox, 1988). It can grow on hard coral substrates with little sediment cover, and can be found on muddy substrates at low tide (Short *et al*., 2010). Numerous algae and epiphytes grow thickly on its leaves. The leaves also are a food source for animals that eat seagrass such as small grazing creatures, such as snails, dugongs and green turtles. Therefore, it is also sometimes called Dugong grass or Turtle grass (McKenzie *et al*., 2007).

Phenology responses of *Thalassia hemprichii* are approximately synchronous in the same season in the Indo-Pacific region (Walker *et al*., 2001). In Thailand, *Thalassia* is in seagrass beds in Haad Chao Mai National Park, the largest seagrass beds with the highest species diversity for a single area in Thailand (Green and Short, 2003). This area also is claimed to be the habitat of the last dugong herds in the country (Hines *et al*., 2005; Supanwanid and Lewmanomont, 2003). *Thalassia hemprichii* is one of the most dominant species in the seagrass beds of Thailand (Nakaoka and Supanwanid, 2000). Most studies of *T. hemprichii* in Thailand have focused on ecology and distribution (Poovachiranon *et al*., 1994; Nakaoka and Supanwanid, 2000), while morphological, anatomical and phenological studies are still limited. This research aimed to investigate the morphological and anatomical characteristics of the reproductive organs and phenology of *T. hemprichii* at different water depths. Information about the reproductive organs and phenology relating to water depth and season are important for understanding adaptation to an oceanic lifestyle and long-term restoration and conservation of seagrass beds.

MATERIAL and METHODS

Morphological study

The specimens of *Thalassia hemprichii* were collected from Haad Chao Mai National Park, Trang Province, southern Thailand, facing the Andaman Sea. The collection site was at 7°23.4'N to 7°23.4'N latitude and 99°20.16'E to 99°19.64'E longitude. Different parts of the reproductive organs, including staminate flowers, carpellate flowers and fruits were collected monthly. Different stages of the seedlings were obtained from seeds planted in an aquarium. The fresh specimens were observed under stereo microscope for morphological studies.

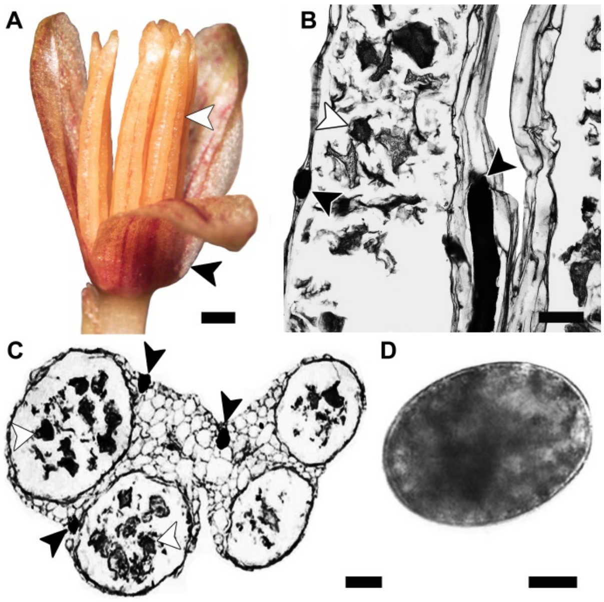

Fig. 1. Morphology and anatomy of ***Thalassia hemprichii*** staminate flower. **A**: A staminate flower showing tepals (black arrow) and anthers (white arrow). **B**: Longitudinal section of an anther showing pollen grain (white arrow) and secretory cells (black arrow). **C**: Transverse section of an anther showing 4 pollen sacs with pollens (white arrows) and secretory cells (black arrows). **D**: A spherical pollen grain. Scale bar: A = 1.0 mm; B = 50 µm; C = 100 µm; D = 10 µm.

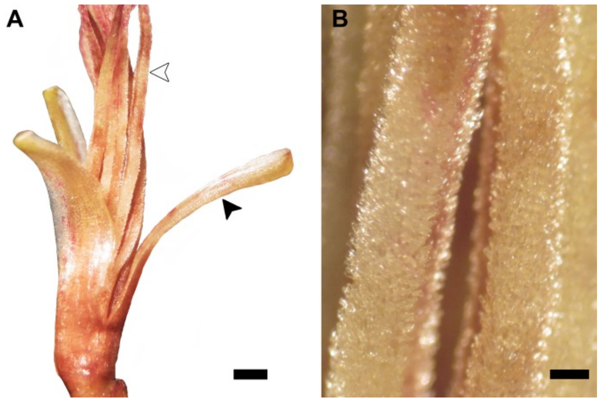

Fig. 2. Morphology of ***Thalassia hemprichii*** carpellate flower. **A**: A carpellate flower showing tepals (black arrow) and stigmas (white arrow). **B**: Stigmas showing long papillae. Scale bar: A = 1.0 mm; B = 0.2 mm.

Anatomical study

The specimens were fixed in a weak chromium acetic acid solution for 24 h at room temperature. Permanent slides were prepared by the paraffin method (Johansen, 1940; Kermanee, 2008). The specimens were stained with 1% safranin and 0.5% fast green prior to mounting with permount.

Developmental study

Seeds of *Thalassia hemprichii* were planted in an aquarium. The culture medium consisted of sterilized artificial seawater and sandy loam collected from the seagrasses beds.

Phenological study

The occurrences of reproductive organs were recorded monthly for one year in quadrats of 1.0×1.0 m. The sampling sites were at 1.5, 1.1, 0.9, and 0.7 meters above the lowest low water (LLW). The percentage of reproductive organs and shoots of *T. hemprichii* were counted from 4 quadrats at each sample site. For data analysis, differences in various parameters were tested with one way ANOVA followed by Tukey test ($P<0.05$).

RESULTS

Reproductive organ characteristics

Staminate flowers are solitary, actinomorphic, slightly reddish white, and unscented. The flower develops on a vertical underground stem. The peduncle is 1-3 cm long. The spathe is broadly lanceolate, **2-3** cm long, and 2-branched at the acuminate apex. The imbricate tepals are 0.8-1 cm long and 3-4 mm wide and consist of a single layer of epidermis. Two large air lacunae are in each tepal. Numerous secretory cells were observed in the mesophyll of the tepals. The androecium consists of 9 stamens. The stamens, with short filaments, are 0.5-0.9 cm long and 0.8-1 mm wide (Fig. 1A). The anthers have a single vascular bundle surrounded by connective parenchyma. The anther has secretory cells in the wall layers. Long secretory cells were found in the connective parenchyma. Circular secretory cells were found in the epidermis and outer endothecium (Figs. 1B, 1C). The anther consists of 4 pollen sacs with numerous pollen grains (Fig. 1C). The pollen grains are spherical, 30-50 µm in diameter and without an aperture (inaperturate) (Fig. 1D).

The whitish carpellate flowers are solitary and actinomorphic. The peduncle is **1.1-1.5** cm long. The spathe is lanceolate. The reddish tepal is oblong, 1-1.2 cm long and 2-2.5 mm wide, glabrous. The apex is hook shaped. The tepals have a single layer of epidermis and lack secretory cells. The stigma is 1.4-1.6 cm long and 0.4-0.6 mm wide. (Fig. 2A). Each pistil has six slender styles terminated by 2-branched stigmas. Numerous long papillae were observed on the stigmas (Fig. 2B). The peduncle consists of one layer of epidermis. The vascular cylinder is in the center of the cortex. Other vascular bundles are surrounded by ground tissue. The cortex contains numerous air lacunae and secretory cells. The secretory cells are scattered in the cortex (Figs. 3A-3C). The gynoecium is a syncarpous inferior ovary with 6 carpels and is covered by a spathe. The ovary wall is covered with numerous short trichomes. Abundant secretory cells were observed in the spathe and ovary wall. Each ovary contains one to nine anatropous ovules on a parietal placenta (Figs. 3D, 3E).

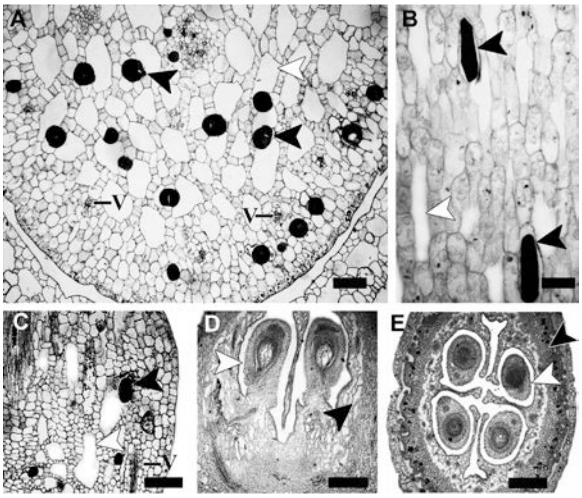

Fig. 3. Anatomy of ***Thalassia hemprichii*** carpellate flower. **A**: Transverse section of a peduncle showing numerous lacunae (white arrow), vascular bundles (v) and secretory cells (black arrows). **B**: Longitudinal section of a peduncle showing air lacunae (white arrow), and secretory cells (black arrows). **C**: Longitudinal section of a peduncle showing vascular bundles (v), air lacunae (white arrow), and secretory cells (black arrows). **D**: Longitudinal section of an ovary showing anatropous ovules (white arrow) and ovary wall (black arrow). **E**: Transverse section of an ovary showing 4 ovules (white arrow), and ovary wall (black arrow) which covered by a spathe. Scale bar: A = 100 µm; B = 100 µm; C = 200 µm; D = 500 µm; E = 500 µm.

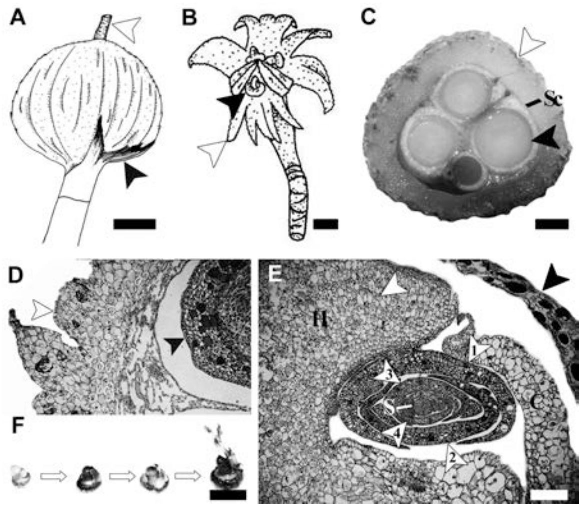

Fig. 4. Fruit and seed of ***Thalassia hemprichii***. **A**: An illustration of a mature fruit with a persistent spathe at the base (black arrow) and long beak at the apex (white arrow). **B**: An illustration of ripening fruit showing viviparous seed (black arrow) and pericarp (white arrow). **C**: Transverse section of fruit showing pericarp (white arrow), and seeds (black arrow) which are covered by a soft seed coat. **D**: Transverse section of pericarp (white arrow) and seed showing seed coat (black arrow). **E**: Transverse section of seedling showing numerous starch grains (white arrow) and four leave in different stage of development (white arrow with Arabic numerals, 1 = 1st leaf, 2 = 2nd leaf, 3 = 3rd leaf, and 4 = 4th leaf) are protected by seed coat (black arrow) with secretory cells. **F**: Development of seedling, 1st day, 2nd day, 4th day, and one week, respectively. Abbreviations: C, cotyledon; H, hypocotyl; S, shoot; Sc, seed coat. Scale bar: A = 5 mm; B = 5 mm; C = 3 mm; D = 250 µm; E. = 500 µm; F = 6 mm

The fruit is oblate to ovoid and prickly, 1.5-2.5 cm long and 1-3 cm wide. There are one to nine seeds per fruit. The spathe is persistent at the base of the fruit, which has an apical beak 1-1.5 mm long (Fig. 4A). The pericarp is decomposes after ripening (Fig. 4B). The exocarp develops thick spines. The outer layer of the exocarp has 10-20 lobes. Secretory cells were observed in the spines. The mesocarp contains scattered vascular bundles, ground parenchyma and secretory cells. Abundant starch grains were observed in the ground parenchyma of the mesocarp. The endocarp consists of parenchyma with large air lacunae (Figs. 4C, 4D). The seeds germinate before they are released from the fruit (viviparous seeds). Mature viviparous seeds are 3-5 mm long and 4-6 mm wide. The viviparous seeds have a seed coat which large air lacunae and secretory cells. The embryo has a distinct leaf primordium that is protected by a cotyledon and enlarged hypocotyls. Secretory cells and starch grains were found in hypocotyl tissue (Fig. 4E).

Mature seeds from ripened fruit were cultured in an aquarium under sterile conditions. The first leaf was observed within 2 days after planting. Seedlings with 4 leaves were obtained in one week. The horizontal rhizome was developed in four weeks. A vertical rhizome emerged from the horizontal rhizome, after which numerous adventitious roots developed from the rhizome. The hypocotyl and radicle were no longer developed (Figs. 4F, 5-6).

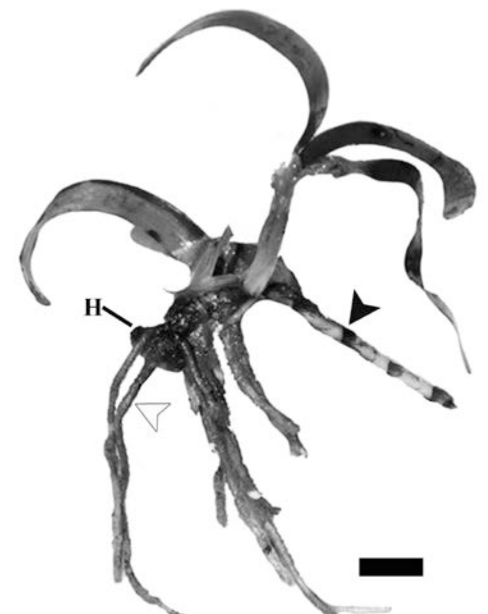

Fig. 5. Morphology of ***Thalassia hemprichii*** plantlet after one month showing numerous adventitious root which produced from horizontal rhizome (black arrow), primary root (white arrow), and brunette hypocotyl. Abbreviations: H, hypocotyl. Scale bar = 9 mm.

Table 1 Number of shoots and reproductive organs of ***T. hemprichii*** monthly recorded at Haad Chao Mai National Park, the Andaman sea.

Year	Month	Number of shoots and reproductive organs (mean±S.E./m^2)			
		Shoots	Staminate flowers	Carpellate flowers	Fruit setting
2015	August	888.25±17.06^{a}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}
	September	878.50±47.37^{a}	22.00±12.00^{bc}	103.25±49.51^{c}	54.50±20.99^{c}
	October	921.75±46.82^{a}	66.50±16.50^{b}	127.25±62.71^{bc}	99.75±46.27^{bc}
	November	935.00±23.25^{a}	16.25±6.25^{bc}	66.50±42.39^{c}	63.75±30.69^{bc}
	December	878.75±50.31^{a}	84.75±21.92^{b}	85.50±49.85^{c}	66.50±32.39^{bc}
2016	January	861.25±50.68^{a}	199.00±86.28^{a}	205.00±69.53^{ab}	74.00±38.85^{bc}
	February	953.00±59.96^{a}	246.25±99.03^{a}	311.25±77.20^{a}	201.25±43.85^{ab}
	March	936.00±38.65^{a}	87.75±34.86^{b}	297.75±99.90^{ab}	266.25±78.01^{a}
	April	817.75±35.81^{a}	0.00±0.00^{c}	0.00±0.00^{d}	107.25±34.82^{bc}
	May	823.50±29.15^{a}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}
	June	852.25±47.95^{a}	44.25±15.55^{bc}	55.25±35.25^{c}	22.25±12.25^{cd}
	July	878.50±47.24^{a}	82.50±37.07^{b}	121.25±64.94^{bc}	88.75±42.13^{bc}
Total		10625	849	1373	1044

Letters (superscript) attached to values indicate significant differences (Tukey test, P<0.05) among month.

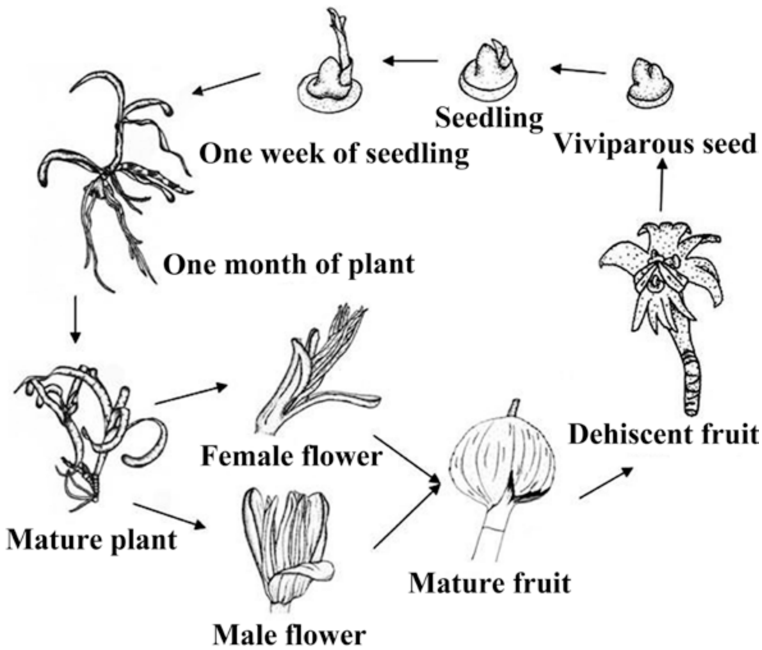

Fig. 6. Development of vegetative and reproductive organs of ***Thalassia hemprichii*** showing different stages of reproductive organs to grow into mature plant which is capable of both sexual reproduction and asexual propagation.

Phenology

Studies of the timing of phenophases of flowering and seasonal changes of reproduction in *Thalassia hemprichii* found that staminate and carpellate flowers tend to increase from October to February, and decrease from March to May. The average number of shoots and reproductive organs at various months are shown in Table 1. The maximum percentage of staminate and carpellate flowers was 27.82 and 35.17 percent, respectively. Fruit set tends to increase from January to March and is sparse in April. Maximum fruit set was 30.08 percent. Average data of temperature and depth of the water in meters in Trang province in the Andaman sea in each month were compared with production of reproductive organs (Fig. 7). We found that temperature fluctuated in each month. Production of reproductive organs tended to increase when temperature was in the range of 24-26 ^{c}o. Production of reproductive organs was steadily increased during the lowest low tides (December to March).

The percentage of reproductive organs at various water depths throughout the year revealed that staminate flower and carpellate flowers and fruit tend to decrease when the depth of the water increases (Table 2, Fig. 8). The percent of staminate flowers was 19.05, 10.36, 2.40 and 0.16 percent at 1.5, 1.1, 0.9 and 0.7 meters above the lowest low water (LLW), respectively. The percent of carpellate flower was 23.23, 17.91, 8.71 and 1.85 percent at 1.5, 1.1, 0.9 and 0.7 meters above LLW, respectively. Percent of fruit above LLW were 18.21, 12.89, 7.04 and 1.18 percent at 1.5, 1.1, 0.9 and 0.7 meter, respectively (Fig. 8). The average number of

Table 2 Number of shoots of ***T. hemprichii*** and reproductive organs at various water depths above lowest low water (LLW) at Haad Chao Mai National Park, the Andaman sea.

Water depth above the lowest low water	Number of shoot and reproductive organs (mean±S.E./m²)			
	Shoots	Staminate flowers	Carpellate flowers	Fruit setting
1.5 meter	987.42±17.46[a]	168.67±47.05[a]	205.67±41.23[a]	161.17±31.16[a]
1.1 meter	875.08±21.72[b]	91.75±39.39[ab]	158.50±47.66[a]	114.08±32.77[a]
0.9 meter	862.42±14.82[b]	21.25±15.57[b]	77.08±38.79[ab]	62.33±27.84[ab]
0.7 meter	816.58±14.85[b]	1.42±0.83[b]	16.42±9.85[b]	10.50±7.13[b]

Letters (superscript) attached to values indicate significant differences (Tukey test, P<0.05) between water depth.

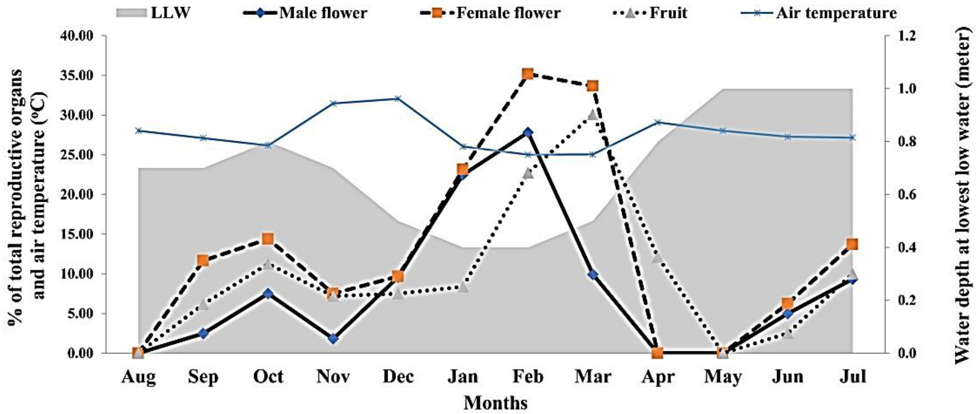

Fig. 7. Seasonality in reproductive organs abundance of ***Thalassia hemprichii*** at Haad Chow Mai National Park, the Andaman Sea, Thailand, expressed as percent of the total number of staminate and carpellate flowers, and fruit setting. Average data of temperatures and heights of water predicted in meters at the lowest low water in each month are shown.

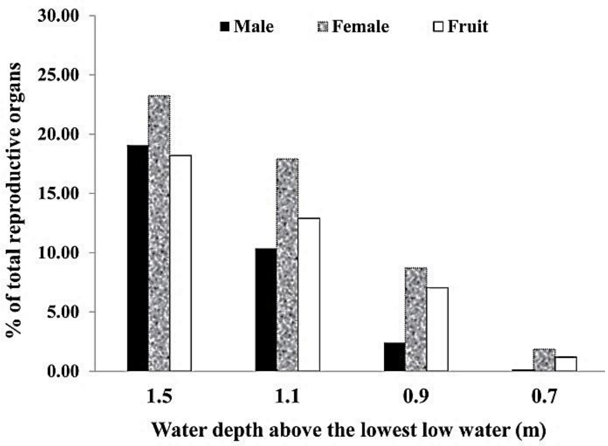

Fig. 8. Percentage of the total number of reproductive organ occurrence of ***T. hemprichii*** in various water depths above the lowest low water at Haad Chow Mai National Park, the Andaman Sea, Thailand.

shoots and reproductive organs at various water depths above LLW are shown in Table 2. There was significant difference in shoot density at different water depths ($p<0.05$; Table 2). The highest annual mean density was found at 1.5 meters above the lowest low water and the lowest at 0.7 meter above the lowest low water, 987.42±17.46 and 816.58±14.85 m^{-2}, respectively. The number of staminate flowers, carpellate flowers and fruit tend to decrease as the water becomes deeper (Table 2).

DISCUSSION

Numerous secretory cells were found in the tepals and anthers. The secretory cells may relate to high light intensity at low tide. Because the staminate flowers develop on vertical underground stems, and are raised in the water on a long peduncle, they are not protected by a leaf from desiccation at low tide. This finding is consistent with the findings of Short and Duarte (2001), who reported that high light intensity increases the percentage of secretory cells in the leaves and tepals of *T. hemprichii*. In addition, long secretory cells were observed in the connective parenchyma of the anthers and circular secretory cells in the epidermis and outer endothecium. Long secretory cells develop from single secretory cells, which elongate extensively, along with laticifers, during plant growth. The secretory cells may help in bearing the fragile stamens and prevent wave or water surges that may cause detachment of immature anthers. In addition, Jillian *et al.* (2008) reported that laticifers are vital for the defense of plants against herbivores. The perishable tepals of carpellate flower do not have secretory cells. We observed that carpellate flowers are buried in mud or sediment in the seagrass beds and only the stigma is raised above the ground to trap pollen grains.

Plants of *Thalassia hemprichii* are dioecious. Pollinators were not observed. Staminate flowers do not form distinct petals and sepals, but have one undifferentiated whorl comprising structures called tepals. Three tepals protect the anther before it opens and support them after flowering. They are unscented and apparently are pollinated by seawater, multiple petals may be a barrier to pollen dispersal. In addition, *T. hemprichii* has a mechanism to achieve pollination by forming large air lacunae in the tepals of staminate flowers. The staminate flower is held on a long peduncle. This enhances the staminate flower to float on water and freely disperse after detachment from staminate plant when the water is higher. At high tide the pollen grains drift through the water, and are trapped by the numerous long papillae on the stigmas of carpellate flowers, which are above the ground while ovary is below the ground. The ovary is at the base of the shoot and hidden by a leaf sheath and sediment for defense from waves and herbivores.

Consequently, in seagrass beds, the seeds of *T. hemprichii* are buried in mud and compressed by sediment. This finding contradicts Lacap *et al.* (2002), who reported that fruit and seeds of *T. hemprichii* traveled at 0.47 km h^{-1} and flotation time was 55 hours. We observed that the fruit and seeds of *T. hemprichii* in the mud cannot drift. The fruit are sometimes detached from the peduncle and occur above ground by severe storms or animals in the seagrass beds. Kuo *et al*. (1991), reported that numerous dehiscent fruits were found on the shore in Queensland.

Anatomical studies found that the fruits of *T. hemprichii* have numerous secretory cells in the pericarp. The contents of the cells may protect the fruit from herbivory. The taste is quite caustic and distinctly astringent. The short spines on the pericarp of the fruit penetrate the sediment during growth. The pericarp decomposes immediately after ripening. After ripening, the seeds are released from the fruit and grow abundantly near the parent plant. In addition, parent plants have numerous, compact horizontal rhizomes that form a network. Lacap *et al*. (2002) and den Hartog (1970), reported that it is the dominant seagrass in mixed seagrass beds in the Indo-Pacific. The seeds of *T. hemprichii* begin to grow before they detach from the fruit (viviparous seed). The viviparous seeds have an embryo with a distinct leaf primordium which is protected by a hypocotyl. The hypocotyl has numerous starch grains and secretory cells in the base portion. The seeds remain vertical in the water and the straight taproot pierces into mud when the seed drops. Secretory cells in the hypocotyl tissue may help defend against insects and creatures in the soil. We observed that seeds of *T. hemprichii* have a soft seed coat covering the seed. The seed coat decomposes immediately after fruit ripening. However, in Queensland, Australia, Kuo *et al*. (1991) reported that seeds of *T. hemprichii* lack a distinct seed coat, but are covered by a soft pericarp on certain portions.

The timing of phenophases of flowering and seasonal changes of reproduction in *T. hemprichii* show that reproductive organs tend to increase when the temperature is in the range of 24-26 ^{o}C, and peak in the range of the lowest low tide. However, they do not peak during high tide period when temperatures are 24-26 ^{o}C. The findings show that *T. hemprichii* responds to a variety of tide levels during flower production. Flowering in *T. hemprichii* was greatest when the lowest tides occurred at night and temperatures were low (December to March). In contrast, reproductive organs of *T. hemprichii* were low when the lowest tides occurred during the daylight and temperatures were high (April to August).

Although, previous phenological studies on *T. hemprichii* focused on seasonal changes and temperature (Phillips *et al*., (1983) and McMillan (1982) noted that temperature is a major influence of seagrass physiology, distribution, flowering and seed germination. Furthermore, McMillan (1980) reported that *T. hemprichii* did not flower at high temperatures (27-31 ^{o}C), but flowered after transfer from a higher temperature to a lower one of 24-26 ^{o}C. Temperature is a critical factor in flower production, as shown in experimental cultures, not in situ. Our study indicates that tide probably plays a key role in sexual reproduction. The phenology of seagrasss in various places occurs at different seasons. This process is probably directly related to the lowest tide in each zone.

The number of reproductive organs at various water depths tends to decrease when the depth of water increases and peaks at 1.5 m above LLW. Shoots production showed different trends, but was rather constant. The results apply only to the study site (Haad Chao Mai National Park, Thailand). Since *T. hemprichii* is able to reproduce both sexually and asexually, horizontal and vertical rhizomes are developed all the time and can grow all year round. This is one of the reasons that *T. hemprichii* in seagrass beds in Andaman Sea, Thailand, were not damaged after the tsunami disaster (quality of seawater and total suspended solids were normal) (Department of marine and coastal resources, 2005; Munprasit *et al*., 2006; Department of marine and coastal resources, 2013), and it still widely distributed in seagrass beds in Thailand.

ACKNOWLEDGEMENTS

We would like to thanks the Department of National Parks, Wildlife and Plant Conservation for research permits. This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, and the Graduate School, Kasetsart University, Chatuchak, Bangkok, 10900, Thailand.

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