

Morphological and anatomical responses of the common mangrove *Rhizophora mucronata* seedlings to flooding**Surangkana Phandee^{1,*}, Sutthinut Soonthornkalump¹ and Pimchanok Buapet^{1,2}**¹*Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla 90110, Thailand*²*Coastal Oceanography and Climate Change Research Center, Prince of Songkla University, Songkhla 90110, Thailand*

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Abstract

Mangrove forests play an important role for both humans and animals especially as a food source and habitat. *Rhizophora mucronata* is a dominant species in many mangrove areas. When flood occurs, the seedlings which are in the most sensitive stage, might be significantly affected. A purpose of this study was to investigate the morphological and anatomical response of *R. mucronata* seedlings to the two levels of flooding: waterlogging and submergence. An experiment consisted of three treatments including control (regular drainage), waterlogging (containing 10 cm of water above the soil surface) and complete submergence that lasted for 20 days. Our results show a rapid leaf abscission and an adjustment of leaf angle to more vertical position in the submerged seedlings. Both responses are common in the flooded plants and are related to hormonal regulation. In addition, plant roots in flooded treatment were visibly darker than the controls, possibly due to a damage in highly reducing rhizosphere. In terms of anatomical change, we found the enlarged aerenchyma tissues and lenticel formation in root periderm of the flooded plants. These results suggest that the two flooding levels may induce a hypoxia and consequently stimulated an alteration of anatomical characteristics which plays an important role in conveying and storing oxygen within roots. However, there is no significant difference in anatomical features of leaf tissue in this study. Further studies in physiological adaptation are required to better understand the responses of the mangrove seedlings to flooded condition.

Keywords: Waterlogging, Submergence, Hypoxia, Aerenchyma formation**Introduction**

Mangroves forests provide a habitat and food source for many wild animals and humans. It is also a nursery for juvenile aquatic animals and provides a natural barrier protecting the coastal communities from wind, tides and storms as well as facilitates the sedimentation processes (Kamali & Hashim, 2011; Lu, 2013). *Rhizophora mucronata* is one of the dominant species in many areas of mangrove forests including the southern part of Thailand (Thampanya, 2006; Wan Juliana, 2014; Clarisse, 2016). A small mangrove seedling is an important part for expand and establish mangrove areas, but the seedling stage is susceptible to changing environment (Lewis, 2019) Currently, mangrove forests are facing a rapid deterioration as a result of anthropogenic disturbances such as clearing for aquaculture, urbanization and charcoal production for agriculture (Kamali & Hashim, 2011; Lovelock, 2015). In addition, global warming imposed a number of stressors on mangrove ecosystems, such as increasing temperature, more frequent severe storms, changes in a pattern of ocean circulation and sea level rise (Gilman, 2008; Lu, 2013). The rise of sea level in the Indo-Pacific region has been estimated to be about 0.6-1.2m by 2100, which has a significant impact on the areas with low tidal range and low sediment deposition (Lovelock, 2015). In 2010-2015, the high levels of flooding in mangrove forests have been reported from a short period as a few days to a long period up to three weeks (Duke, 2017). Long-term flooding can lead to a reduction in

oxygen content (hypoxia) both in water and sediment phases and may lead to mangrove die-off (Chambers, 2016). In addition, previous studies reported that *R. mucronata* seedlings showed that there was a decrease in biomass and plant height with increasing inundation level and salinity (Hoppe-Speer, 2011).

Survival of plants under flooding depends on their adaptation and tolerance. Previous studies showed that plants respond to flooding via several mechanisms. Plants may alter their energy allocation to the major organ for survival and growth (Bailey-Serres, 2016) as well as exhibit some morphological and anatomical changes to avoid the submergence and the associated stress, such as an increase in height (Voesenek, 2004), rapid re-positioning of leaf to upward direction (Cox, 2004), the formation of adventitious root (Vidoz, 2010), the generation of lenticel on their stem and root (Shimamura, 2010) as well as the expending of lacunae airspace in cortex aerenchyma area (Longstreth & Borksenious, 2000). It is clear that most of the adaptations previously observed are for the purpose of increase gas exchange to avoid hypoxia. Although most of the wetland species have been adapted to periodic waterlogging, different species respond differently to inundation (Hoppe-Speer, 2011; Phukan, 2015). Small seedlings may be more susceptible to flooding as a complete submergence may occur. This study aims to investigate the morphological and anatomical responses of *R. mucronata* seedlings to the two levels of flooding: waterlogging and submergence. These results would improve our understanding in the changes of leaf and root structure under flooding condition and also possibly apply to the management of mangrove habitats.

Materials and methods

One month old seedlings of *Rhizophora mucronata*, obtained from a mangrove rehabilitation center in Songkhla Province, were grown in a semi-control environment (in the plastic boxes with a size of 41 x 59 x 33 cm³) using the sediment collected from natural setting. During an acclimation period, the seedlings were watered with ½ Hoagland nutrients solution every three days and the salinity was gradually adjusted from 2 to 8 psu that is similar to natural habitat (Hoppe-Speer, 2011). The experiment consisted of three treatments (three seedlings per treatment):

- 1) Control: Seedlings were grown in a regular drainage condition.
- 2) Waterlogged condition: Seedlings were grown without drainage with the water level of 10 cm above the soil.
- 3) Submerged condition: Seedlings were grown without drainage with the water level above the leaf apex.

Roots and leaves were collected at the end of the experiment on day 20 in total 18 samples. For the root samples, soil and sediment were removed by washing in de-ionized water and the taproot samples were collected at 4 cm from the root tip (approx. 1 cm long). The leaf samples (0.5X1.5 cm containing midrib) were collected at the center of leaf. All plant tissue samples were fixed in formalin-acetic acid-alcohol II (FAA II) solution containing 50% ethanol, 4% paraformaldehyde and 5% glacial acetic acid for 48h. The fixed sections were transferred to dehydration in a graduated tert-butyl alcohol (TBA) series (70%, 95% and 100%, with 2 h for each concentration). After that, the dehydrated samples were infiltrated and embedded with paraffin wax. Embedded samples were sectioned (6-8 µm) using rotary microtome and then affixed on glass slide. The prepared slide was stained with Safranin and fast green staining (Ruzin, 1999).

The measured parameters associated with the leaves are lamina thickness, upper epidermis thickness, hypodermis thickness, mesophyll layer thickness, lower epidermis thickness and area of air cavity per lamina. The measured parameters associated with the roots are root thickness, cortex thickness, number of cell layers in cortex, number of cell layers in periderm, ratio of thickness between pith and cortex, ratio of thickness between aerenchyma and periderm and area of air cavity per cortex. All parameters were observed within 100 µm² of randomly chosen area with 3 replicates each. A comparison was made between the controls and flooded seedlings. The mean and standard error values of the three

replicates measurements were compared and analyzed by one-way ANOVA test at the level of $p \leq 0.05$ using STATISTICA ver.10 program.

Results

Morphological changes

We documented some morphological changes as well as the visible alterations in *Rhizophora mucronata* seedlings throughout the experimental period. Abscission of leaves older than the second leaf pair occurred rapidly after five days in the complete submergence treatment. The submerged seedlings showed an adjustment of leaf angle (Fig.1A, pointed arrow). The initial angle between leaf pair in the submerged seedlings was approximately 99.5 ± 7.0 degree. After 20 days of the experiment, the angle between submerged leaves pair decreased by almost 42% at about 57.53 ± 1.2 degrees. In addition, the color of the leaves and stems were not different both the initial and the end of the experiment (Fig.1 A) In terms of roots, there was a difference in root color (Fig.1B). We observed that the color of flooded roots are darker than the control.

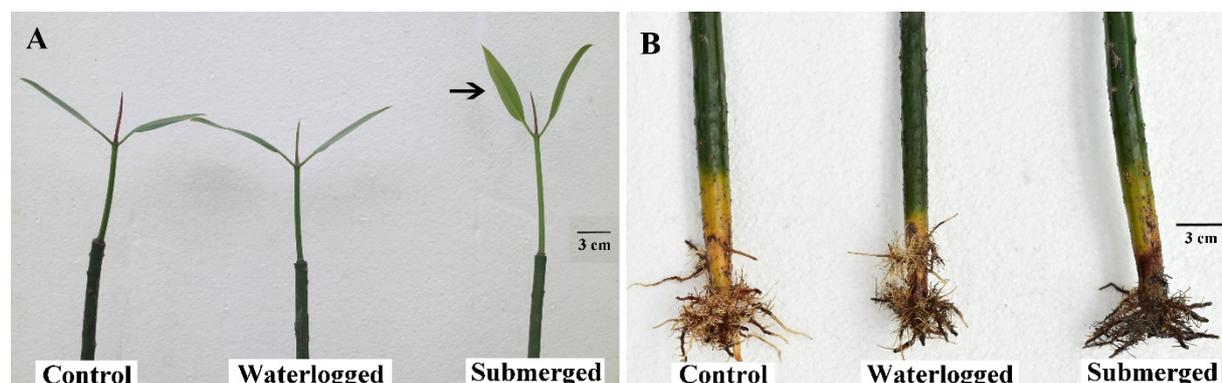


Figure 1 The visible character changes in leaves (A) and roots (B) of *Rhizophora mucronata* seedlings after 20 day of control, waterlogged and submerged treatments.

Anatomical changes

Leaf anatomical features

The leaf structure of *Rhizophora mucronata* seedlings consists of five major tissue layers in a lamina (Fig. 2, A-C): the upper epidermis, hypodermis, palisade mesophyll, spongy mesophyll and the lower epidermis, which was covered with a thin wax and contained typical stoma (Fig.2B, pointed arrow). A vascular bundle is distributed throughout the leaf width. Several parameters showed no statistically significant difference between the controls (drained) and flooded (waterlogged and submerged) seedlings, including lamina thickness or thickness of each tissue contributed to lamina thickness as well as the air cavity per laminar area of $100 \mu\text{m}^2$ (Table 1).

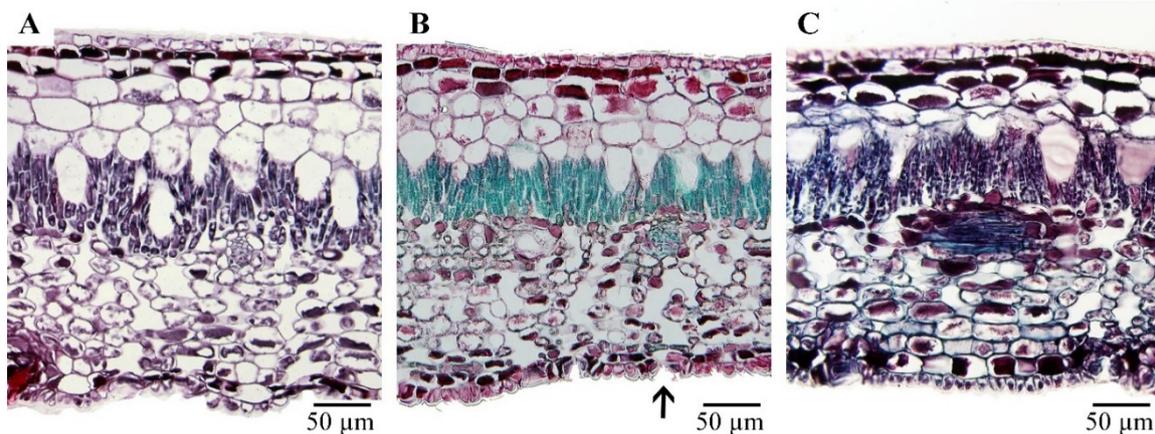


Figure 2 The anatomy of *Rhizophora mucronata* leaf (A; control, B; waterlogged and C; submerged seedling): the cross-section of the leaf blade showing the layer of palisade parenchyma and the mesophyll containing multilayers of hyperdermis, palisade parenchyma, vascular bundle of netted vein, spongy mesophyll, and stomata in the adaxial (arrow) (B).

Root anatomical features

The parameters such as root thickness, cortex thickness, number of cell layers in cortex, number of cell layers in periderm, and ratio of thickness between pith and cortex, were not statistically different between treatments (Table 1). However, the histological feature demonstrated that normal arrangement in the layer of periderm which indicated that the necrosis of periderm and lysed cell were absented. In addition, we detected the response related to tissue aeration induced by flooding. Waterlogged and submerged seedlings developed aerenchyma tissues with irregular cell shape and large lacunae (Fig.3E, F). Furthermore, the proportion of air cavity area per cortex of $100 \mu\text{m}^2$ in submerged seedlings was significantly higher than the controls (Table 1, $P \leq 0.05$). We also observed a formation of lenticel according to loose arrangement of hypodermis in the submerged seedlings (Fig.3C, pointed arrow). In addition, the ratio of thickness between aerenchyma tissue and periderm was significantly higher in the waterlogged treatment compared to the controls (Table 1, $P \leq 0.05$).

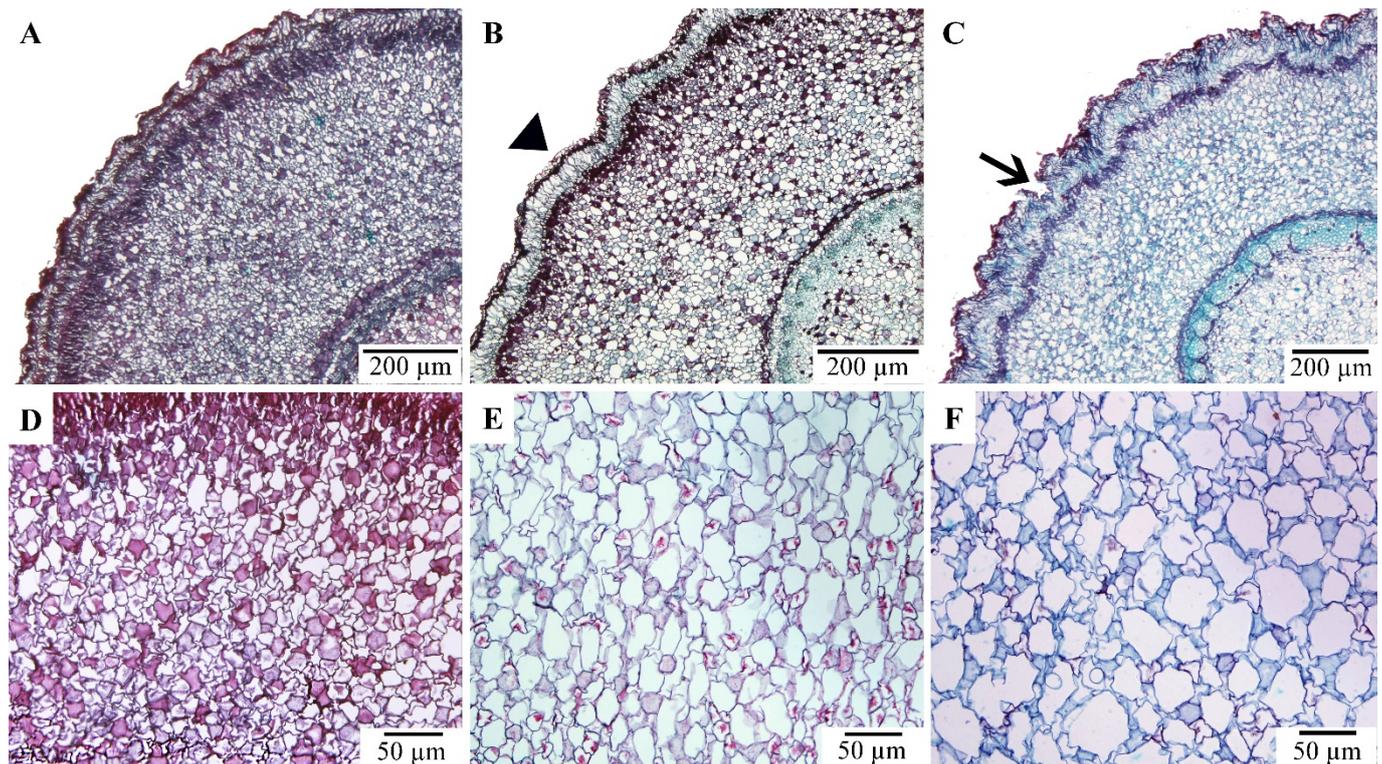


Figure 3 The root X.S. of *Rhizophora mucronata* seedlings after 20 days in the different growth conditions. The drained treatment (A) showed the densely cortex parenchyma with small air cavity (D), waterlogged root (B) the visible of cortex aerenchyma expansion was observed (E) and submerged root (C) showed the lenticel formation (arrow) and increased of aerenchyma space in cortex (F). The arrow head shown the normal arrangement in the layer of periderm (B).

Table 1 The anatomical parameters of leaves and roots of *Rhizophora mucronata* seedlings under drained, waterlogged and submerged conditions (n=3)

*Different alphabets represent significant differences.

Parameter	Drained	Waterlogged	Submerged
	Mean±SE	Mean±SE	Mean±SE
Leaf			
Laminar thickness (µm)	264.07±19.56 ^a	244.95±6.53 ^a	272.84±22.27 ^a
Upper epidermis thickness (µm)	8.64±0.36 ^a	8.95±0.69 ^a	8.73±0.44 ^a
Hypodermis thickness (µm)	92.09±9.65 ^a	86.61±3.41 ^a	96.09±8.77 ^a
Mesophyll layer thickness (µm)	123.86±8.57 ^a	127.10±10.33 ^a	119.51±7.54 ^a
Lower epidermis thickness (µm)	10.48±0.61 ^a	10.00±0.62 ^a	10.54±0.65 ^a
Area of air cavity per laminar of 100 µm ²	6.04±0.91 ^a	7.34±1.40 ^a	4.90±1.09 ^a
Root			
Root thickness(µm)	1255.04±97.93 ^a	1353.49±94.86 ^a	1274.18±16.19 ^a
Cortex thickness (µm)	463.08±38.64 ^a	517.42±28.51 ^a	550.29±39.91 ^a
Number of cell layer in cortex	21.72±6.16 ^a	26.61±2.90 ^a	17.61±3.74 ^a

Parameter	Drained	Waterlogged	Submerged
	Mean±SE	Mean±SE	Mean±SE
Number of cell layer in periderm	6.61±1.32 ^a	4.11±0.20 ^a	8.78±1.53 ^a
Ratio of thickness between pith and cortex	0.50±0.05 ^a	0.47±0.04 ^a	0.45±0.02 ^a
Ratio of thickness between aerenchyma and periderm	1.55±0.57 ^a	3.96±0.57 ^b	1.59±0.32 ^a
Area of air cavity per cortex of 100 μm^2	12.58±1.68 ^a	31.27±8.10 ^{ab}	35.72±5.59 ^b

Discussion and conclusions

Essentially, the negative effect of flooding environment is reducing gas exchange, leading to the reduction of carbon fixation as well as causing to the low oxygen level (hypoxia). In case of complete submergence, it does not only generate low gas exchange but also generate the light depleted condition by the water body reaching to low plant photosynthetic rate (Phukan, 2015). If flooding continuous in time, the reduction of photosynthesis capacity will be occur that effect can lead to a low growth rate depending on the level of flooding stress (Striker, 2012; Phukan, 2015). In addition, the faster response of the leaf abscission was found in this study. Several works on woody flooded plant have shown the early leaf abscission combined with higher ethylene level (Voeselek & Blom, 1989; Grichko & Glick, 2001; Negin & Moshelion, 2016; Polko, 2015). In addition, one of the reasons for the occurrence of leaf abscission is that plants under submerged condition may experience energy shortage due to low oxygen and light, resulting in energy allocation from older leaves to younger leaves (Kreuzwieser, 2004). Previous studies reported that plants increase an accumulation in both ethylene and abscisic acid as an early response to flooding. These two hormones are involved in the regulation of leaf abscission and epinastic leaf movement (Cox, 2004; Vidoz, 2010; Negin & Moshelion, 2016). A modulation of leaf angle to a more vertical position in the complete submerged plants observed in our study may be one of the strategies to approach sufficient light and increase contact with the air. The upward bending of leaves and the elongation of petioles called hyponastic response have been shown in other plants under complete submergence and have been suggested to play a role in order to maximize the leaf surface above the water level to restoring gas exchange (Voeselek & Blom, 1989; Cox 2004; Polko, 2015) Darker root color in the flooded treatments was possibly due to a reducing soli in flooded treatment. A reduction in soil redox potential is often found in concomitant with flooding. Such a change in physicochemical properties alters the availability of various essential nutrients and induces a production of phytotoxins such as Fe and Mn in reducing forms, lactic, formic, acetic, and butyric acid, ethanol, H₂S, etc. (Pezeshki & De Laune, 2012).

Previous studies reported that mangrove and wetland species can develop aerenchyma tissue rapidly especially in flooded and hypoxia condition (Youssef & Saenger, 1996; Purnobasuki & Suzuki, 2005; Teakle, 2011). In our results, the enlargement of cortex aerenchyma and the increase of lenticel formation in periderm in the submerged roots reflected hypoxia condition induced by flooding at both levels. This indicated that aerenchyma plays a crucial role in conveying and storing oxygen within the roots (Youssef & Saenger, 1996; Longstreth & Borksenious, 2000; Purnobasuki & Suzuki, 2005). These characteristics are important for gas diffusion between inner layers of plant tissue and the surface under flooded and low oxygen environment (Shimamura, 2010). However, we did not find significant difference in anatomical features of leaf tissue between the controls and the flooded seedlings even under the complete submergence. It is possible that the duration of this experiment may not sufficient to elicit any impact on leaf anatomy and as the leaves we observed were not in the fully developmental stage, an alteration in anatomical development may be minor (Herrera, 2013).

In conclusion, *Rhizophora mucronata* seedlings showed both morphologically and anatomically alteration in response to flooding. The responses observed in this study related to light depletion and aeration, suggesting that these are the two main responses occurring during the floods. Nevertheless, we did not observe any mortality, indicating the flood tolerance of *R. mucronata*, which are usually subjected to sea level fluctuations in their natural habitats (Youssef & Saenger, 1996; Kozlowski, 1997). However, this study gives an estimate of the optimal level of the flood that will reach an optimal growth of *R. mucronata* seedling and also shows the flood tolerance of *R. mucronata* seedling. Such information can be used to select suitable areas and environments for using this species in the process of effective mangrove rehabilitation.

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