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Oxidative stress as an indicator of niche-width preference of mangrove *Rhizophora stylosa*

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ABSTRACT

Inundation, elevation gradient and salinity fluctuations are considered the major abiotic drivers that influence the growth and distribution of mangroves. In certain conditions, these environmental stressors trigger excessive generation of reactive oxygen species (ROS), affecting mangrove physiology and homeostasis, leading to oxidative stress and mortality if the condition remains exacerbated. As a natural defense to quench the deleterious effects of ROS, mangroves have developed an antioxidant system to scavenge the toxic effects of excessive ROS. This study investigated environmental stress due to salinity, inundation, and elevation gradient using the biochemical responses in the leaves of *Rhizophora stylosa* as an oxidative stress indicator in greenhouse experiments and field conditions. From the variations of biochemical responses and levels of oxidative stress, the niche-width preference was extrapolated. The results of the study showed that the observed environmental factors significantly induced the generation of high H_2O_2 concentrations in the leaves of *R. stylosa*, which in return activated the antioxidant defense system. Inundation of the whole plant imposed a higher-order oxidative stress compared with the effect of salinity in the greenhouse condition, as shown by the significant increase in H_2O_2, and even caused sublethal damage, as manifested by the chlorotic leaves when prolonged. In the field, rare inundation and high elevation are also considered stressful to *R. stylosa*, as shown by the significantly higher H_2O_2 levels compared with those in the frequently inundated (but plants not submerged) areas. The long-term negative effects of high H_2O_2 at the plant and community levels were manifested in the reduction of growth rate in plants cultured in the greenhouse and the reduction of height in the 30-year-old *R. stylosa* plantation. Integrating the levels of oxidative stress induced by salinity, inundation and elevation from both greenhouse experiments and field conditions, it appears that relatively low oxidative stress results in a preference of the niche-width of *R. stylosa* in inundated areas as long as the leaves remain emerged, even during the spring tide. Thus, this species typically dominates middle intertidal areas. These findings could have valuable implications for the selection of areas appropriate for mangrove rehabilitation.

1. Introduction

Mangroves are halophytic plants inhabiting the intertidal zones of tropical and subtropical regions. Being at the interface between land and sea, the survival and establishment of seedlings are under the continuous influence of different environmental drivers, specifically salinity and tidal inundation (Tomlinson, 2016). Salinity is one of the primary abiotic drivers in mangroves, and the effect of salinity has been extensively studied (Ball, 1988a; Lugo and Snedaker, 1974). However, conflicting views remains unresolved concerning whether mangroves are facultative or obligate halophytes (Krauss and Ball, 2013; Wang et al., 2011). Different mangrove species show different salinity preferences and achieve optimum growth in varying salinity levels. A study by Jayatissa et al. (2008) showed optimum growth of *Sonneratia caseolaris* at low salinity (3–5 ppt), while Aziz and Khan (2001) reported that *Ceriops tagal* had optimum growth at 50% seawater. Most studies have found that seedlings grow best at 25% seawater, while high salinity or a total lack of salt (i.e., freshwater) adversely affect growth (Clough, 1984). Tidal inundation is another dominant abiotic drivers in mangroves and considered to have an important role in the paradigm of mangrove establishment and distribution (Krauss et al., 2008); however, research on tidal inundation and species distribution must also acknowledge that vegetation-inundation linkages are not universally applicable and that the species distribution is multifactorial

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(Friess, 2017).

The establishment of seedlings is the most critical stage in the life cycle of seed plants, and it is rendered difficult for mangroves by the unstable and variable substrates and tidal influence (Tomlinson, 2016). As an adaptation to inhabit the inundated conditions, most mangroves have developed a large propagating structure called the propagule (or seedling). While still attached to the parent tree, the embryo of the propagule is developed, or termed viviparous - typical among *Rhizophora* species (Hogarth, 2015). Mangrove vivipary results in considerable parental nutrients and energy investment in the early growth of seedlings (Hogarth, 2015; Tomlinson, 2016), providing ample nutrients and energy to support the early growth under nutrient-limited and salt-stressed conditions (Farrant et al., 1992; Krauss et al., 2008).

Mangroves are highly productive forests and known to host a rich and diverse associated marine fauna and provide considerable services to humans (Barbier et al., 2011). However, despite its ecosystem services, mangroves have suffered high decimation in the past decades arising from agriculture and aquaculture conversion (Primavera and Esteban, 2008; Richards and Friess, 2016). In response, mangrove rehabilitation initiatives have attracted a large amount of attention from different sectors to foster mangrove recovery and biodiversity. Most rehabilitation programs have utilized only a single species of *Rhizophora* (Primavera and Esteban, 2008; Samson and Rollon, 2008), creating a monospecific plantation with no postplanting management plan (Asaeda et al., 2016; Barnuevo et al., 2017). The results of the intensive efforts of mangrove rehabilitation programs are stories of mixed successes and failures. These efforts were often unsuccessful because of the high mortality of the planted seedlings due to inappropriate site selection (Primavera and Esteban, 2008; Samson and Rollon, 2008), and they failed to consider the niche-width preference or the area, which is less stressful to the planted species. Niche-width is a space, a segment of a community or a range of a condition that a species can inhabit and successfully survive (Van Valen, 1965). Mangrove forests worldwide naturally exist in a raised and sloped platform above the mean level, inundated approximately 30% or less by the tidal waters (Lewis III, 2005). More frequent inundation causes stress and eventually mortality. A field study by He and Lai (2009) in China showed that the survival rate of *Rhizophora stylosa* sharply decreased from 88.9% to 44.0% as the tidal flat elevation decreased. In the Philippines, there is a widespread tendency to plant mangroves in lower intertidal areas that result in low survival of 10–20% (Primavera and Esteban, 2008). Kodikara et al. (2018) showed that seedlings cultured in high salinity had significantly lower survival rates. Mangora et al. (2014) stress that the submergence time and water salinity affect the sustainability of mangrove habitats and that the areas experiencing prolonged submergence with saline water may be the most severely affected.

The combined effects of different abiotic factors affect the physiology of mangroves and, consequently, cause excessive induction of reactive oxygen species (ROS), leading to oxidative stress (Sreenivasulu et al., 2007; Wang et al., 2014). ROS are highly reactive oxygen derivatives that are formed as byproducts of various metabolic pathways localized in different cellular compartments, specifically in chloroplasts, mitochondria and peroxisomes (Nakano and Asada, 1981). In a biological context, ROS, specifically hydrogen peroxide (H_2O_2), act as signaling molecules; however, in extreme conditions, their levels significantly increase and damage cellular functions, eventually leading to mortality if the unfavorable condition persists (Sharma et al., 2012). As a response, mangroves have developed a species-specific natural defense system, specifically antioxidant enzymes (AOX) (including ascorbate peroxidase, catalase and guaiacol peroxidase) to scavenge the deleterious effects of ROS, and a range of physiological mechanisms (Das et al., 2016; Jaleel et al., 2009). This study determined the niche-width preference of *Rhizophora stylosa* Griff. by investigating its oxidative stress responses to salinity, water level and inundation, both in field and greenhouse conditions. An understanding of the niche-width of this species provides important insights and guidelines for policy-makers and stakeholders in selecting rehabilitation sites to ensure the high survival of plantations.

2. Methodology

2.1. Propagule collection and experimental design

Mature propagules of *Rhizophora stylosa* were collected from Olango Island, Lapu-lapu City, Cebu, in the central Philippines on March 2016 and transferred to Saitama University, Japan for culture in the greenhouse. Propagules were considered mature if they were easily detached from the parent tree after gentle shaking of the branch (Robert et al., 2015). The collected propagules were wrapped in a moist paper towel and transported to Japan by airplane. Immediately upon arrival, the propagules were randomly divided into three and placed in bins with water of three different salinities (0, 20, 35 ppt) prepared from Instant Ocean, Aquarium Systems (Krauss et al., 2006; McKee, 1996; Ye et al., 2005). The propagules were acclimatized for two weeks at an ambient temperature of 27 ± 5 °C. After the acclimatization period, the propagules were individually planted in seedling bags (110 × 250 mm) filled with 2 L of mixed washed river sand and vermiculite at a 4:1 ratio (Yates et al., 2002; Zhu et al., 2012). The seedlings were kept inside the three aquarium tanks irrigated with different salinities (0, 20, 35 ppt). Inside the tank, the cultured seedlings were arranged on top of a platform made from a pile of bricks, subjecting the plants to irrigation at water depths of 3–5, 13–15 and 33–35 cm to simulate the different water levels corresponding to low water (LW), mid-water (MW) and high water (HW), respectively (Fig. 1, Experiment 1). The number of seedlings cultured per water depth was replicated into three groups. The leaves of cultured plants were above water level or in an emergent condition. Every two days, the salinity was adjusted by adding tap water to compensate for water loss due to evapotranspiration, and the water was replaced once a month to prevent the salinity from becoming stale and to control the growth of algae. A greenhouse condition was maintained with a 12-h photoperiod and at 27 ± 5 °C. The heights of the cultured plants were measured monthly with a ruler, and the growth rate was determined based on the difference between the final and initial height divided by the culture period. After 5 months, on August 25, 2016, the cultured seedlings (with 3–4 pairs of leaves) were harvested from 11:00 to 13:00 when the light intensity peaked. For every harvested seedling, the light intensity was measured using a portable quantum flux meter (Apogee, MQ-200, USA). The leaf samples were immediately analyzed for reactive oxygen species (ROS), specifically hydrogen peroxide, antioxidant enzymes (AOX) including catalase, ascorbate peroxidase and guaiacol peroxidase, pigments (chlorophyll *a* and b), carotenoids and the Fv/Fm ratio, as detailed below.

Another set of seedlings were cultured in separate aquarium tanks filled with 3-cm-deep saline water (0 20, 35 ppt). After 15 months of greenhouse culture, from June to August 2017, the plants were subjected to varying submergence and inundation periods (semidiurnal, diurnal, permanently submerged) simulating the tidal cycles for seven days at three different salinity levels (0, 20, 35 ppt) corresponding to the same salinity in which the plants were cultured (Fig. 1, Experiment 2). For semidiurnal inundation (SDI), the plants were submerged twice a day for three hours (i.e., three hours submerged and then three hours emerged, equivalent to the culture conditions, followed by another three hours submerged and then a return to the emerged or culture conditions). For diurnal inundation (DI), the plants were submerged for 6 h per day, and for the permanently submerged (PS) conditions, the plants were submerged for 24 h. For SDI and DI, the submersion experiment started from 10:00 to 10:30. In the emerged condition, the experimental tank was drained up to a depth of only 3 cm water (a similar water depth in which the plants were cultured), while in the inundated or submerged condition, the topmost pair of leaves was 15 cm below the water surface. The numbers of seedlings in the