Growth and biochemical stress responses of Rhizophora stylosa Griff. to salinity, water depth and inundation

（ヤエヤマヒルギの塩分、水深、湛水期間に対する生長及び生化学的ストレス応答

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埼玉大学大学院理工学研究科（博士後期課程）
理工学専攻（主指導教員　浅枝　隆）

ABNER P. BARNUEVO

SAITAMA UNIVERSITY
Saitama, Japan

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By

ABNER P. BARNUEVO
15DE056

Prof. Takashi Asaeda
Supervisor

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DEDICATION

There are a number of people without whom this thesis might not have been written, and to whom I am greatly indebted.

I dedicate this to my loving and supportive Tatay and Nanay,
whose love, encouragements and prayers,
enabled me to get another success and milestone.

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who have been constant sources of support and encouragements
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For I know the plans I have for you,
declares the Lord,
plans to prosper you and not to harm you,
plans to give you hope and a future.

Jeremiah 29:11

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

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ABSTRACT

Mangroves are trees and shrubs that inhabit the coastal areas in the tropical and subtropical countries. They are known to provide important ecosystem services that contribute to human wellbeing. But, despite of its functions and values, they are one of the valuable habitats that have suffered widespread habitat loss due to increasing population density and continuing economic development in the coastal areas. The continuous degradation of mangrove habitats has encouraged government and multi-lateral sectors to undertake rehabilitation initiatives to foster the recovery and biodiversity of these areas. However, some rehabilitation initiatives suffer high mortality because of incorrect species-site matching and failure to recognize the ecophysiology of mangrove species with the environmental stressors. Inundation, elevation gradient and salinity fluctuations are considered the major abiotic drivers that influence the survival, growth and distribution of mangroves. In extreme 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 (AOX) system to scavenge the excessive ROS. This study investigated the effects of salinity, water depth and inundation on the growth, biochemical stress responses, and ecophysiology of *Rhizophora stylosa* in greenhouse microcosm experiments and field studies.

The field study was conducted in Olango and Banacon Islands, in the central part of the Philippines where *R. stylosa* plantations was established over 60 years ago. Transect line plot method was employed to assess the mangrove forest structure perpendicular to the shoreline. The leaves of *R. stylosa* were collected from natural and planted forests within the sampled transect line-plot. For every sampled tree, the light intensity and the pore water salinity was measured in situ and the elevation gradient was estimated based on mean water level at neap tide. The collected samples were immediately frozen with dry ice inside a thermal box and transported to the laboratory. On the other hand, for microcosm experiments, mature propagules of *R. stylosa* were collected in Olango Island and two experimental set up were conducted. In the first set up, the propagules were individually planted in seed bag and cultured in aquarium tanks filled with

different salinity treatments: low (LS) – 0 ppt, moderate (MS) – 20 ppt and high salinity (HS) – 35 ppt. The seedlings were arranged on the top of a platform and irrigated with low water (LW, 3-5 cm), mid-water (MW, 10-13 cm) and high water (HW, 30-33 cm). The developments of the first leaves were monitored, and the average height, biomass and leaf tissues were measured and sampled at 5 and 10 months. In another set up, the seedlings were cultured at the low-water level in three different salinity treatments. After 15 months, the seedlings were subjected to inundation hydroperiod simulating the tidal cycle as semi-diurnal inundation (SDI), diurnal inundation (DI) and permanent submersion (PS) for one week. These microcosms simulated emerged and inundated conditions, mimicking intertidal inundation that seedlings would experience. Leaf samples from field and experiments were analyzed for hydrogen peroxide (H_2O_2), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD), pigments, carotenoids, Fv/Fm and proline. Statistical analyses were done using XLSTAT Premium.

The mangrove forest structure (density, species diversity, average height, girth size) showed a significant difference between the natural and planted forests. The planted forest had lower structural complexity than the reference natural forest. Even 60 years after the forest was created in Banacon Island, it still lacked the understory of young cohorts. While a forest has been created, it does not mimic a natural forest. Future mangrove restoration programs should consider planting several species and maintain sufficient spacing for growth in order to reproduce in the rehabilitated areas some of the key ecosystem characteristics of natural mangrove forests and to achieve the best outcome and functionality of the restored habitat. Results of the biochemical analyses showed that the H_2O_2 and AOX activities for samples collected at the higher elevation areas with rare inundation have significantly higher values compared with the samples collected at the inundated areas. On the other hand, the chlorophyll a and b for samples at the inundated areas showed an increasing with salinity, while those in higher elevation and rarely inundated areas showed a decreasing trend. 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.

The results of the greenhouse experiment showed that salinity significantly influenced the early growth and biomass production of *R. stylosa*. At 5 months, low salinity provided the optimum condition for relative growth rate (RGR), while at 10 months, the optimum condition and higher RGR shifted towards the moderate salinity. Salinity also influenced the biomass allocation and partitioning as shown in the root/shoot ratio. At 5 months, the seedlings cultured in high salinity had higher allocation for root biomass while those cultured in low salinity had higher allocation for shoot biomass. At 10 months, the biomass allocations shifted, and the seedlings cultured in high salinity had higher allocation for shoot biomass while those in low salinity had higher allocation for root biomass. This temporal shift in salinity preference and optimum condition implies an adaptive morphological plasticity with salinity stress. On the other hand, the proline content of the leaves showed a significant increase for seedlings cultured in moderate and high salinity at 10 months compared with at 5 months. This increase in proline accumulation signifies physiological adaptations to saline conditions, which is consistent with the observed changes in growth and biomass production. Results of the biochemical analyses of leaf tissues showed that levels of ROS and AOX activities were significantly lower in the emerged condition than those in an inundated condition. Periodic inundation (SDI and DI) imposed a higher order of stress as indicated by ROS levels compared with the effect of salinity, while prolonged inundation (PS) caused sublethal damage as manifested by the chlorosis of the leaves in moderate and high salinity treatments. The chlorotic leaves developed relatively faster in high salinity (4 days after) than in moderate salinity (5 days after); whereas in low salinity, chlorosis was not observed even at the end of the experimental period. The pigments (Chl a and b), carotenoids and Fv/Fm showed a significant reduction relative to the inundation hydroperiod in all salinity treatments, and the PS showed the highest reduction. Inundation and salinity both significantly influenced the reduction, but the inundation was the most influential factor.

Extrapolating ecophysiology of *R. stylosa*, this species had low tolerance to inundation stress (high ROS and AOX, reduced pigments). Translating this low tolerance to field conditions, in the frequently inundated areas (i.e. seafront mangroves fringes) that are subjected to longer inundation at spring tides, this species may suffer from oxidative stress, stunted growth and consequently low survival.

CHAPTER I

I. Introduction

1.1 General 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 that the optimum growth of *Sonneratia caseolaris* was 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). The influence of tidal inundation on mangroves species distribution has long been recognized concept in mangrove ecology as early as 1920s by J.G. Watson, described as the inundation classes referring to the flooding frequency at which different mangrove vegetation species could be found (Friess, 2017). But, 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, and not dependent on inundation alone (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., 1992a; 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). But 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). From 1980 to 2005, around 19% of the world's mangroves were lost, and the Southeast Asia region contributed the highest mangrove degradation (FAO, 2007). Subsequently, mangrove rehabilitation initiatives have attracted a large amount of attention from different sectors to foster mangrove recovery and biodiversity. The restoration programs received a renewed impetus after the 2004 Indonesia tsunami and the 2013 Typhoon Haiyan in the Philippines due to the highly-valued mangrove ecosystem services as buffer and bioshield along coastlines (Wolanski and Elliott, 2015). However, 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 to 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.

1.2 Statement of the problem

Located at the interface between the land and sea, the establishment and survival of mangrove seedlings are under the continuous influence of the interplay of different environmental drivers (Figure 2). Tidal inundation, land elevation and salinity are considered the major abiotic drivers that influence the survival, growth and distribution of mangroves. In extreme conditions, these environmental stressors tigger excessive generation of oxygen species (ROS), affecting mangrove physiology and homeostasis, leading to oxidative stress (Sharma et al., 2012) and mortality if the condition remains exacerbated (Figure 3). ROS are highly reactive oxygen derivatives that are formed as a by-product of various metabolic pathways localized in different cellular compartments, specifically in chloroplast, mitochondria and peroxisomes (Nakano and Asada, 1981). However, during times of stressful conditions, ROS are significanstly generated and result to damage in cell structures and its functions. As a response to quench the deleterious effects of ROS, mangrove have developed an antioxidant (AOX) defense system including the ascorbate peroxidase, catalase and guaiacol peroxidase to scavenge the excessive ROS and a range of physiological mechanisms (Das et al., 2016; Jaleel et al., 2009). Mangroves were reported to have expressed gradients of ecophysiological responses in organ construction, metabolism, biomass partitioning, and related enzyme activities and hormone levels due to abiotic stress (He et al., 2007; Luzhen et al., 2005; Naidoo, 1985; Skelton and Allaway, 1996).

Understanding the ecology and adaptability of mangroves to the interplay of abiotic stressors requires an interdisciplinary knowledge of botany, physiology, geography,

and more recently, molecular and genetic sciences (Friess, 2017). Mangroves developed species-specific stress tolerances, thresholds and adaptations to inhabit the stressful intertidal conditions. However, the ecophysiology and species-specific niche-width are not yet well established in the paradigm of mangrove ecology. An understanding of the niche-width of this species provides important insights and guidelines for policymakers and stakeholders in selecting rehabilitation sites to ensure the high survival of plantations.

From the observations and lessons-learned from several large-scale plantations of monospecific *R. stylosa*, the following questions should be asked: 1) Why did several plantations suffer high mortality? 2) What is the niche-width preference of this species? 3) Where should this species be planted relative to the intertidal region? To answer these practical questions, understanding the ecophysiology or how the environment interacts with the physiology of the species *R. stylosa* is necessary.

1.3 Objectives of the study

This study determined the niche-width preference *Rhizophora stylosa* Griff., a dominant and iconic mangrove species for rehabilitation, by investigating its ecophysiological respoonses to salinity, water depth, elevation gradient and inundation hydroperiod in the greenhouse microcosm experiments and field conditions.

Specifically, this study measured the:

1) growth and biomass of cultured seedlings in relation to salinity and water depths;
2) leaf pigments, carotenoid and Fv/Fm ratio of greenhouse cultured seedlings and field samples;
3) hydrogen peroxide (H_2O_2) of both the greenhouse cultured seedlings and field samples as a proxy to measure the reactive oxygen species (ROS) and serves as an in index of stress;
4) activities of the antioxidant enzymes (AOX) including the ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) for both experimental seedlings and field samples; and

5) quaternary osmolytes specifically the foliar proline in the experimental seedlings.

The changes and differences of ROS and AOX were compared when in emerged, inundated and submerged condition and the optimum condition and niche-width preference was extrapolated.

1.4 Significance of the study

The determination of the ecophysiology and levels of oxidative stress of *R. stylosa* when in an emerged, periodically inundated and submerged conditions and in field condition could serve as basis in understanding the niche-width preference of this species. Knowledge of the species-specific niche-width could have valuable implications for restoration ecology as a basis for selecting areas appropriate for mangrove rehabilitation to ensure the high success of the plantation.

Additionally, the role of environmental drivers (i.e. salinity and inundation) in mangrove ecology has long been recognized, however, the ecophysiology is not yet fully understood. Knowledge of the species-specific ecophysiology could be a new input into the paradigm of mangrove ecology and could provide novel insight among the policymakers for future knowledge-based rehabilitation programs.

1.5 Scope and limitations

The study focused only on one mangrove species – the *R. stylosa*. This species is the commonly selected species for mangrove planting initiatives and rehabilitation projects due to the availability of pits ropagules throughout the year, convenience of collection and it does not require nusery culture period. The intertidal condition of typical mangrove area was simulated in a microcosm in greenhouse condition to determine its growth and biochemical responses to salinity, water depths and inundation hydroperiod. Observations for the growth and biomass were carried out up ten months only. For field work, the same species was sampled perpendicular to the intertidal gradient – from seaward to landward. The result of both the microcosm experiment abd field study was extrapolated to identify the niche-width of *R. stylosa*.

For reactive oxygen species (ROS) stress indicator, the H_2O_2 was analyzed, whereas for antioxidant enzymes (AOX), catalase activity (CAT), ascorbate peroxidase activity (APX) and peroxidase activity (POD) were analyzed. Other biochemical analyses conducted were on the pigments (Chl a and b), carotenoids, fluorescence and the quaternary osmolytes specifically the proline.

CHAPTER II

2. Review of Related Literature

2.1 Mangrove basic biology and adaptations

Mangroves are trees and shrubs that inhabit in inundated and saline coastal areas. High salinity, wave action and fluctuating water levels present problems that are rarely experienced in terrestrial and freshwater water habitat (Hogarth, 2015). A total of 124 countries and areas were identified as containing one or more true mangrove species (Saenger et al., 1983; Tomlinson, 2016). Mangroves have overcome the inhospitable environmental conditions and successfully colonized the intertidal areas by developing mechanism and adaptations to: salinity, inundation and water-logged soil. Generally, mangroves deploy a variety of means to cope with this harsh environment. The principal mechanisms are exclusion of salt by the roots, tolerance of high salt concentrations, elimination of excess salt secretion and development of reproductive organ known as the propagule.

The underground tissues of any plant require oxygen for respiration. In soils, that are water logged, gas diffusion between soil particles can supply this need. In a waterlogged soil, the spaces between soil particles are filled with water. Even when water is saturated with oxygen, the concentration is far below that of air, and the diffusion rate of oxygen through water is roughly 10,000 times less than through air (Ball, 1988a). Oxygen movement into waterlogged soil is therefore severely depleted. Moreover, oxygen that is present is soon depleted by the aerobic respiration resulting to anaerobic soil condition. Mangroves have adapted so such unpromising conditions by developing various forms of aerial roots and pneumatophores. The roots of most trees are branch off the trunk underground. In well-oxygenated soil, there is little difficulty in getting the oxygen needed for respiration. In waterlogged soils, special aerating structures are required. In *Rhizophora* species, roots from branches from the main trunk as much as 2 m above the ground, elongate and penetrate the soil some distance away from the mains stem. A different form of root architecture is developed by other species like in *Avicennia* and *Sonneratia*. Shallow horizontal roots radiate outwards known as the pneumatophores. Pneumatophores supply the respiratory

needs of the underground roots in anoxic soil (Hogarth, 2015). When exposed at low tide, aerial roots readily acquire sufficient oxygen for respiration. Air passes into the roots through numerous tiny pores, or lenticels, which are particularly abundant close to the point at which the column root enters the soil surface and then pass along roots through air spaces.

Mangroves typically grow in an environment whose salinity is between that of fresh water and sea water, seawater comprising approximately 35 g salt/l. This means an osmotic potential of -2.5 MPa, and water must be taken against this pressure (Hogarth, 2015). The principal mechanisms in coping with saline water are exclusion of salt by the roots, tolerance of high tissue salt concentrations and elimination of excess salt by secretion. However, the interplay of these mechanisms is complex and not clearly understood.

All mangroves disperse their offspring by water. A distinctive feature of most species is that they produce large propagating structures called the propagules. This term is used because in most mangroves species what leaves the parent tree is a seedling, not a seed or a fruit. After pollination, the growing embryo remains on the parent and is dependent on it for period that often extends to several many months or known as vivipary.

2.2 Mangrove species distribution and coverage

Mangrove forests are distributed in the inter-tidal region between the sea and the land in the tropical and subtropical regions of the world between approximately 30° N and 30° S latitude. Their global distribution is believed to be delimited by major ocean currents and the 20° C isotherm of seawater in winter (Alongi, 2009).

Some 15.2 million hectares of mangroves are estimated to exist worldwide as of 2005, down from 18.8 million hectares in 1980 (FAO, 2007). The most extensive mangrove area is found in Asia, followed by Africa and North and Central America (Figure 4). Five countries (Indonesia, Australia, Brazil, Nigeria and Mexico) together account for 48% of the total global area and 65 percent of the total mangrove area is found in just ten countries. The largest extent of mangroves is found in Asia (42%) followed by

Africa (20%), North and Central America (15%), Oceania (12%) and South America (11%). Approximately 75% of mangroves are concentrated in just 15 countries (Table 1) (Giri et al., 2011).

Based on the report of FAO (2007), human pressure on coastal ecosystems is often high, with land competition for aquaculture, agriculture, infrastructure and tourism. The consequent conversion of mangrove areas to other uses over the past decades has been alarming. However, although mangroves still face major threats, the rate of loss has recently been decreasing – from some 187,000 ha lost annually in the 1980s (–1.04% per year) to 102,000 ha annually (–0.66% per year) during the 2000 to 2005 period. The figures suggest that during the past 25 years, about 3.6 million hectares have been lost, corresponding to some 20% of the global mangrove area in 1980. At the regional level, Asia suffered the largest net loss where more than 1.9 million hectares since 1980, mainly due to changes in land use from 1980 to 1990. North and Central America and Africa also contributed significantly to the decrease in mangrove area at the global level, with losses of about 690,000 and 510,000 ha respectively over the last 25 years.

2.3 Mangrove functions and values

Mangrove forests provides a number of important functions and ecosystem services that contribute to human wellbeing, including provisioning (e.g., timber, fuel wood, and charcoal), regulating (e.g., flood, storm and erosion control; prevention of salt water intrusion), habitat (e.g., breeding, spawning and nursery habitat for commercial fish species; biodiversity), and cultural services (e.g., recreation, aesthetic, non-use) (FAO, 2007; Spaninks and van Beukering, 1997). Mangroves were also often used for the production of tannin suitable for leather work and for the curing and dyeing of fishing nets (FAO, 1994). Many of these ecosystem services have the characteristics of ‘public goods’ such that the people who benefit cannot be excluded from receiving the service provided (e.g., habitat and nursery service supporting fisheries); and that the level of consumption by one beneficiary does not reduce the level of service received by another (e.g., coastal protection and storm buffering) (FAO, 2007).

Since the 2004 Indian Ocean tsunami, there has been strong interest globally in both restoring mangrove ecosystems and in their ability to protect coastlines and people from damaging storms. There is also an ongoing debate over whether or not the cost of mangrove restoration is higher than the value of the coastal protection service provided by these ecosystems (Sandilyan and Kathiresan, 2015). Successful mangrove restorations were relatively expensive and variable, ranging from US$225 to US$216,000 per ha, excluding the costs of the land (Lewis III, 2005). However, most estimates are around US$5000 to US$10,000 per ha; for example, in the Caribbean, restoration site costs are US$5077 per ha (Adame et al., 2015), and in Thailand US$8812 to US$9318 per ha (Barbier, 2007). In addition, mangrove ecosystems provide other important economic benefits other than storm protection, including carbon sequestration, collected wood and non-wood products, and support for off-shore fisheries (Barbier, 2007; Barbier et al., 2011; Huxham et al., 2015).

The protective value of mangrove ecosystems is directly related to their ability to attenuate, or reduce the height, of the storm surges and waves as they approach shorelines. This wave attenuation function derives from the vegetation and root structure of mangroves, which are an important source of friction to moving water and sediment (Bao, 2011; Gedan et al., 2011; Koch et al., 2009; Massel et al., 1999; Mazda et al., 1997; McIvor et al., 2012). In addition, mangrove trees also have the capacity to buffer winds (Das and Crépin, 2013; McIvor et al., 2012). The value of mangroves in providing such protection against high-speed and damaging winds is an often over-looked, but nonetheless very important, benefit. The growing evidence indicating that mangroves have significant wave attenuation and wind buffering functions has led to interest in valuing their storm protection benefit, and also provided better understanding of the underlying ecological structure and functions contributing to this benefit, including how it varies across mangrove landscapes and different tide levels (Barbier, 2016; Koch et al., 2009; McIvor et al., 2012).

2.4 Environmental stressors and biochemical responses

Mangrove habitats are subjected to various stress conditions that include both abiotic and biotic. The abiotic stressors include salinity, temperature fluctuations, heavy metal, low oxygen, flooding, UV-B, water logging condition (Figure 2) contributing

towards anaerobic environment (Das et al., 2016). The mangrove plants are also confronted by biotic stresses that include microbial pathogens such as fungi, nematodes and bacteria along with insects and herbivores.

Salinity is a major abiotic stress in mangrove environment exemplified by high content of soluble salts mainly NaCl. High salt depositions in the soil generate a low water potential zone in the soil, making it increasingly difficult for the plant to acquire both water as well as nutrients. Therefore, salt stress essentially results in a water deficit condition in the plant and takes the form of a physiological drought (Mahajan and Tuteja, 2005). The major adverse effects of salinity stress on plants can be summarized as hyper ionic stresses leading to ion imbalance, deleterious effect on the functioning of some enzymes, osmotic imbalance, membrane disorganization, reduction in growth, inhibition of cell division and expansion, decrease in photosynthesis and increase in production of ROS (Doganlar et al., 2010; Kosová et al., 2013; Zhu, 2002). A primary response in salt stressed plants is decrease in plant water potential leading to the overall toxic damages and yield reduction (Cha-Um and Kirdmanee, 2009).

Tidal flooding and inundation posed another stress to mangroves. Mangroves are exposed to a range of oxygen concentrations during their life that can vary from a fully aerobic state (normoxia) to oxygen deficiency (hypoxia) or the total absence of oxygen (anoxia) (Das et al., 2016). Plants generally met with anoxic conditions during winter or ice encasement, flooding condition and seed imbibition among others. Excessive generation of ROS is an integral part of low oxygen stress (Gout et al., 2001). Microarray analyses carried out under different low oxygen conditions on different plants have reported the activation of ROS-regulated genes or those associated with ROS (Banti et al., 2013).

Although mangroves are adapted to periodic waterlogging, different species respond differently to the duration of inundation (Luzhen et al., 2005). Prolonged inundation can cause mangrove die-back in Kosi Estuary in KwaZulu Natal, South Africa when the mouth of the estuary was closed and water level increase (Hoppe-Speer et al., 2011).

2.5 Mangrove status and threats

Despite of the many services and benefits provided by mangroves, these coastal forests have often been undervalued and viewed as wastelands and unhealthy environments. The high population pressures frequently present in coastal zones have in some places led to the conversion of mangrove areas for urban development (FAO, 2007). In order to increase food security, boost national economies and improve living standards, many governments encouraged the development of shrimp and fish farming, agriculture, and salt and rice production in mangrove areas. Mangroves have also been fragmented and degraded through overexploitation for wood forest products and pollution. Indirectly, habitats have been lost because of dam construction on rivers, which often diverts water and modifies the input of sediments, nutrients and freshwater. Even though dense mangrove forests can be important in coastal protection, natural disasters should also be listed among the possible causes of degradation: several tropical countries are frequently hit by cyclones, typhoons and strong winds, and the trees in the front lines may be damaged and/or uprooted during these catastrophes.

Mangroves have traditionally been widely used and exploited in the past in the majority of countries in which they exist. Knowledge of their current and past extent, condition and uses is essential for forest managers and policy- and decision-makers. The planning of sustainable forest management at the local and national levels depends largely on this information, and the lack of data on the status and distribution of mangroves makes it difficult to prepare successful plans for their conservation. FAO (2007) estimated that some 15.2 million hectares of mangroves exist in 2005, down from 18.8 million hectares in 1980. The world has thus lost some 3.6 million hectares of mangroves over the last 25 years, or 20% since 1980. However, some areas showed significant improvement. From about 185,000 hectares of mangrove loss every year in the 1980s, the net loss dropped to some 118,500 hectares per year in the 1990s and to 102,000 hectares per year (or a loss of 0.66 percent annually) during the 2000-2005 period (FAO, 2007).

The most extensive mangrove area is found in Asia, followed by Africa and North and Central America. Five countries (Indonesia, Australia, Brazil, Nigeria and

Mexico) together accounted for 48% of the global area. FAO (1994) reported that Asia supports around 39% of the world's total mangrove, down from 42% a decade ago. The edaphic and coastal features of south and Southeast Asian countries, together with the high rainfall and significant riverine inputs, are particularly favorable to the development of well-structured mangrove forest.

The region of Southeast Asia contains the greatest diversity of mangrove species and more than one-third of the world's mangrove forest extent (Richards and Friess, 2016). Estimates of historical mangrove deforestation are unreliable in many instances, but Asia may have lost more than one-third of its mangrove area between the 1980s and 1990s. Such deforestation has had substantial negative impacts on biodiversity, with 16% of the world's mangrove vegetation species now at an elevated risk of extinction. Mangrove deforestation also has implications for the provision of ecosystem services.

2.6 Lessons-learned from mangrove rehabilitations

The result of the intensive efforts of mangrove restoration programs were met with mixed successes and failures stories. These efforts were often unsuccessful, largely because of the high mortality of the seedlings, inappropriate site selection, predation, overtopping by waves and barnacle infestation (Primavera et al., 2011; Samson and Rollon, 2008; Wolanski and Elliott, 2015). Most of the rehabilitation programs utilized only single species of *Rhizophora* (Asaeda et al., 2016; Barnuevo et al., 2017; Primavera and Esteban, 2008; Samson and Rollon, 2008), creating a monospecific plantation with no post-planting management plan. Although it is almost impossible to restore the estuarine ecosystems to their original state, the principal goal of restoring it is generally to reproduce as much as possible the characteristics and functioning of natural wetlands (Duarte et al., 2009). In cases where some successes were achieved, these restoration and creation efforts were seldom assessed to test to what level the created mangrove wetlands delivers the desired ecosystem services (Elliott et al., 2007). Despite of the proliferation of projects aiming to restore and rehabilitate mangroves, there is still little evaluation of their effectiveness. Therefore, our study assessed the structural development and complexity of the large-scale

plantations in the central part of Philippines and compared these with the adjacent natural stand as reference.

Mangrove conservation and management guidelines in the Philippines were promulgated in the 1980s and subsequently mangrove rehabilitation initiatives attracted much attention from different sectors to foster its recovery and biodiversity (Primavera and Esteban, 2008; Walters, 2005). Government-led mangrove reforestation was implemented with financial aid from government and international agencies, though community-initiated mangrove reforestation traced back as early as 1930s in Bais Bay, Negros Oriental and in 1950s in Banacon Island, Getafe, Bohol (Primavera, 2000) in the central part of the Philippines primarily for coastal protection and for firewood supply (Walters, 2000). The mangrove restoration programs in tropical and subtropical regions throughout the world received a renewed impetus after the 2004 Indonesia tsunami, in order to provide highly-valued ecosystem services (Wolanski and Elliott, 2015), and the 2013 Typhoon Haiyan in the Philippines, which highlighted the importance of mangroves as buffer and bioshield along coastlines.

CHAPTER III

3. Methodology

The studies were conducted in two phases: 1) field survey and 2) greenhouse microcosm experiments. The first phase of the study involved processing of the government permits, ocular survey of the study sites and aerial and ground assessment of the mangrove community structure to compare the natural and planted forests. This serves as the baseline information of the succeeding surveys. In the succeeding field works, leaf samples and propagules or seedlings were collected and transported to Saitama University for laboratory analyses and greenhouse culture. The second phase of this study involved the greenhouse microcosm experiments. The collected seedlings were cultured in the greenhouse and subjected to different experimental treatments as detailed below.

3.1 Part 1 – Field work

3.1.1 Field sampling and mangrove forest assessment

Field study was conducted in Olango and Banacon Islands, in the central part of Philippines on November 2015 (Figure 5) (Asaeda et al., 2016; Barnuevo et al., 2017). Olango Island is larger in area (4482 ha) than Banacon Island (425 ha) and the island has been declared as the the first Ramsar site in the Philippines. It is surrounded by broad sandy beaches and rocky shorelines, inshore flats, seagrass beds, coral reefs, mangrove forests and mudflats. The southern end of the island forms a shallow bay covered by limestone sediment of coral origin. For the last 30 years, various NGOs have planted *R. stylosa* at high density (0.5 x 0.5 m), in the intertidal areas of lower elevations along the eastern shoreline. On the other hand, Banacon Island is a low-lying island with 96% of the intertidal area covered by mangrove forests and only 15 ha dry land is used as residential area. It is often showcased as a success story of community-based initiatives in mangrove planting since the 1950s due to high survival rate of the plantation and without government-led intervention (Walters, 2005), and this practice is continuing. The local residents of the island have planted *R. stylosa* (0.5 x 0.5 m) in about 500 parcels and the size of each patch varies

from 100 to 40,000 m^2 (Walters, 2003) which were tightly clustered forming into a contiguous mosaic of different stands that are separated by narrow boat pathways.

An aerial survey was conducted using drone which traversed the natural and planted forests to identify and determine the distribution of the planted and natural mangrove areas. This survey served as basis for the selection of the plots in subsequent ground sampling as well as the sampling for leaves for biochemical analyses. Random sampling was used and the transect line plot (100 m^2) method was employed to assess the mangrove vegetation structure. The transect line was laid perpendicular to the shoreline and the plots (~50 m apart) were demarcated along the transect traversing from seaward fringe, middle and to landward zone. A total of 44 plots in natural forest and 20 in planted forest were assessed. The unequal number of sampling plots between natural and planted forest is due to the observed homogeneity of planted forest stand in terms of height and diameter at breast height (DBH). The DBH taken approximately 1.3 m above the ground and height of mangroves within the plots were taken with a DBH tape and a laser distometer respectively. Plants were then categorized based on their DBH and height as trees (DBH greater than 0.04m and height above 1 m), saplings (DBH of less than 0.04 m and height above 1 m) and seedlings (height less than 1 m). Species were identified following (Primavera et al., 2004) and the mangrove vegetation structure was analyzed following (English et al., 1997).

3.1.2 Studied species and sampling of leaves and propagules or seedlings

The species of *R. stylosa* Griff. (Figure 1a) is one of the common and dominant species in the study sites (Asaeda et al., 2016; Barnuevo et al., 2017). This species is commonly found and widely distributed in the Southwest Pacific and Australia (Figure 6). The habitat is frequently found at the mouth of estuaries and also be likely found in open seawater on fairly exposed shores, including on live reef and sandy shores. It can grow up to 30 m tall but is more common at 5-10 m (Ellison et al., 2010).

The leaves of R. *stylosa* were collected during low tide across the intertidal gradient perpendicular to the shoreline. The elevation of the sampling sites ranged with an

approximately 1 m difference between the level inundated every day and that rarely or never inundated in normal conditions. Samples were collected from natural and planted forests (old and young) and the leaves were not submerged even during spring tide. Samples were collected in the part of the upper canopy directly exposed to sunlight from 09:00 to 16:00. The heights of the sampled trees were measured with a LASER disto-meter. The salinity of the pore water ranged from 29 to 36 ppt. The collected samples were immediately frozen with dry ice inside a thermal box and transported to the laboratory for analyses of ROS, antioxidant enzymes and pigments as indicators of oxidative stress and habitat preference.

As an adaptation to inhabit the inundated conditions, most mangroves have developed a large propagating structure called the propagule (or seedling) (Figure 1b). 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., 1992a; Krauss et al., 2008).

The mature propagules of *R. stylosa* were collected from Olango Island, Lapu-lapu City, Cebu in the central Philippines (Figure 5) 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. **A**ll the government permits both from the Philippines and Japan were processed and complied before importing the propagules of *R. stylosa* to Japan for the greenhouse culture.

3.2 Part 2 - Greenhouse culture, experiments and laboratory analyses

3.2.1 Greenhouse acclimatization and bagging

Immedaiately upon arrival, the propagules were randomly divided into three and placed in bins with water of three different salinities (0, 20, 35 ppt) (Figure 7a)

prepared from Instant Ocean, Aquariums Systems (Krauss et al., 2006; McKee, 1996; Ye et al., 2005). The propagules were acclimatized fro two weeks at an ambient temperature of 27±5^{o}C. The greenhouse condition was kept at 12 hours photoperiod and with temperature of 27±5 ^{o}C. After the acclimatization period, the propagules were individually planted in seedling bags (Figure 7b) (110 x 250 mm) filled with 2 l of mixed washed river sand and vermiculite in a 4:1 ratio (Yates et al., 2002; Zhu et al., 2012).

3.2.2 Experiment 1 – effects of salinity and water depths

The seedlings were then kept inside the three aquarium tanks (Figure 8) with 120 x 45 x 45 cm dimension irrigated with three different salinities (0, 20 and 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 be 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 (Figure 8b). In LW, only the soil pot is flooded with water, in MW, approximately 50% of the propagule is flooded with water, and in HW, only 2-4 cm tip of the propagules emerged (or 95% of the propagules flooded with water). The number of seedlings cultured per water depth was replicated into three groups. The leaves of cultured plants were above the 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 appearance and development of the leaves were monitored daily and the average height and relative growth rate (RGR), leaf size stem diameter, and internode length were measured monthly. After 5 and 10 months, on August 2016 and February 2017 respectively, the cultured seedlings were harvested (Figure 9) 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 assayed for ROS, specifically hydrogen peroxide, antioxidant enzymes, pigments, proline and Fv/Fm ration.

3.2.3 Experiment 2 – effects of salinity and inundation hydroperiod

Another set of seedlings were cultured in separate aquarium tanks filled with 3-cm deep saline water (0 20, 35 ppt) (Figure 10) in the same greenhouse. After 15 months of greenhouse culture, in June to August 2017, the seedlings were subjected to varying submergence and inundation periods (semi-diurnal, diurnal, permanently submerged) simulating the tidal cycles for seven days at three different salinity levels (0, 20, 35 ppt) corresponding to the same salinity where the plants were cultured (Figure 11). For semi-diurnal inundation (SDI), the seedlings were submerged twice a day for three hours (i.e. three hours submerged and then three hours emerged, equivalent to in culture conditions, followed by another three hours submerged and then a return to emerged or culture conditions). For diurnal inundation (DI), the seedlings were submerged for 6 hours per day, and for the permanently submerged (PS) conditions, the seedlings were submerged for 24 hours. For the 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 seedlings were cultured), while in the inundated or submerged conditions, the topmost pair of leaves was 15 cm below water surface. The seedlings were checked daily for the directly observable changes in the leaf color and to check the water levels and salinity. The number of seedlings and the experimental tank were assessed in three relicates, and the inundation was conducted in a time series. At the end of the experiment, leaf tissue samples were collected and assayed as deataile dbelow.

3.2.4 Measurement of plant growth and biomass

The heights and internodes of the cultured seedlings were measured with a ruler, and the relative growth rate (RGR) were obtained from the difference between the final and initial height divided by the culture period. The leaf size or area was determined by the taking the scaled photograph of the leaves and then processed using ImageJ processor to determine the leaf area. The stem diameter was measured with a digital caliper.

The biomass of the harvested cultured seedlings at 5 and 10 months was determined by drying in an oven at the temperature of 80^{o}C for 72 hours or until constant weight was obtained. The dried samples were then partitioned into above-ground biomass (AGB), or the shoots and below ground-biomass (BGB), or the roots.

3.2.5 Extraction and determination of pigments and carotenoids

Approximately 25-50 mg of fresh leaf tissues were prepared in three replicates with a leaf tissue punch. The pigments were extracted with N,N-dimethylformamide for 24 hours and measured with a spectrophotometer (Shimadzu, UV Mini 1210). Chlorophyll a and b and the carotenoid concentrations were calculated based on Wellburn (1994).

3.2.6 Fv/Fm measurement

Chlorophyll fluorescence was measured using a chlorophyll fluorescence imaging technique (FC 1000-H; Photon Systems Instruments, Czech Republic) with auto image segmentation. Initially, plant segments were dark-adapted for 20 minutes, and the maximum quantum efficiency of photosystem II photochemistry (Fv/Fm) was calculated following DeEll and Toivonen (2003).

3.2.7 Extraction and enzyme assays for H_2O_2, APX, CAT and POD

Analyses for hydrogen peroxide (H_2O_2), catalase activity (CAT), ascorbate peroxidase activity (APX) and peroxidase activity (POD) were performed by grinding the fresh leaf samples (300 - 500 mg) using a mortar and pestle and liquid nitrogen with an ice-cold 50 mM phosphate buffer, pH 6.0. Polyvinylpyrrolidone (PVP) was added to the ground tissue sample to mask the effects of phenolic compounds. The extracts were centrifuged at 3000 × g and 4°C for 15 minutes, and the supernatant was separated for subsequent enzyme assays. The prepared tissue extracts were done in three replicates.

The concentration of H_2O_2 was determined based on Jana and Choudhuri (1982). The reaction mixture contained 750 µL of the enzyme extract mixed with 2.5 mL of 0.1% titanium sulfate in 20% H_2SO_4 (v/v). The mixture was centrifuged at 5000×g for 15

minutes at room temperature, and the intensity of yellow color development was measured at 410 nm using a spectrophotometer. The H_2O_2 was calculated using the standard curve prepared with known concentration of H_2O_2 and expressed as µmol/g FW.

The CAT activity was measured according to Aebi (1984). The reaction mixture was prepared with 100 µL of 10 mM H_2O_2 and 2.00 mL of 100 mM potassium phosphate buffer (pH 7.0) in the cuvette. An aliquot of 500 µL of the enzyme extract was then added to the cuvette to start the reaction. The absorbance reduction was measured with a spectrophotometer at 240 nm using a spectrophotometer for every 10 seconds for three minutes. The CAT activity was calculated using an extinction coefficient of 40 mM^{-1} cm^{-1} and expressed as µmol/min/g FW.

The APX activity was measured according to Nakano and Asada (1981). The reaction mixture contained 100 µL of enzyme extract, 200 µL of 0.5 mM ascorbic acid in 50-mM potassium phosphate buffer (pH 7.0) and 2.0 mL of 50-mM potassium phosphate buffer (pH 7.0). The reaction was started by adding 60 µL of 1 mM H_2O_2, and the reductionin in absorbance was measured at 290 nm using a spectrophotometer every 10 seconds for three minutes. The APX activity was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1} and expressed as µmol/min/mg FW.

The POD activity was measured based on MacAdam et al. (1992). The reaction mixture contained 3.0 mL of 50 mM potassium phosphate buffer (pH 6.0), 40 µL of 30 mM H_2O_2 and 50 µL of 0.2 M guaiacol. The reaction was started by the addition of 100 µL of tissue extract, and the increase in absorbance was measured at 420 nm using a spectrophotometer every 10 seconds for 3 minutes. The POD activity was calculated using an extinction coefficient of 26.6 mM^{-1} cm^{-1} and expressed as µmol/min/g FW.

3.2.8 Extraction and determination of proline

Proline contents in leaves were measured according to Bates et al. (1973). Approximately 300 mg of dried leaves were homogenized in 5 mL of 3 % aqueous sulfosalicylic acid and centrifuged (12,000 rpm, 15 minutes, 4 ^{o}C). The mixture of

supernatant (2 mL), acid-ninhydrin (2 mL) and glacial acetic acid (2 mL) was incubated at 100 ^{o}C for 45 minutes and then extracted with toluene. The absorbance of toluene was measured at 520 nm using a spectrophotometer. The amount of proline was calculated from standard curve prepared with known concentration of proline.

3.3 Data processing and statistical analyses

Statistical analyses were carried out in XLSTAT Premium. For the greenhouse data set, the response variables (growth, biomass, ROS, AOX, pigments, carotenoids, proline, Fv/Fm) were evaluated for normal distributions as well as for homogeneity of the variances. Two-way analysis of variance (ANOVA) were then performed to examine the effects of salinity, water depths and inundation period on all the response variables at a significance level of $p<0.05$. For the field data, the t-test was performed to compare the differences in response variables with the intertidal level (inundated and not inundated).

CHAPTER IV

4. Results

4.1 Experiment 1 – effects of salinity and water depths

4.1.1 Propagule development, growth and biomass

Salinity influenced the initial establishment and growth of the cultured *R. stylosa* propagules. The first pair of leaves unfurled relatively faster for the seedlings cultured in low salinity or in freshwater (75.5±12.2 days) than for those in moderate (90.5±10.0 days) and high salinity (93.3±11.5 days) (Figure 12). LS provided favorable conditions for the initial growth development of the cultured seedlings. Salinity had a significant effect on observed differences in the initial development of the cultured seedlings (Table 2).

After five months, the highest average height was observed in LS with 17.6±1.8 cm followed by those in MS (15.3±1.1 cm), while those in HS (10.2±1.1cm) had the lowest. However, at 10 months, the highest average height was observed in those in MS and LS (24.4±1.6 cm and 23.2±2.2 cm, respectively) while those in HS had the lowest (17.7±1.6 cm) (Figure 13). The relative growth rate (RGR) showed that the LS provided the best condition for the first five months with average RGR of 3.52±0.09 cm/month followed by those in MS with 3.05±0.05 cm/month while those in HS had the lowest with only 2.04±0.11 cm/month (Figure 14). However, at 10 months, the optimum condition shifted towards the MS with RGR of 2.34±0.04 cm/month and LS with 2.32±0.05 cm/month while those in HS had the lowest with only 1.77±0.05 cm/month. There was a shift in favorable condition wherein the cultured seedlings were adapted to MS after 10 months. Salinity had a significant effect on the differences of the average height at five and 10 months culture period, while the water depth and the interaction of salinity and water depth have no effect (Table 2 and 3). Although the water level per salinity treatment showed a slight difference in average heights, the differences however were not statistically significant.

Salinity significantly influenced the AGB production for both the seedlings cultured for 5 and 10 months, while the water depth and the interaction of salinity and water depth have no effect (Table 2 and 3). At five months, higher AGB productions was observed in LS and MS cultured seedlings (1.74±0.12 g DW and 1.76±0.08 g DW, respectively), while those in HS cultured had the lowest with only 0.88±0.01 g DW (Figure 15a). The same trend was also observed for AGB production at ten months. The AGB production monthly increment for the first five months showed highest increments in LS and MS with average values of 0.35±0.02 g DW for both treatments, while those in HS had the lowest (0.18±0.002 g DW). At 10 months, the highest AGB monthly increment was observed in MS (0.49±0.01 g DW), followed by those in LS (0.43±0.01 g DW), while those in HS had the lowest with only 0.39±0.01 g DW (Figure 15b). The difference in the AGB between 5 and 10 months showed that the HS cultured seedlings had the highest increment (0.21 g DW), followed by those in MS (0.13 g DW), while those in LS had the lowest (0.08 g DW). This result means that the favorable conditions for AGB production shifted to saline conditions and impoies implies an adaptation to salinity.

Similarly, salinity also significantly influenced the below-ground biomass (BGB) or root biomass for both the seedlings cultured for 5 and 10 months while the water depth and the interaction of salinity and water depth have no effect (Table 2 and 3). At 5 months, the seedlings cultured in HS had the highest average BGB (1.11±0.04 g DW), followed by those in MS (0.95±0.02 g DW), while those in LS had the lowest (0.76±0.02 g DW) (Figure 16a). At 10 months, the favorable condition was shifted and the highest values was observed for seedlings cultured in LS (3.23±0.08 g DW), followed by those in MS (2.51±0.09 g DW), while those in HS had the lowest (1.46±0.07 g DW). At five months, the monthly BGB increment was highest for seedlings cultured in HS (0.22±0.01 g DW/mo.), followed by those MS (0.19±0.003 g DW/mo.) while those in LS had the lowest (0.15±0.005 g DW/month) (Figure 16b). The difference between 5 and 10 months BGB productions showed that the LS cultured seedlings had the highest increment (0.34 g DW) followed by those in MS (0.12 g DW), while those in HS had the lowest increment.

In terms of biomass partitioning, at 5 months, the cultured seedlings in HS had a higher allocation for BGB, while those in LS had a higher allocation for AGB, as

shown by the root-to-shoot ratio (Figure 17). However, at 10 months, the biomass partitioning shifted. Those cultured in HS had a higher allocation for AGB while those in LS had a higher allocation for BGB. This shift in optimum conditions signifies an adaptation towards MS and HS.

4.1.2 Leaf proline

Proline is one of the major organic osmolytes that accumulate in the cytosol in response to environmental stressors such as salinity. Its accumulation is an important mechanism for osmotic regulation when under salt stress. The concentration of proline in the leaves showed an increasing trend with the increasing salinity for both 5 (R^2 = 0.886) and 10 months (R^2 = 0.896) cultured seedlings (Figure 18). Salinity had significant effect on the variability of proline while water depth and the interaction of salinity and water depth have no effect (Table 4 and 5). There was a significant increase in proline accumulation in the MS and HS cultured seedlings at 10 months compared to the values at 5 months. This signifies physiological adaptation towards saline conditions, which is consistent with the observed changes in growth and biomass productions.

4.1.3 Pigments, Fv/Fm and biochemical responses in emerged condition

The H_2O_2 in the leaves of *R. stylosa* showed an increasing trend with salinity for both the 5-month (R^2 = 0.854) and 10-month (R^2 = 0.917) culture periods (Figure 19a). The H_2O_2 values showed no significant change and remained comparable between the 5 and 10 months. The seedlings cultured in LS had the lowest, followed by those in MS, while those in HS had the highest H_2O_2 concentration. Salinity had a significant effect on the variability of H_2O_2 production for both the seedlings cultured over 5 months ($F = 51.737$, $p<0.0001$) and 10 months ($F = 98.823$, $p<0.0001$) while the water depth and the interaction of salinity and water depth have no effect (Table 4 and 5). The high H_2O_2 concetnration retard the growth and biomass production of the cultured *R. stylosa* as shown by its negative relationship with average height (Figure 19b) and biomass (Figure 19c) for both the 5 and 10 months cultured periods.

Salinity had a significant effect on the induction of the activities of antioxidant enzymes including the APX (5 months: $F = 1{,}798.52$, $p<0.0001$; 10 months: $F = 2{,}979.12$, $p<0.0001$); CAT (5 months: $F = 17.62$, $p<0.001$; 10 months: $F = 42.72$, $p<0.0001$) and POD (5 months: 65.77, $p<0.0001$; 10 months: $F = 73.04$; $p<0.00001$) (Figure 20a, b and c). While the water depth and the interaction of salinity and water depth have no effect on the variability of AOX activites (Table 4 and 5). Furthermore, the H_2O_2 concentration showed a positive correlation with the AOX activities. As H_2O_2 increases, the AOX activities also increases to scavenge the H_2O_2 and lessen the effects.

The pigments (Chl a and b) and carotenoids exhibited temporal variations between 5 and 10 months. At 5 months, it showed a decreasing trend with salinity, and the seedlings cultured in LS had the highest values, followed those in MS, while those in HS had the lowest (Figure 21 a and b). However, at 10 months, the trend was reversed and showed an increasing trend with salinity. The seedlings cultured in LS had the lowest values, followed by those in MS, whereas those cultured in HS had the highest. At 10 months, the seedlings cultured in LS showed a sifnificant reduction compared with the values at 5 months, whereas those in MS and HS showed increments. Salinity had a significant effect on the variability of the Chl a (5 months: $F = 36.275$, $p<0.0001$; 10 months: $F = 10.119$, $p<0.0001$) and the Chl b (5 months: $F = 76.529$, $p<0.0001$; 10 months: $F = 51.969$, $p<0.0001$), while the water depth and the interaction of the salinity and water depth have no effect (Table 4 and 5). Similarly, carotenoids also followed the same trend as the pigments (Figure 21c). At 5 months, carotenoids showed a negative correlation with salinity, while at 10 months, the trend reversed. At 10 months, the seedlings cultured in LS exhibited a significant reduction compared with the values at 5 months, whereas those in MS and HS had significant increments. Salinity had a significant effect on the variability of carotenoids for both the seedlings cultured over 5 and 10 months, while the water depth and the interaction of the salinity and water depth had no effect (Table 4 and 5).

On the otherhand, the Fv/Fm ratio slightly decreased with increasing salinity (Figure 22), however the difference is not statistically significant for both the 5 months (Table 4) and 10 months (Table 5) culture period.

4.2 Experiment 2 - effects of salinity and inundation hydroperiod

4.2.1 H_2O_2 and activities of antioxidant enzymes

Periodic inundation (SDI and DI) and prolonged inundation (PS) induced a higher order magnitude of stress than the effects of salinity (Table 6). The PS even caused sublethal damage as manifested by the chlorosis of the leaves (Figure 23) in MS and HS treatments. The chlorotic leaves developed faster in HS than in MS, appearing after four days in the HS treatment and after five days in the MS treatment; whereas in LS, chlorosis was not observed even at the end of the experimental period.

The periodic inundation caused a significant increase in the generation of H_2O_2 both for SDI and DI, whereas the generation significantly decreased for the PS (Figure 24). However, the reduction of H_2O_2 concentration in the PS condition was due to the sublethal damage manifested by the yellowing of leaves (Figure 23), indicating that the plants could no longer produce the ROS. Based on the 2-way ANOVA, salinity, inundation and the interaction of salinity and inundation had significant effects on the H_2O_2 variations ($F = 7.30$, $p<0.05$; $F = 62.15$, $p<0.0001$; $F = 4.73$, $p<0.05$ respectively). Among these variables, inundation was the most influential factor (Table 6).

Similarly, the periodic inundation significantly induced the activities the activities of APX ($F = 1{,}058.79$, $p<0.0001$), CAT ($F = 197.15$, $p<0.0001$) and POD ($F = 460.06$, $p<0.0001$) (Figure 25 and Table 6). Salinity and the interaction of salinity and inundation had also significant effect on the variation in AOX activities; however, the effect of inundation was a higher order of magnitude. By contrast, for the PS, all the AOX activities were significantly reduced relative to the reduction in H_2O_2, which is attributed to the sublethal damage of the leaf tissues.

4.2.2 Pigments, carotenoids and Fv/Fm ratio

The Fv/Fm ratio (Figure 26), pigments (Chl a and b) and carotenoids (Figure 27) showed a significant reduction relative to the inundation period in all of the salinity treatments, and the PS condition showed the highest reduction. For pigments and

carotenoids, inundation and salinity both significantly influenced the reduction, but the inundation was the most influential factor (Table 6). However, for the Fv/Fm ratio, only inundation had a significant effect (Table 6). As previously stated, this reduction in pigments, carotenoids and Fv/Fm ratio was attributed to the observed morphological damage of the leaf tissues manifested by the yellowing of the leaves and implied that *R. stylosa* seedlings had low tolerance to prolonged underwater stress.

4.3 Integrating both the greenhouse experiments and the field data

Salinity showed a strong correlation with the H_2O_2 for both the greenhouse cultured seedlings and field samples (Figure 28). The levels of H_2O_2 concentration for the greenhouse-cultured seedlings at 5 months ranged from 38.6±3.2 to 64.3±4.1 μmol/g FW, while for the field samples, the H_2O_2 levels ranged from 36.3±0.86 to 77.9±6.1 μmol/g FW, with the highest values determined for samples collected in areas with high salinity. The field samples collected in the inundated areas, where soil was covered with water during spring tide but plant leaves emerged above water level, had lower H_2O_2 concentrations (mean = 45.20±5.69 μmol/g FW) compared to samples collected at the higher elevation with rare or no inundation (mean = 63.46±7.27 μmol/g FW) (Figure 29). This differences in H_2O_2 levels between the inundated and not inundated areas is statistically significant ($p<0.05$, Table 7). Similarly, the foliar AOX activities collected in the inundated areas had relatively lower mean values (15.54±1.99 μmol/min/g FW) compared with the samples collected at the higher elevations with rare or no inundation (26.35±2.82 μmol/min/g FW). The difference in AOX activities between the inundated and not inundated areas was statistically significant ($P<0.05$, Table 7).

The pigments (Chl a and b) increased slightly with increasing salinity in the greenhouse-cultured plants and field samples (Figure 30). For the greenhouse-cultured seedlings at 5 months, the pigment content ranged from 694.30±49.82 to 865.90±74.40 μg/g FW, while for the field samples, it ranged from 699.77±30.68 to 901.24±27.88 μg/g FW (Figure 31). For field samples, the pigments in the inundated areas slightly increased with increasing salinity, while in the higher elevation and

rarely inundated areas, Chl a and b gradually declined with increasing salinity. This differences in the pigments between the inundated and not inundated areas is statistically significant ($p<0.05$, Table 7). Similarly, the carotenoids followed the same trend of the pigments. The carotenoids values for seedlings cultured at 5 months ranged from 139.73±9.56 to 181.08±11.13 µg/g FW, with the highest values observed for seedlings cultured in low salinity. For the field samples, carotenoids showed an increasing trend with salinity and elevation. Samples at the higher elevation and rarely inundated sites demonstrated higher values compared with samples at the inundated areas (mean values of 197.81±15.72 µg/g FW and 183.32±15.00 µg/g FW respectively); however, this difference was not significant ($p>0.05$, Table 7).

CHAPTER V

5. Discussion

The role of abiotic drivers specifically the salinity, elevation and inundation has been extensively studied. (Ball, 1988a; Friess et al., 2012; Watson, 1928). While earlier studies focused on the effects of salinity on the growth rate and biomass and the influence of inundation in the species distribution and survival of the young cohorts, there is still a gap of knowledge linking the biochemical stress indicators into the paradigm of mangrove ecophysiology that could serve as a blueprint for rehabilitation and restoration guidelines. This study provides a platform in further understanding the niche-width preference and ecophysiology of the commonly selected mangrove species for rehabilitation – the *R. stylosa* to salinity, water depth, inundation and elevation by integrating the growth and biochemical stress indicators into the paradigm of mangrove ecology and restoration strategy.

Mangroves are one of the valuable habitats that have suffered widespread decimation stemming from increasing population density and continuing economic development, with approximately 44% of the world population living within 100 km of the coast (Butchart et al., 2010). From 1980 to 2005, approximately 20% of global mangroves were lost FAO (2007), and the southeast Asia region contributed the highest mangrove decline primarily because of agriculture and aquaculture conversion (Primavera and Esteban, 2008; Richards and Friess, 2016). As a response to the alarming rate of mangrove decline, rehabilitation programs receive much attention to restore the damaged habitat. However, for several mangrove restoration programs, the results include stories of mixed successes and failures (Asaeda et al., 2016; Barnuevo et al., 2017). These efforts were often unsuccessful, largely because of the high mortality of the seedlings due to the failure to recognize the species-specific tolerance thresholds of mangroves in relation to the environmental conditions (Oh et al., 2017; Primavera and Esteban, 2008; Samson and Rollon, 2008).

5.1 Adaptive morpho-physiological plasticity as a respond to salinity

Adaptive morphological plasticity was exhibited in the early establishment and growth of the greenhouse-cultured *R. stylosa* seedlings. At 5 months, the highest average height and growth rate was observed in seedlings cultured at low salinity, whereas at 10 months, the highest average height was observed in moderate salinity. This implies a temporal shift in favorable conditions between the 5 and 10-month culture periods. Another morphological plasticity was exhibited in the biomass partitioning between the 5 and 10-month culture periods. Comparing the AGB and BGB allocations, at 5 months, those cultured in moderate and high salinity had higher allocations for BGB, while those in low salinity had higher allocations for AGB, as shown by the root-to-shoot ratio. However, at 10 months, there was a shift in biomass allocations. The seedlings cultured in moderate and high salinity had higher allocations for AGB, while those in low salinity had higher allocations for BGB.

The relationship of mangroves and salinity has long been studied (Ball, 1988a; Lugo and Snedaker, 1974), however, there is still have no consensus on the salinity preferences of mangroves. The study of Patel et al. (2010) showed a negative relationship between the emergence rate of *A. marina* seedlings and the salt content; the study of Ye et al. (2005) showed that the increasing salinity delayed the root initiation of *Acanthus ilicifolius*, while low salinity stimulated the growth of *A. marina* (Ball and Anderson, 1986; Clough, 1984). Studies on the growth and development of different mangrove species have shown differences in salinity preferences. The species of *Ceriops tagal* had optimum growth at 50% seawater (Aziz and Khan, 2001); the species of *Sonneratia caseolaris* had optimum growth at low salinity (3-5 psu) (Jayatissa et al., 2008), and the species of *Excoecaria agallocha* achieved its optimum growth below 5 psu (Chen and Ye, 2014b). Differences in the apparent knowledge on salinity preferences of mangroves could be attributed to the age of the experimental seedlings and duration of the study period. Some studies were conducted in few weeks to months, thus, overlooked the shift in the optimum condition. This study has shown that *R. stylosa* shifted its salinity preference from low salinity during the early stage of development to saline conditions.

The observed morphological plasticity of *R. stylosa* seedlings cultured in saline conditions helped them cope with the stressful environment (Vovides et al., 2014). It has been reported that plants tend to change their root to shoot ratio in response to biotic and abiotic factors (Ericsson, 1995). Mangroves that were exposed to stressful conditions had been reported to invest more in root production for tolerance (Lovelock et al., 2009). The study of McKee (1995) on three mangrove species (*R. mangle*, *A. marina* and *Laguncularia racemosa*) showed that the low nutrient and or light availability caused lower investment in root biomass, whereas high nutrient availability resulted in greater investment in leaf area and number of leaves. Furthermore, biomass accumulation and stem elongation of *Sonneratia alba* and *S. lanceolata* was stimulated by low salinity and inhibited by further increasing salinity (Ball and Pidsley, 1995). The nutrient enrichment study of Naidoo (1987) on *A. marina* showed that the addition of 14 mg/l N had a significant effect on root production at 0.5 M NaCl and significantly increased the shoot production at 0.1 M and 0.3 M NaCl. In the field study in Richard Bay, South Africa, enrichment with N and N + P shifted the resource allocation to shoots from 38% to 55% and increased dry biomass accumulation by over 500% compared to the control (Naidoo, 2009).

Physiological adaptation was exhibited in the proline accumulation in the leaves between the 5- and 10-month culture periods. The proline content of the leaves showed a significant increase for seedlings cultured in the moderate and high salinity at 10 months compared with at 5 months. This increase in proline accumulation signifies physiological adaptations to saline conditions, which is consistent with the observed changes in growth and biomass production. Plants exposed to salt stress undergo changes in their environment, and its ability to tolerate salt is determined by multiple biochemical pathways that facilitate the retention and acquisition of water, protect chloroplast functions and maintain ion homeostasis (Parida and Das, 2005). Essential pathways include those that lead to the synthesis of osmotically active metabolites and certain free radical scavenging enzymes that control ion and water fluxes and support the scavenging of oxygen radicals. Proline is an important component of salt-stress responses of plants (Khedr et al., 2003) and is considered one of the major organic osmolytes that accumulate in the cytosol in response to environmental stressors, including salinity (Burg and Ferraris, 2008). To accommodate the ionic balance in the vacuoles, the cytoplasm accumulates

compatible solutes that doesn't interfere with the normal biochemical reactions (Parida and Das, 2005; Yancey et al., 1982), but instead replace the water in biochemical reactions. With the accumulation proportional to the change of external osmolarity within species-specific limits, the osmolytes functions in the protection of structures and osmotic balance, supporting continued water influx (or reduced efflux) (Hasegawa et al., 2000). Additionally, other studies have reported that proline improved the salt tolerance of *Pancratium maritimum* by protecting the protein turnover machinery against stress-damage and up-regulating stress protective proteins (Khedr et al., 2003).

Another physiological plasticity was observed in the temporal variation of pigments and carotenoids between the 5- and 10-month periods. At 5 months, the temporal variation showed a negative correlation with salinity and a positive correlation at 10 months. The seedlings cultured in low salinity had a significant reduction at 10 months, whereas those in moderate and high salinity treatments showed a significant increment. The increment in saline conditions supports the observed higher RGR at 10 months of the culture period. Higher pigments translate into higher photosynthetic capacity and energy production and therefore a faster growth rate. Earlier studies reported that chlorophyll and carotenoids decreased under salt stress; however, the ages of studied seedlings were not clearly specified. Salinity or NaCl treatment caused significant decreases in Chl a, Chl b, and carotenoids in the leaves of *B. parviflora* (Parida et al., 2002) and in the leaves of *Aegiceras corniculatum* (Pritinanda et al., 2013). The total chlorophyll (Chl a+b), Chl a, and b carotene decreased with NaCl stress in the leaves of tomato (Khavari-Nejad and Mostofi, 1998), and the leaf pigments of nine studied genotypes of rice under salinity stress generally decreased, but relatively high pigment levels were found in six genotypes (Alamgir and Ali, 1999). However, Wang and Nii (2000) have reported that the chlorophyll content increased under conditions of salinity in *Amaranthus*, and in the study of Takemura et al. (2000), the Chl a and Chl b of the mangrove *B. gymnorrhiza* increased with a NaCl treatment.

Other studies on mangroves have demonstrated that high salinities lowered the net photosynthesis due to a reduction in stomatal conductance (Ball, 1988b) as well as higher rates of dark respiration relative to assimilation to meet the increasing costs of

intracellular ion compartmentalization (Lopez-Hoffman et al., 2007). Generally, the photosynthesis of mangroves, as in other vascular woody plants, ranges between 5 to 20 μmol CO_2 m^2/s (Krauss et al., 2008; Naidoo et al., 2002), but at low salinity, the rate of photosynthesis can exceed 25 μmol CO_2 m^2/s. The tolerances and responses of different mangrove species to different abiotic factors resulted from the integrated events occurring at all organization levels from morphological to physiological and biochemical levels.

5.2 Do mangroves need salt?

The question as to whether mangroves need salt for growth and reproduction remains poorly understood. Contemporary knowledge classified mangroves as either facultative or obligate halophytes. Some reports classified mangroves as facultative halophytes because they are capable of growing both in freshwater and saline water, while other reports classified mangroves as obligate halophytes; salt is necessary for their growth and they cannot survive in freshwater permanently (Krauss and Ball, 2013; Wang et al., 2011). In this study, the low salinity or freshwater provided the optimum conditions for the initial growth and biomass production. However, as its growth progressed, the favorable conditions shifted to saline conditions accompanied by higher growth rate and biomass production and increments in pigments and proline accumulation. This result signifies that mangroves' physiological adaptation and tolerance to salinity shifted with age.

Whereas most of the earlier studies on the effects of salt stress on different mangrove species have shown that low salinity or freshwater provided the best conditions for growth, some studies reported that moderate salinity provided optimum conditions. This contradicting view about the salinity preferences of mangroves could be attributed to the differences of the ages of the experimental plants, and most experiments were done after few weeks of culture. Hence, earlier studies have failed to capture the temporal physiological shift and adjustment to salinity treatments over time.

Generally, woody plants are relatively salt tolerant during seed germination, changed to be more sensitive during the seedling stage, and become progressively more

tolerant with age through to the reproductive stage (Wang et al., 2011). Specifically, for viviparous mangroves including *R. stylosa*, their seedling stage is the period of higher salt tolerance, primarily because its propagules store large amounts of nutrients and energy (Ball, 2002; Tomlinson, 2016). The vivipary and crypto-vivipary are reproductive strategies of mangroves to store considerable parental nutrients and energy investments for the seedlings (Hogarth, 2015) in order to support the metabolic needs during early growth under nutrient-poor or salt-stressed conditions (Farrant et al., 1992b; Wang et al., 2002). Additionally, the propagules contain large amounts of Na^+ and Cl^- (Bhosale and Shinde, 1983; Clough, 1984; Downton, 1982), allowing them to grow in freshwater for a certain period of time with sufficient levels of these ions in their tissues (Atkinson et al., 1967; Ball et al., 1987). Thus, the viviparous nature of some species of mangrove, including *R. stylosa*, provides their seedling with the ability to grow in high or in low salinity or even in freshwater for an extended period (Wang et al., 2011).

The apparent understanding of the salt tolerance of mangroves, specifically the viviparous species, is based on short-term growth experiments with seedlings for a few weeks to months (Wang et al 2011); therefore, the tolerance and preference of the studied mangroves has been overlooked in many earlier reports. Based on this study, the salinity preference of the mangrove *R. stylosa* is age-dependent, as shown in the shift of RGR and biomass and by the variation of proline, pigments and carotenoids between the 5 and 10-month cultures.

5.3 Comparison of oxidative stress between emerged and submerged conditions

The periodic inundation caused a significant increase in the generation of H_2O_2 compared with the emerged condition in both SDI and DI, whereas the generation significantly decreased for the PS (Figure 32). However, the reduction in H_2O_2 concentration in the PS condition was due to the sublethal damage manifested by the yellowing of leaves, indicating that the plants could no longer produce the ROS. Salinity, inundation and the interaction of salinity and inundation had significant effects on the H_2O_2 variations ($F = 7.30$, $p<0.05$; $F = 62.15$, $p<0.0001$; $F = 4.73$, $p<0.05$, respectively). Among these variables, inundation was the most influential factor (Table 6). Similarly, the periodic inundation significantly induced the activities

of APX (F = 1,058.79, P<0.0001), CAT (F = 197.15, P<0.0001) and POD (F = 460.06, P<0.0001) in the inundated condition compared with the emerged condition (Figure 33). Salinity also significantly affected the variation in activities of these enzymes; however, the effect of inundation was a higher order of magnitude than that of salinity (Table 6). By contrast, for the PS, all the AOX were significantly reduced relative to the reduction of H_2O_2, which was attributed to the sublethal damage of the leaf tissues. The pigments (Chl a and b) and carotenoids showed a significant reduction relative to the inundation hydroperiod in all salinity treatments, and the PS condition showed the highest reduction (Figure 34). Inundation and salinity both significantly influenced the reduction, but the inundation was the most influential factor (Table 6). Salinity had no significant on the Fv/Fm ratio when in an emerged condition (Figure 35). However, when in an inundated condition, the values reduced significantly reduced (Table 6). As previously stated, this reduction in pigments, carotenoids and Fv/Fm ratio was attributed to the observed morphological damage of the leaf tissues manifested by the yellowing of the leaves and implied that *R. stylosa* seedlings had low tolerance to prolonged underwater stress.

Although the importance of tidal flooding and inundation in mangroves have long been reported (Watson, 1928), much of the literature on establishment and early development of mangroves have either ignored the effects of flooding within laboratory settings or have failed to quantify tidal inundation in the field. Deriving the deviation (as induction or reduction) of H_2O_2 and AOX values when in inundated and submerged condition from the emerged condition (pooled average of LW, MW and HW) as reference, the periodic inundation (SDI, DI) showed a significant induction while the prolonged PS showed a significant reduction (Figure 32). In the SDI, the H_2O_2 increment ranged from 23.5 to 42.1% and in DI, the increment ranged from 29.2 to 62.2% with the low salinity treatment showed highest; while in PS condition, the H_2O_2 showed a significant reduction in both the moderate (6.6%) and high (34.9%) salinity; while in low salinity showed an increment of 25.0%. The reduction H_2O_2 in moderate and high salinity under PS condition is the attributed to the sublethal damage in the leaf tissues. The same trend followed by the antioxidative enzymes (Figure 33). As the H_2O_2 increases, the enzyme activities also increased under the SDI and DI while decreased for the PS condition. The AOX functions as ROS scavenging system to lessen the oxidative stress, however, under extreme condition (i.e. PS),

plant tissues can no longer the defense enzymes. Additionally, the pigments (Chl a and b) and carotenoid (Figure 34) and the Fv/Fm ratio (Figure 35) also significantly reduced when in submerged condition vis-à-vis emerged condition. When under water, the processes of photosynthesis and the photosystem I and II were adversely affected.

High salinity caused osmotic stress and reduced the availability of water (Chen and Ye, 2014a), resulting in stomatal closure and a reduced supply of carbon dioxide (Li et al., 2008). In addition, salt stress also induced ion toxicities such as membrane disorganization, production of reactive oxygen species, and disturbance of nutrient balance. Increases in salinity reduced nitrogen accumulation in *Kandelia candel* (Kao et al., 2001) and inhibited the uptake of K^+ resulting in damage to the photosynthetic apparatus (Patel et al., 2010). Earlier studies have shown that periodic submersion and tidal water logging influenced the photosynthetic processes and the growth of several species of mangroves. A study by Luzhen et al. (2005) showed that the increasing duration of immersion significantly reduced the photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO_2 of *K. candel*. The long periods of tidal immersion significantly inhibited the photosynthesis of *K. candel*. The relative growth rate of *B. gymnorrhiza* decreased significantly with waterlogged time, with the highest value found for drained plants and the lowest in waterlogged plants under 12 weeks (Ye et a; 2005). The same trend was reported by Hoppe-Speer et al. (2011) on the adverse effects of inundation and salinity on the growth and biomass of *R. mucronata*. Additionally, Hoppe-Speer et al. (2011) found that plants exposed to high salinity and continuous inundation treatments showed symptoms of stress such as leaf shedding, excessive salt secretion, reduced leaf production and leaf necrosis. The submergence study of Mangora et al. (2014) on *A. marina*, *B. gymnorrhiza* and *Heritiera littoralis* seedlings showed that the survival and photosynthetic rates declined with increasing salinity and submergence time.

5.4 The relationship of H_2O_2 with antioxidant enzymes and pigments

Being at the interface of land and sea, mangroves are continuously subjected to inhospitable and complex abiotic and biotic factors (Das et al., 2016). These factors exert adverse effects on plants by disrupting the ionic and osmotic equilibrium of the

cells, leading to a decrease in plant growth and development. To mitigate the adverse effects, the antioxidant system works cooperatively to control the cascades of uncontrolled oxidation and to protect plant cells from oxidative damage by scavenging the excessive ROS produced from different abiotic and biotic stresses (Das et al., 2016).

In this study, antioxidant activities exhibited a positive correlation with increasing H_2O_2 concentration as a response to salinity, submersion and solar radiation. The periodic submersion imposed a significantly higher magnitude of stress as reflected by the high H_2O_2 concentration, which was reduced in the permanently submerged condition primarily due to the prevalent sublethal stress. The physiological condition had already been damaged, affecting the plant's ability to generate ROS and antioxidant enzymes. The high activities of APX, CAT and POD in the leaves of *R. stylosa* signify that they are continually involved in rebalancing the equilibrium and mitigating the deleterious effects of ROS.

The antioxidant system works in concert to scavenge the excessive ROS and to protect the plant cells from oxidative damage. Specifically, CAT, a heme-containing enzyme, catalyzes the oxidation of H_2O_2 into water and oxygen. CAT activity in *A. marina* was induced when exposed to salt and light (Jithesh et al., 2006). POD, which is also a heme-containing protein, is actively produced in mangroves to scavenge H_2O_2. Cherian et al. (1999) have reported increased POD activity in root and shoot tissues in *A. marina* under salinity stress. Additionally, the APX, which is a component of the ascorbate-glutathione cycle, is mainly produced in the chloroplasts to scavenge H_2O_2 and to maintain the redox state of the cell (Asada, 2006).

The high levels of antioxidants provided a sufficient resistance and defense system against the oxidative damage, playing a role in effectively lessening injury from ROS. A study by Takemura et al. (2000) reported that salt immediately induced CAT activity and superoxide dismutase (SOD) in *B. gymnorrhiza* when transferred from freshwater to high salinity, while Parida et al. (2004) showed that the activities of antioxidant enzymes in the leaf of *B. parviflora* were significantly enhanced at high concentrations of NaCl. The activities of the antioxidant enzymes, such as CAT, APX, POD, glutathione reductase (GR), and SOD, increased under salt stress in

plants, and a correlation was reported between these enzyme levels and salt tolerance (Gossett et al., 1994; Hernandez et al., 1995; Kennedy and De Filippis, 1999; Lee et al., 2001; Sreenivasulu et al., 2000).

Inhabiting the intertidal region, mangroves are continuously exposed to the interplay of abiotic stressors that alters mangrove physiology and consequently triggers the excessive generation of ROS in the mitochondria, chloroplast and peroxisomes (Sharma et al., 2012). ROS are derivatives of oxygen that are highly reactive and include hydrogen peroxide, hydroxyl radical, superoxide and singlet oxygen. In a biological context, ROS are formed as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress, levels of ROS dramatically increase resulting in significant damage to cellular structures. As a natural response to the increases in ROS, mangrove plants developed an efficient non-enzymatic and enzymatic antioxidant defense system to counter the deleterious effects of ROS (Das et al., 2016). Increases in levels of ROS result in increased relative abundance of several ROS scavenging enzymes such as CAT, POD and APX, among others (Sugimoto and Takeda, 2009). When the ROS production exceeds the scavenging activity of the natural defense mechanism, plants suffer from oxidative stress with consequent effects on physiology, biochemistry, cellular activities and nutrient uptake that result in the reduction of growth and biomass.

5.5 Translating the effects of oxidative stress to the whole plant and community level

Mangrove rehabilitation initiatives attract much attention from different sectors to foster mangrove recovery, and the efforts received a renewed impetus after the 2004 Indonesia tsunami and again after the 2013 Typhoon Haiyan in the Philippines due to the highly valued mangrove ecosystem services specifically as a buffer and bioshield of coastlines (Barnuevo et al., 2017; Wolanski and Elliott, 2015). The results of the intensive efforts of mangrove restoration programs are stories of mixed successes and failures. These efforts were often unsuccessful, largely because of the high mortality of the planted seedlings due to inappropriate site selection, predation, overtopping by waves and barnacle infestation (Primavera and Esteban, 2008; Samson and Rollon,

2008). Most of the plantations were established in the lower intertidal zones and utilized only a single species of *Rhizophora*, creating a monospecific plantation with no post-planting management plan (Asaeda et al., 2016; Barnuevo et al., 2017).

The study of He and Lai (2009) found that prolonged waterlogging induces increased SOD activity in roots, whereas moderate tidal flat inundation inhibits SOD activity in leaves in field conditions. Furthermore, in this study, the biochemical stress indicator (H_2O_2) and the antioxidant activities (APX, CAT, POD) in the leaves of *R. stylosa* increased significantly in the semi-diurnally and diurnally inundated treatments compared with those in leaves in the not inundated and emerged condition. However, prolonged submersion caused sublethal damage as shown by the appearance of chlorotic leaves. This outcome implied that *R. stylosa* had low tolerance to underwater stress. Additionally, inundation caused the reduction of Chl a and b, carotenoids and the Fv/Fm ratio, signifying a reduction in the photosynthetic capacity; thus, seedlings planted in the lower intertidal areas will suffer from retarded growth.

The adverse effects of high H_2O_2 concentration in the greenhouse-cultured *R. stylosa* as well as in the 30-year-old planted trees can be observed at the whole plant and community level, as manifested by the decrease in growth rate and reduction in the average height (Figure 36). At higher H_2O_2 levels, the growth rate of greenhouse-cultured *R. stylosa* was significantly reduced compared with plants with lower H_2O_2 levels. The same observation is also shown in the field condition. Planted *R. stylosa* at the higher elevation have relatively higher H_2O_2 levels and exhibited some extent of dwarfism and stunted growth as manifested by its relatively lower average heights compared with the trees of the same age planted in the inundated areas.

5.6 Species-specific niche-width and implications for mangrove rehabilitation

Extrapolating the results from the microcosm experiment and the field study, it appears that the niche-width of *R. stylosa*, where the H_2O_2 level is relatively low, is limited to the inundated areas as long as their leaves remains emerged even at spring tide (Figure 37a). This finding explains the distribution of this particular species, which typically dominates the middle intertidal areas (Figure 37b). Prolonged inundation is highly stressful to *R. stylosa*. The observed relationships of ROS, AOX,

pigments, carotenoids and Fv/Fm with the underwater stress could have valuable implications for restoration ecology and could serve as basis for selecting areas appropriate for rehabilitation. Based on lessons-learned, several mangrove plantations have suffered high mortality due to incorrect site selection, often planted in the lower intertidal and frequently inundated areas.

Although mangroves are often described as being adapted to seawater flooding, they can cope with only a limited frequency and duration of flooding; otherwise, their growth, physiology, gas exchange, and metabolic activities are affected (Chen et al., 2004; Naidoo et al., 1997; Skelton and Allaway, 1996; Ye et al., 2003; Youssef and Saenger, 1998). Mangrove forests naturally exist in a raised and sloped platform above the mean sea level, inundated approximately 30% of the period or less by tidal waters (Lewis III, 2005). The prolonged inundation hydroperiod in this study caused a significant increase in the H_2O_2 concentration and even led to necrosis of the leaves of *R. stylosa*. Furthermore, in the field, samples collected at the higher elevation and rarely inundated areas had significantly higher H_2O_2 compared with those in the inundated areas as long the leaves were emerged during the spring tides.

The lessons learned from the intensive efforts of mangrove rehabilitation programs are stories of mixed successes and failures (Asaeda et al., 2016; Barnuevo et al., 2017). These efforts were often unsuccessful because of the high mortality of seedlings due to inappropriate site selection (Oh et al., 2017; Primavera and Esteban, 2008; Samson and Rollon, 2008), and they failed to recognize the niche-width preference of the species being planted. A field study by He and Lai (2009) along the Guangxi coast of China showed that the survival rate of *R. stylosa* sharply decreased from 88.9% to 44.0% as the tidal flat elevation decreased. In laboratory conditions, the photosynthetic and survival rates of *Avicennia marina, Heritiera littoralis* and *Bruguiera gymnorrhiza* decline with increasing salinity and submergence period (Mangora et al., 2014). In the Philippines, there is a widespread tendency to plant mangroves in areas that are not their natural habitat, particularly at lower intertidal areas, thus resulting in the low survival of 10 to 20% (Primavera and Esteban, 2008). In this study, when in the emerged condition, the oxidative stress of cultured *R. stylosa* was significantly reduced compared with the inundated or submerged condition. Integrating the levels of oxidative stress for both the greenhouse-cultured

plants and field conditions, *R. stylosa* displayed lower oxidative stress in an emergent condition, while the periodic submersion of the whole plant induced a significantly higher magnitude of oxidative stress.

CHAPTER VI

6. Conclusion

The salinity tolerances of mangroves have been extensively studied for several decades, but differences in views remain unresolved. Some researchers have reported that low salinity or freshwater provided the best conditions for growth, while other studies proved that moderate salinity provided the optimum conditions. Earlier studies, however, were done over a shorter duration and have overlooked and or failed to recognize the temporal shift and age-dependent salinity preferences. This study highlighted the morphological and physiological plasticity of *R. stylosa* as an adaptation to salinity stress with age. In the early stages of growth and development, the low salinity or freshwater provided the optimum conditions for growth, and, as its growth progressed over time, optimum growth shifted towards moderate saline conditions. At 10 months, the relative growth rate and biomass production showed an optimum performance in saline conditions. The morphological plasticity is supported by some physiological variations between the culture periods. The proline content in the leaves of seedlings cultured in saline conditions was significantly increased at 10 months compared with the 5-month period. Significant increments were also observed for pigments (Chl a and b) and carotenoids in the saline conditions, while they decreased for those in freshwater. The observed higher growth rate of the seedlings cultured in low salinity at 5 months could be attributed to its viviparous reproductive strategy. Through its specialized structure – the propagules, it stored large amounts of nutrients and energy that support metabolic needs during its early growth under salt-stressed conditions and allowed them to grow optimally in freshwater for a certain period of time. The result of this study on the morpho-physiological plasticity of *R. stylosa* supports that mangroves are facultative halophytes with optimum condition at moderate salinity, although longer experimental duration in freshwater still required to whether they can survive until reproductive maturity.

This microcosm study mimicking the effect of water depth and tidal inundation on the ecophysiology of *R. stylosa* showed that this species had a low tolerance to inundation stress as shown by the induction of ROS and reduction of the photosynthetic pigments, carotenoids and Fv/Fm ratio. Salinity, inundation and elevation gradient

caused a variation and significant induction of ROS, specifically of H_2O_2, in the leaves of *R. stylosa*. Inundation of the whole plant imposed a higher magnitude of stress, as indicated by ROS levels, compared with the effect of salinity in the greenhouse experiment, and even caused sublethal damage, as manifested by chlorosis of the leaves. This finding implies that the species of *R. stylosa* cannot withstand a prolonged submersion period. In the field study, rare inundation is also stressful to *R. stylosa* as shown by the significantly higher ROS levels and stunted growth of the plants at a higher elevation compared with the inundated areas. Extrapolating the optimum condition from both experiments and translating to field conditions, the frequently inundated areas, specifically at the seafront mangrove fringes subjected to longer hydroperiod inundation at spring tides, may suffer from oxidative stress, stunted growth and consequently poor survival. The biochemical stress responses of *R. stylosa* to salinity and inundation provided a new insight in understanding its ecophysiology and niche-width preference, which is a space or condition with less stress and deemed favorable for growth across the intertidal gradient. Knowledge of the species-specific ecophysiology of mangrove could provide a novel insight among the policymakers for future knowledge-based rehabilitation programs. Additionally, this could have valuable implications for restoration ecology as baseline for the selection of areas that are appropriate for rehabilitation.

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TABLES

Table 1. Countries with large mangrove areas (from Giri et al 2011).

Country	Area (m^2)
1. Indonesia	3,112,989
2. Australia	977,975
3. Brazil	962683
4. Mexico	741,917
5. Nigeria	653,669
6. Malaysia	505,386
7. Myanmar	494,584
8. Papua New Guinea	480,121
9. Bangladesh	436,570
10. Cuba	421,538
11. India	368,276
12. Guinea Bissau	338,652
13. Mozambique	318,851
14. Madagascar	278,078
15. Philippines	263,137

Table 2. Two-way ANOVA showing the effects of salinity, water depth and the interaction of salinity and water depth on the morphology of 5 months cultured *R. stylosa* seedlings.

Response	**Factor**	***F***	***P***	R^2
Ave. height	Salinity	65.046	< 0.0001	0.886
	Water depth	0.735	0.494	
	Salinity x water depth	0.033	0.998	
Leaf area	Salinity	12.135	0.001	0.718
	Water depth	2.664	0.099	
	Salinity x water depth	2.918	0.052	
Stem diameter	Salinity	1.426	0.268	0.304
	Water depth	0.463	0.637	
	Salinity x water depth	0.804	0.539	
Leaf biomass	Salinity	138.528	< 0.0001	0.946
	Water depth	5.088	0.019	
	Salinity x water depth	2.658	0.069	
Stem biomass	Salinity	160.554	< 0.0001	0.952
	Water depth	0.509	0.610	
	Salinity x water depth	0.099	0.981	
Root biomass	Salinity	49.731	< 0.0001	0.862
	Water depth	0.912	0.420	
	Salinity x water depth	0.169	0.951	
AGB	Salinity	157.051	< 0.0001	0.95
	Water depth	2.423	0.119	
	Salinity x water depth	0.928	0.471	

Table 3. Two-way ANOVA showing the effects of salinity, water depth and the interaction of salinity and water depth on the morphology of 10 months cultured *R. stylosa* seedlings.

Response	**Factor**	***F***	***P***	R^2
Ave. height	Salinity	26.353	< 0.0001	0.763
	Water depth	0.475	0.630	
	Salinity x water depth	0.022	0.999	
Leaf area	Salinity	100.255	< 0.0001	0.928
	Water depth	0.306	0.740	
	Salinity x water depth	2.898	0.054	
Stem diameter	Salinity	2.048	0.160	0.311
	Water depth	0.435	0.654	
	Salinity x water depth	0.586	0.677	
Leaf biomass	Salinity	22.915	< 0.0001	0.743
	Water depth	0.578	0.572	
	Salinity x water depth	0.129	0.970	
Stem biomass	Salinity	330.732	< 0.0001	0.976
	Water depth	3.420	0.056	
	Salinity x water depth	1.019	0.425	
BGB	Salinity	134.542	< 0.0001	0.941
	Water depth	1.336	0.289	
	Salinity x water depth	0.063	0.992	
AGB	Salinity	188.777	< 0.0001	0.960
	Water depth	5.564	0.014	
	Salinity x water depth	0.324	0.858	

Table 4. Two-way ANOVA showing the effects of salinity, water depth and the interaction of salinity and water depth on the biochemical responses of the 5 months cultured *R. stylosa* seedlings.

Response	**Factor**	***F***	***P***	R^2
H_2O_2	Salinity	51.737	< 0.0001	0.854
	Water depth	0.546	0.589	
	Salinity x water depth	0.084	0.986	
APX	Salinity	1798.521	< 0.0001	0.995
	Water depth	9.411	0.002	
	Salinity x water depth	1.845	0.164	
CAT	Salinity	17.619	< 0.0001	0.663
	Water depth	0.015	0.985	
	Salinity x water depth	0.048	0.995	
POD	Salinity	65.767	< 0.0001	0.88
	Water depth	0.142	0.869	
	Salinity x water depth	0.096	0.982	
AOX	Salinity	109.698	< 0.0001	0.924
	Water depth	0.306	0.740	
	Salinity x water depth	0.058	0.993	
Chl a	Salinity	36.275	< 0.0001	0.806
	Water depth	0.681	0.519	
	Salinity x water depth	0.187	0.942	
Chl b	Salinity	76.529	< 0.0001	0.896
	Water depth	0.537	0.594	
	Salinity x water depth	0.077	0.988	
Chl a + b	Salinity	66.165	< 0.0001	0.882
	Water depth	0.957	0.403	
	Salinity x water depth	0.156	0.958	
Car	Salinity	46.374	< 0.0001	0.839
	Water depth	0.016	0.984	
	Salinity x water depth	0.174	0.949	
FvFm	Salinity	0.366	0.699	0.044
	Water depth	0.021	0.979	
	Salinity x water depth	0.012	1.000	
Proline	Salinity	66.215	< 0.0001	0.881
	Water depth	0.597	0.561	
	Salinity x water depth	0.064	0.992	

Table 5. Two-way ANOVA showing the effects of salinity, water depth and the interaction of salinity and water depth on the biochemical responses of the 10 months cultured *R. stylosa* seedlings.

Response	**Factor**	***F***	***P***	R^2
H_2O_2	Salinity	98.823	<0.0001	0.917
	Water depth	0.740	0.491	
	Salinity x water depth	0.116	0.975	
APX	Salinity	2979.125	< 0.0001	0.997
	Water depth	12.739	0.000	
	Salinity x water depth	1.474	0.252	
CAT	Salinity	42.716	< 0.0001	0.843
	Water depth	0.642	0.539	
	Salinity x water depth	0.213	0.928	
POD	Salinity	73.040	< 0.0001	0.897
	Water depth	0.853	0.444	
	Salinity x water depth	0.071	0.990	
AOX	Salinity	248.320	<0.0001	0.965
	Water depth	1.522	0.245	
	Salinity x water depth	0.107	0.978	
Chl a	Salinity	10.119	0.001	0.566
	Water depth	0.735	0.494	
	Salinity x water depth	0.152	0.960	
Chl b	Salinity	51.969	< 0.0001	0.865
	Water depth	0.794	0.468	
	Salinity x water depth	0.093	0.983	
Chl a + b	Salinity	16.841	<0.0001	0.664
	Water depth	0.643	0.538	
	Salinity x water depth	0.157	0.957	
Car	Salinity	34.682	<0.0001	0.796
	Water depth	0.104	0.902	
	Salinity x water depth	0.151	0.960	
FvFm	Salinity	0.223	0.802	0.025
	Water depth	0.009	0.991	
	Salinity x water depth	0.001	1.000	
Proline	Salinity	152.479	< 0.0001	0.945
	Water depth	1.212	0.321	
	Salinity x water depth	0.089	0.985	

Table 6. Two-way ANOVA showing the effects of salinity, inundation and the interaction of salinity and inundation on the biochemical responses *R. stylosa* seedlings.

Response	**Factor**	***F***	***P***	R^2
H_2O_2	Salinity	7.30	0.005	0.898
	Inundation	62.15	<0.0001	
	Salinity x inundation	4.73	0.009	
APX	Salinity	1058.79	< 0.0001	0.995
	Inundation	454.74	< 0.0001	
	Salinity x inundation	13.46	< 0.0001	
CAT	Salinity	18.86	< 0.0001	0.963
	Inundation	197.15	< 0.0001	
	Salinity x inundation	0.13	0.970	
POD	Salinity	18.43	< 0.0001	0.983
	Inundation	460.06	< 0.0001	
	Salinity x inundation	1.54	0.234	
AOX	Salinity	78.22	<0.0001	0.985
	Inundation	526.04	<0.0001	
	Salinity x inundation	2.68	0.065	
Chl a	Salinity	41.14	< 0.0001	0.954
	Inundation	120.79	< 0.0001	
	Salinity x inundation	1.39	0.280	
Chl b	Salinity	25.17	< 0.0001	0.935
	Inundation	96.36	< 0.0001	
	Salinity x inundation	0.96	0.455	
Chl a + b	Salinity	38.25	< 0.0001	0.923
	Inundation	119.97	< 0.0001	
	Salinity x inundation	1.24	0.328	
Car	Salinity	48.09	<0.0001	0.889
	Inundation	58.03	<0.0001	
	Salinity x inundation	0.97	0.450	
FvFm	Salinity	3.40	0.056	0.761
	Inundation	23.370	<0.0001	
	Salinity x inundation	0.944	0.461	

Table 7. T-test results comparing the means of field samples taken at the inundated and not inundated areas.

Dependent variables	Group	Mean	SD	*P*
H_2O_2	Inundated	45.20	5.69	<0.0001
	Not inundated	63.46	7.27	
Chl a + b	Inundated	786.15	57.50	0.013
	Not inundated	850.24	37.94	
Car	Inundated	183.32	15.00	0.055
	Not inundated	197.81	15.72	
AOX	Inundated	317.17	53.36	<0.0001
	Not inundated	596.42	79.54	

FIGURES

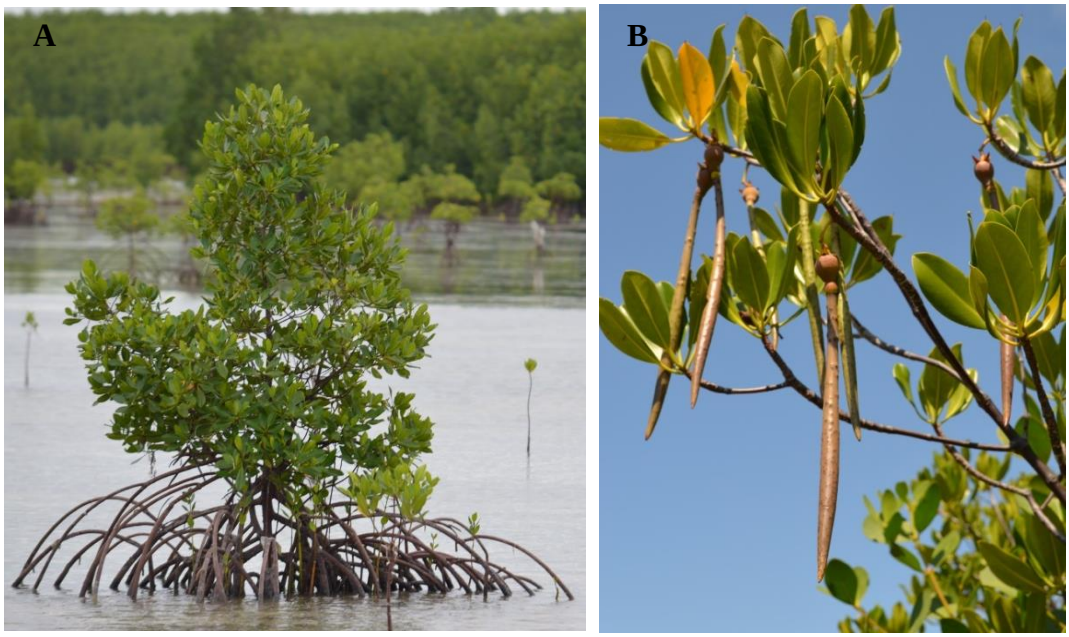


Figure 1. Photo of the studied mangrove species – the *Rhizophora stylosa*. a.) Mature tree of *R. stylosa* and b.) propagules attached to the parent tree.

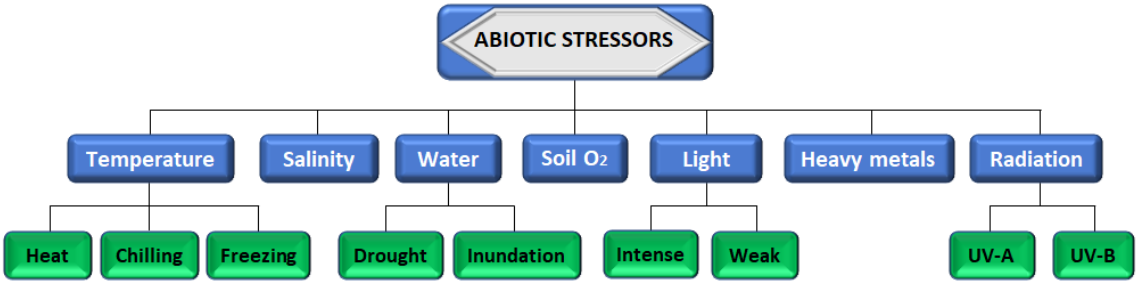


Figure 2. Diagram of environmental and abiotic stressors that continuously influence the mangrove ecosystem.

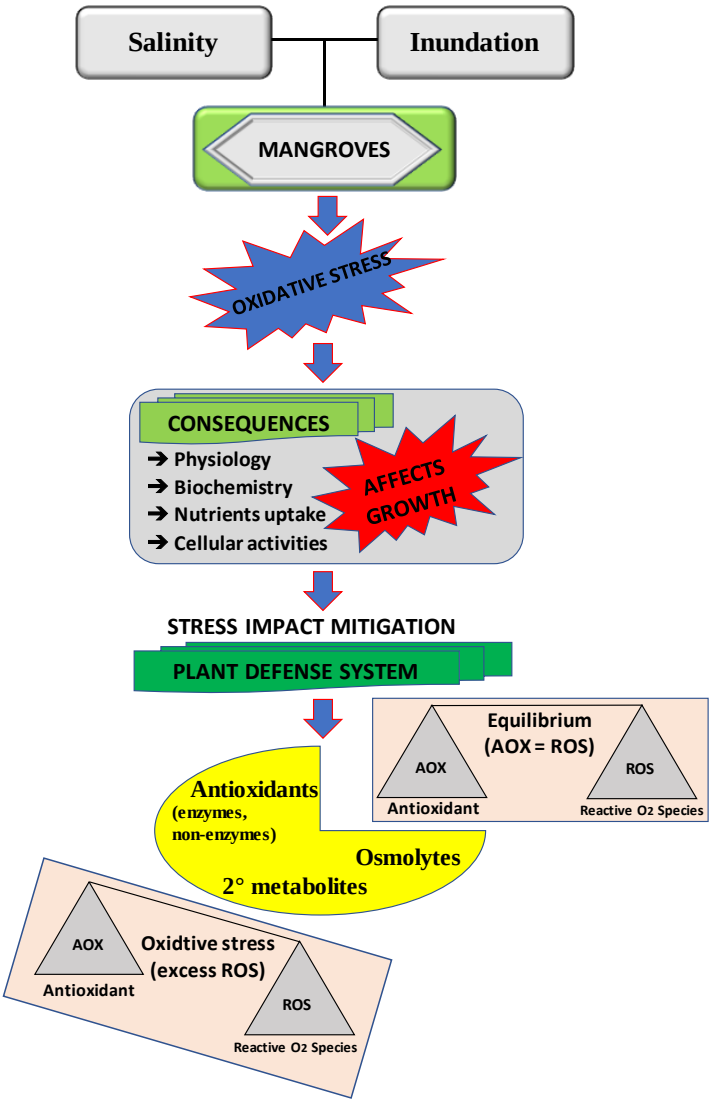


Figure 3. Schematic diagram showing the role of salinity and inundation in the induction of reactive oxygen species and activation of the antioxidant enzymes defense system.

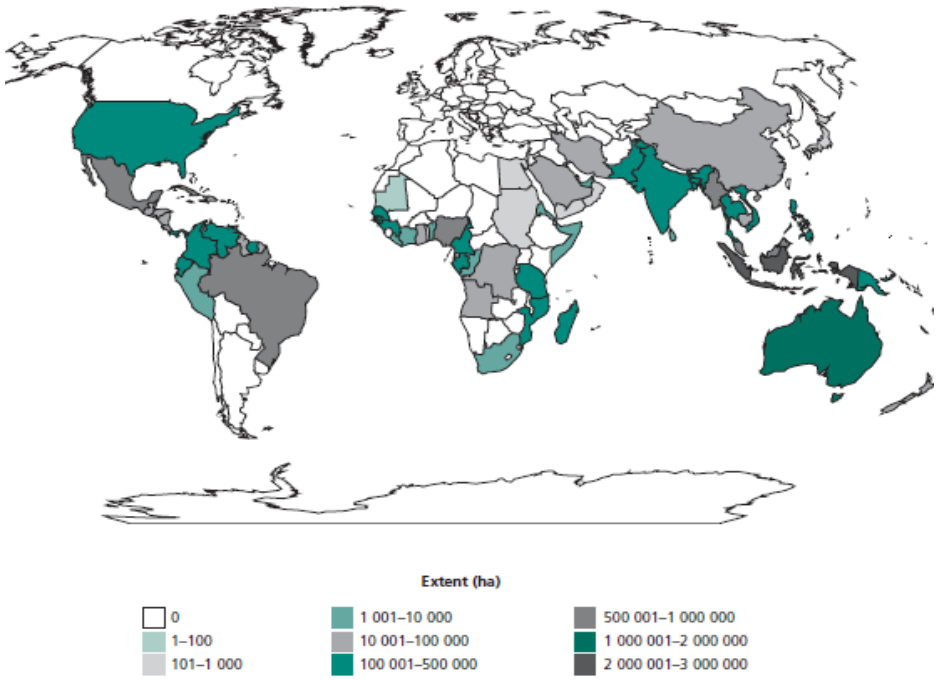


Figure 4. Global distribution of mangroves (from FAO 2007).

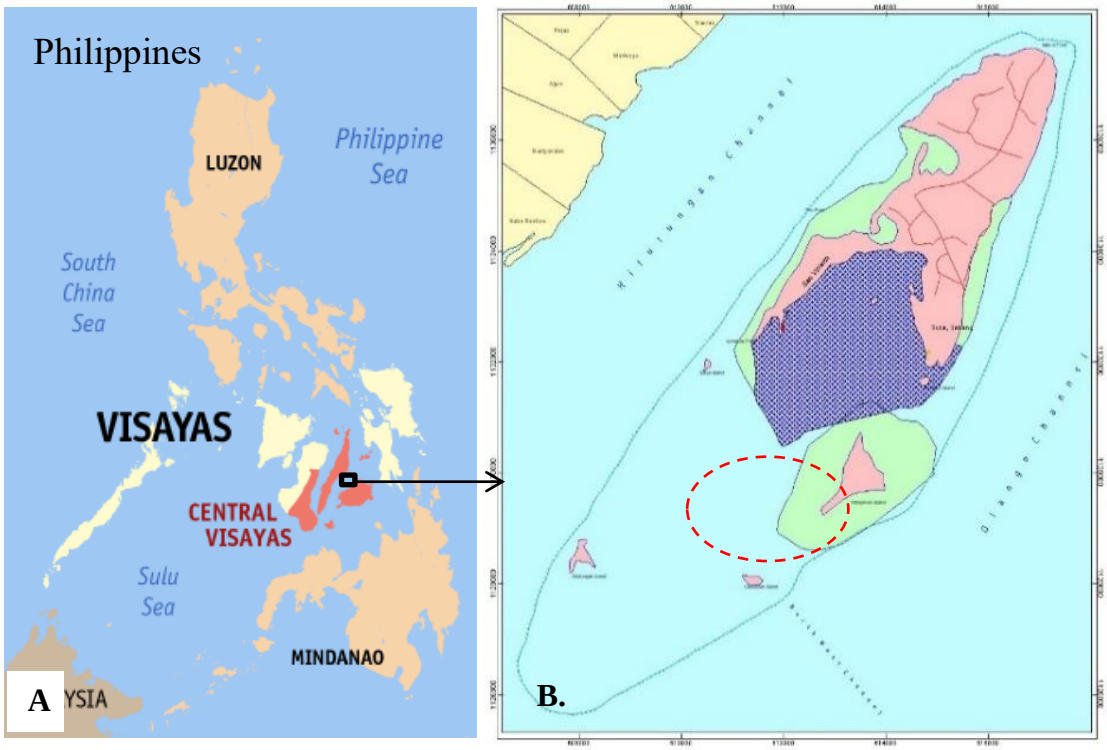


Figure 5. Map of the study sites and source of propagules showing the a) Philippines and b) Olango Island, Cebu.

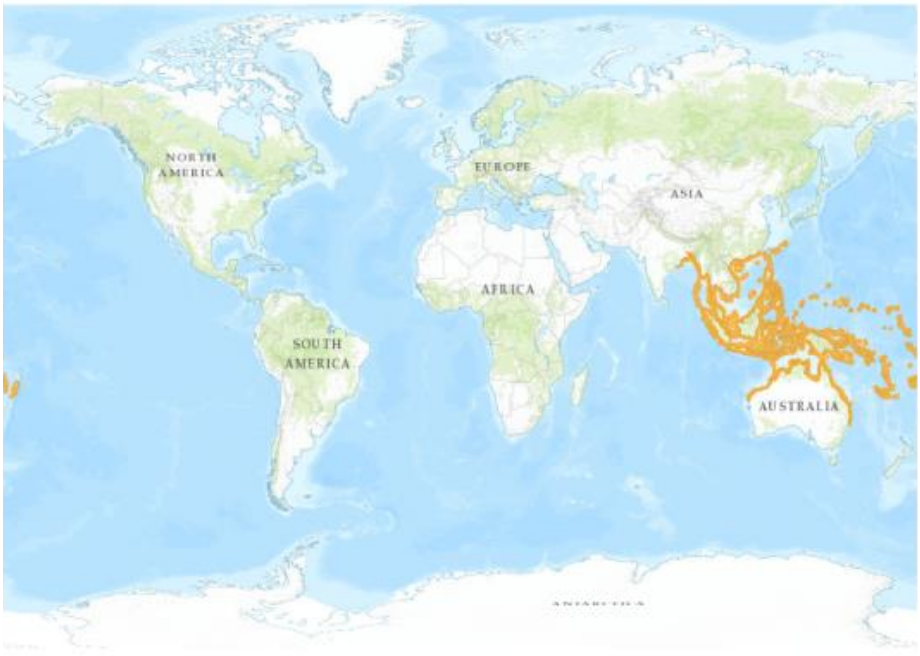
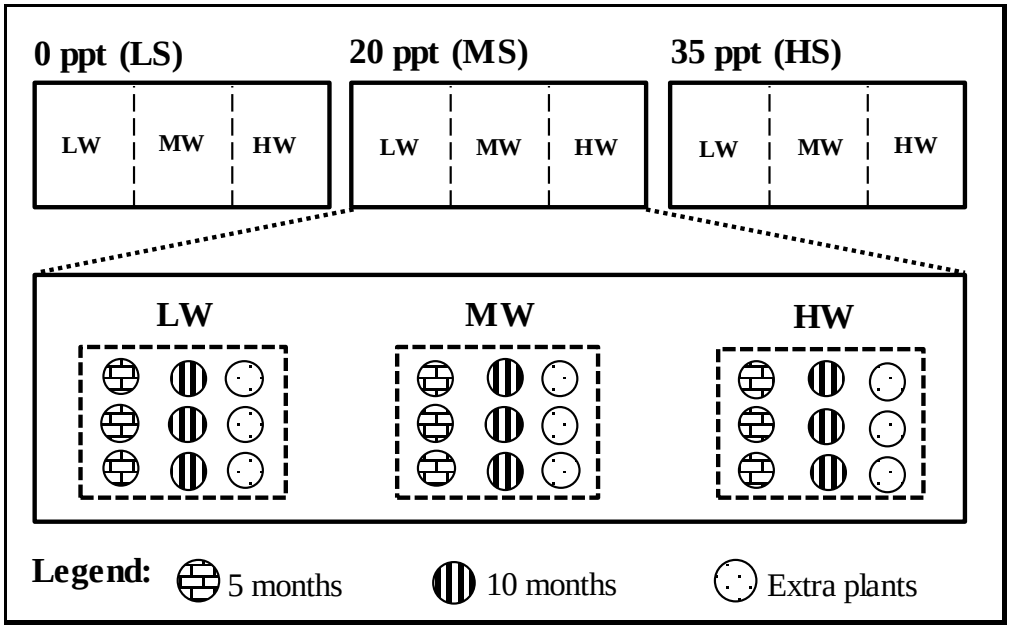


Figure 6. Global geographic distribution of *R. stylosa* mangrove species (Ellison et al. 2010).

Figure 7. Photo of a) collected *R. stylosa* propagules (in bucket) and acclimatized for two weeks and the seedlings bags filled with sand-vermiculite mixture in 4:1 ratio and b) seedlings in aquarium tank irrigated with different water depths.

A



B

Figure 8. Experiment 1 set up – effects of salinity and water depth. a) Diagram of the experimental design, and b) the cultured seedlings in three salinity treatment: 0 ppt - low salinity (LS), 20 ppt - moderate salinity (MS), 35 ppt - high salinity (HS); irrigated with three water depths per treatment: 0-3 cm - low water (LW), 10-13 cm - mid-water (MW), 30-33 cm - high water (HW).

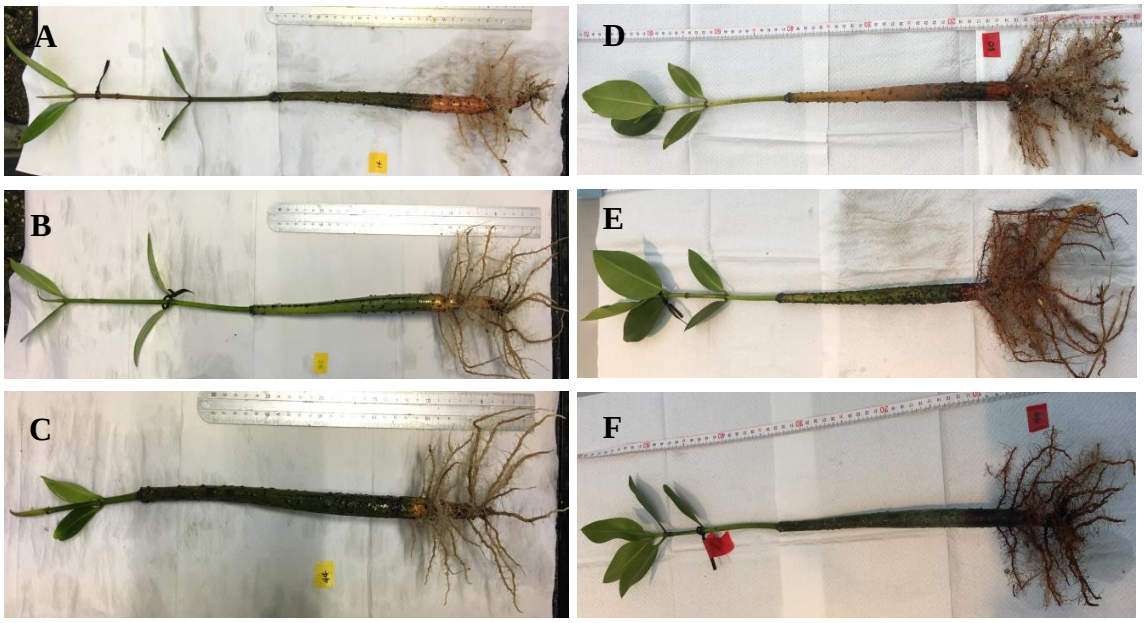


Figure 9. Photo of the harvested cultured seedlings at a) 5 months and at 10 months. (A to C – 0, 20 and 35 ppt respectively; (D to F – 0, 20 and 35 ppt respectively)

Figure 10. Photo of the cultured seedlings in three salinity treatment used for Experiment 2. The aquarium tanks were flooded with 3 cm water depth (similar to LW in Experiment 1) until 15 months.

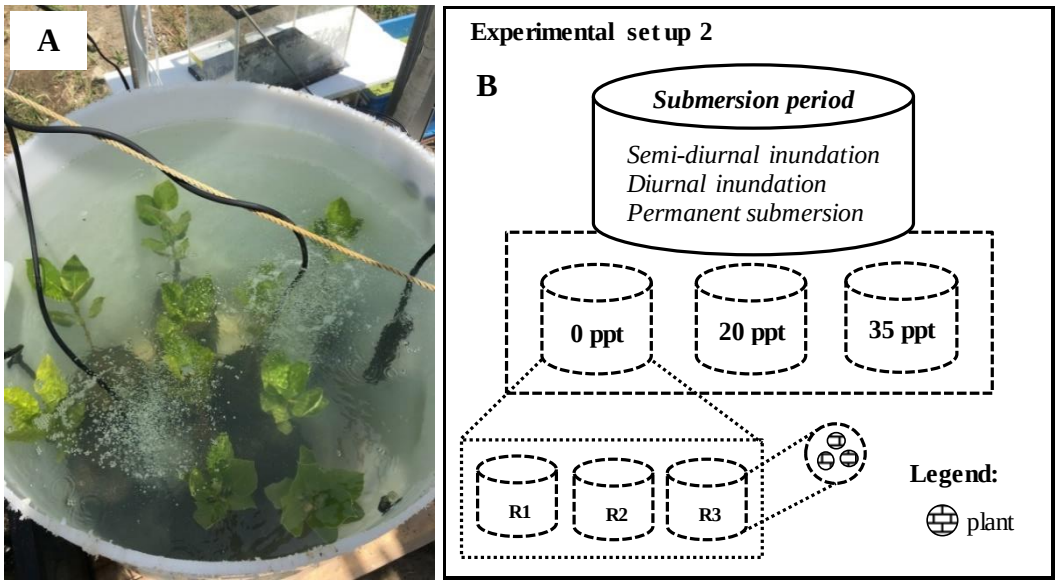


Figure 11. Experimental 2 design - effects of salinity and inundation regimes. a) Photo of the inundated seedlings during the experiment and b) diagram of the experimental design.

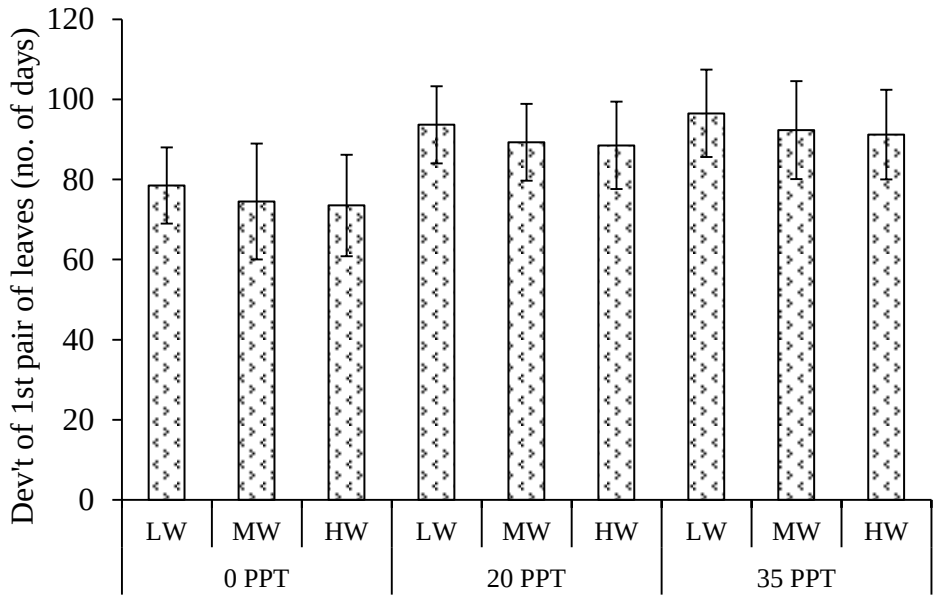


Figure 12. Period of development of first leaves of the cultured seedlings in Experiment 1 (no. of days ±s.d.).

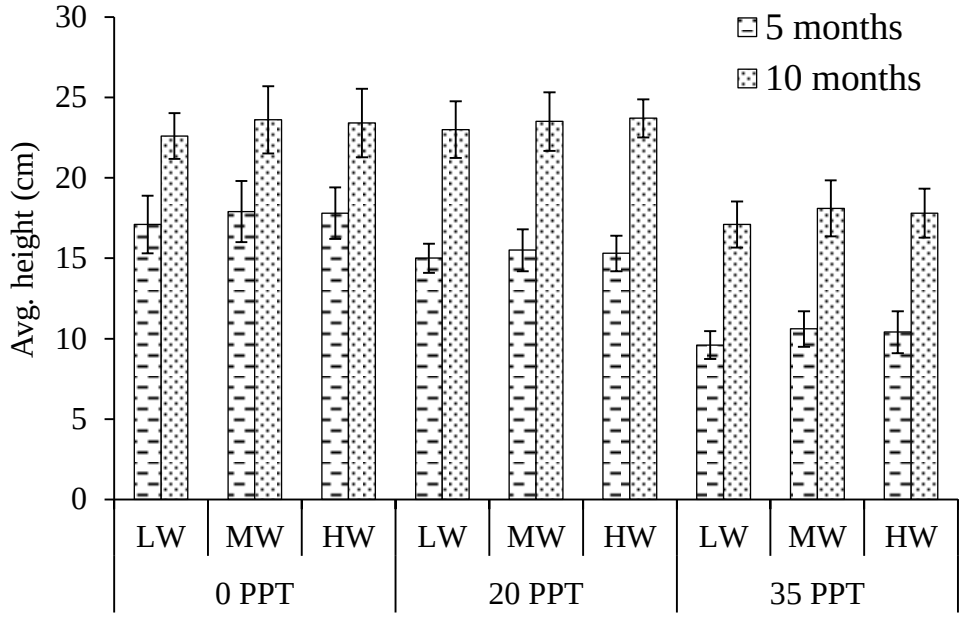


Figure 13. Average height of the cultured seedlings measured at 5 and 10 months culture period (±s.d.).

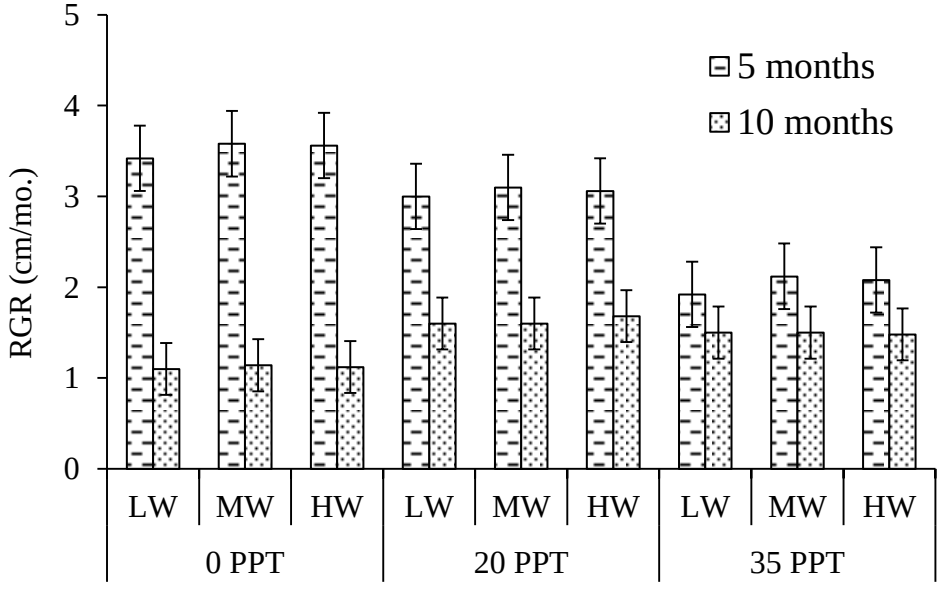


Figure 14. Relative growth rate (RGR) of the cultured seedlings measured at 5 and 10 months culture period (±s.d.).

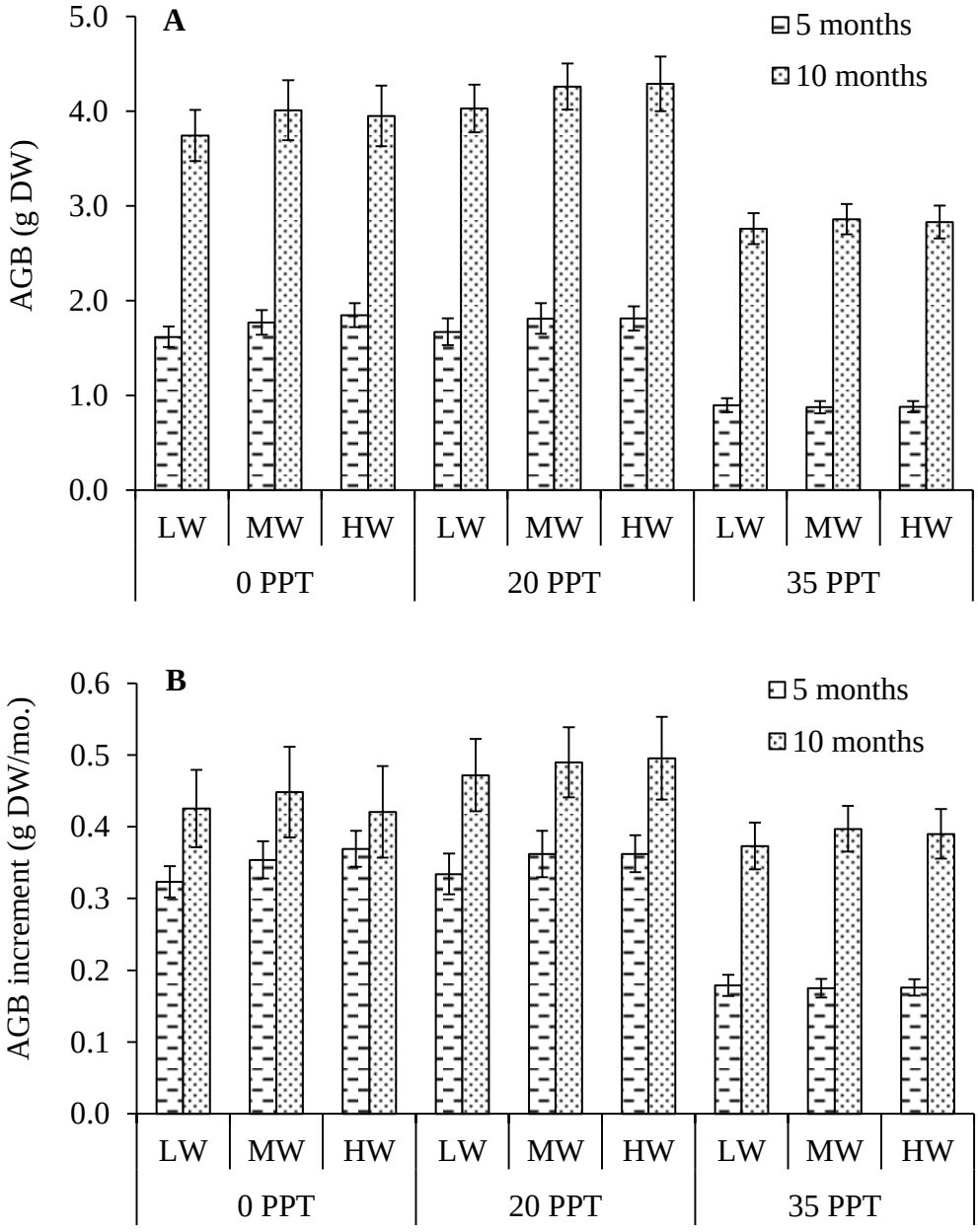


Figure 15. A) Above-ground biomass (AGB) and B) AGB increment of the cultured seedlings harvested at 5 and 10 months (±s.d.).

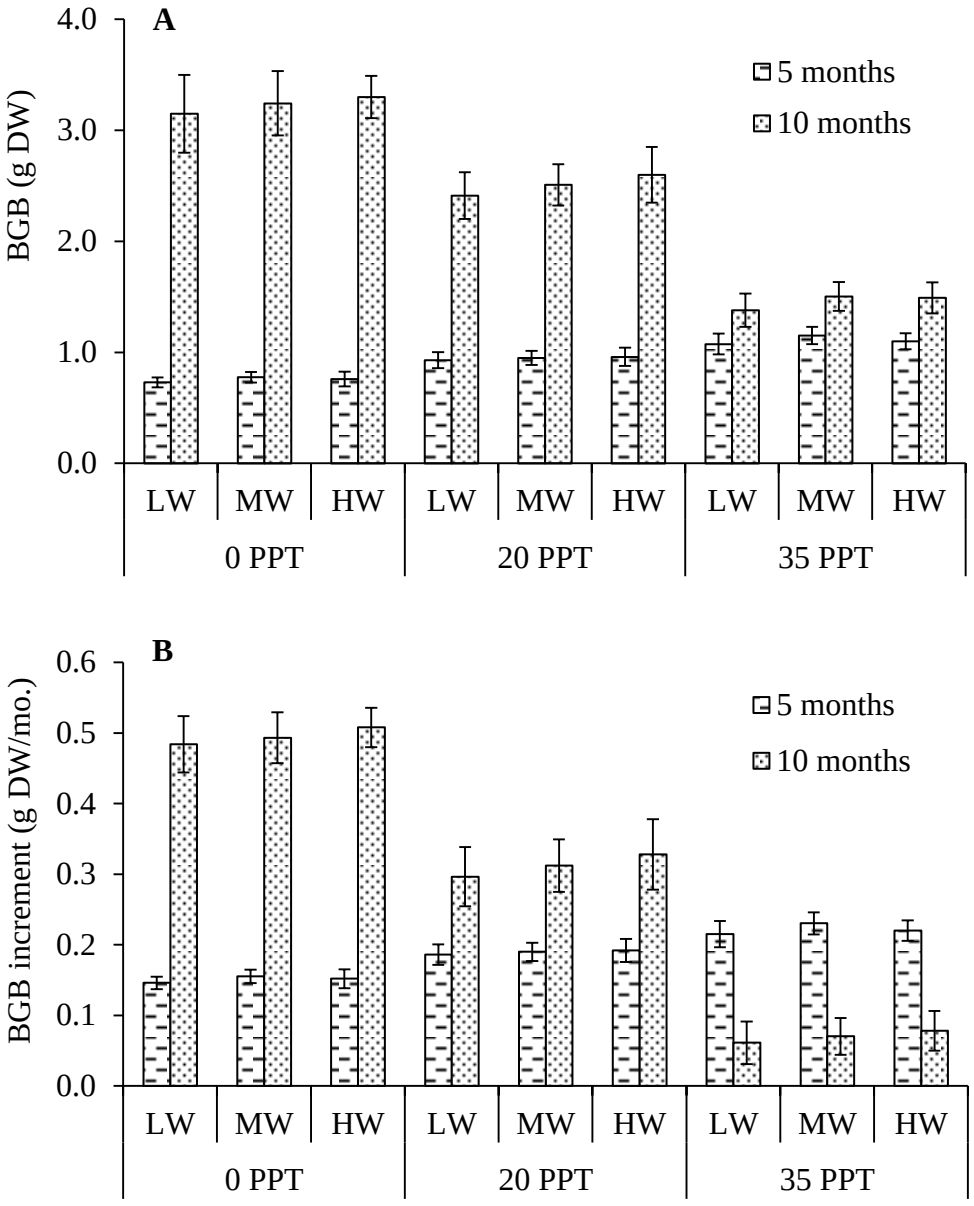


Figure 16. A) Below-ground biomass (BGB) and B) BGB increment of the cultured seedlings harvested at 5 and 10 months (±s.d.).

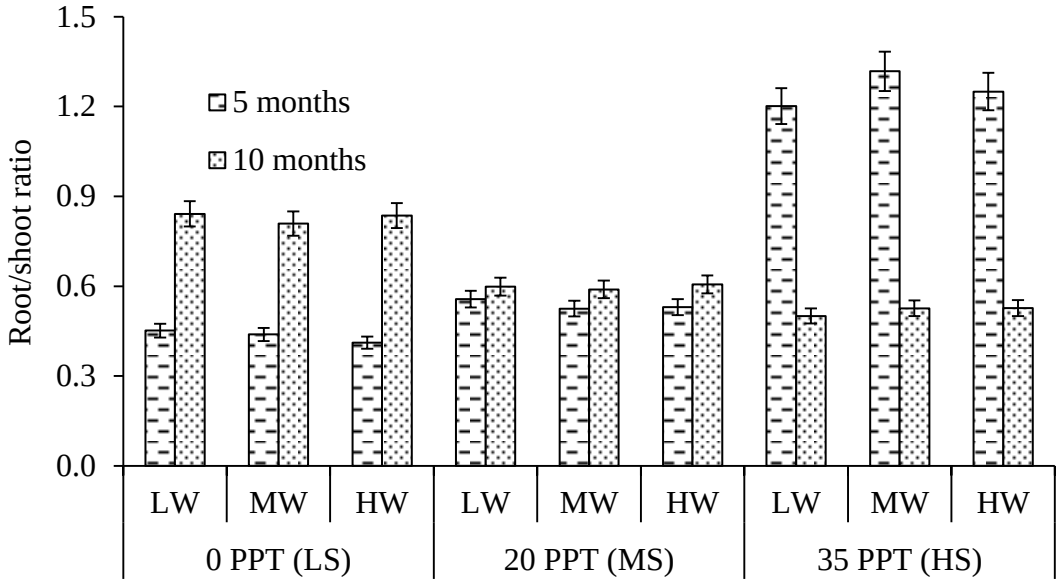


Figure 17. Root to shoot ratio of the cultured seedlings harvested at 5 and 10 months (±s.d.).

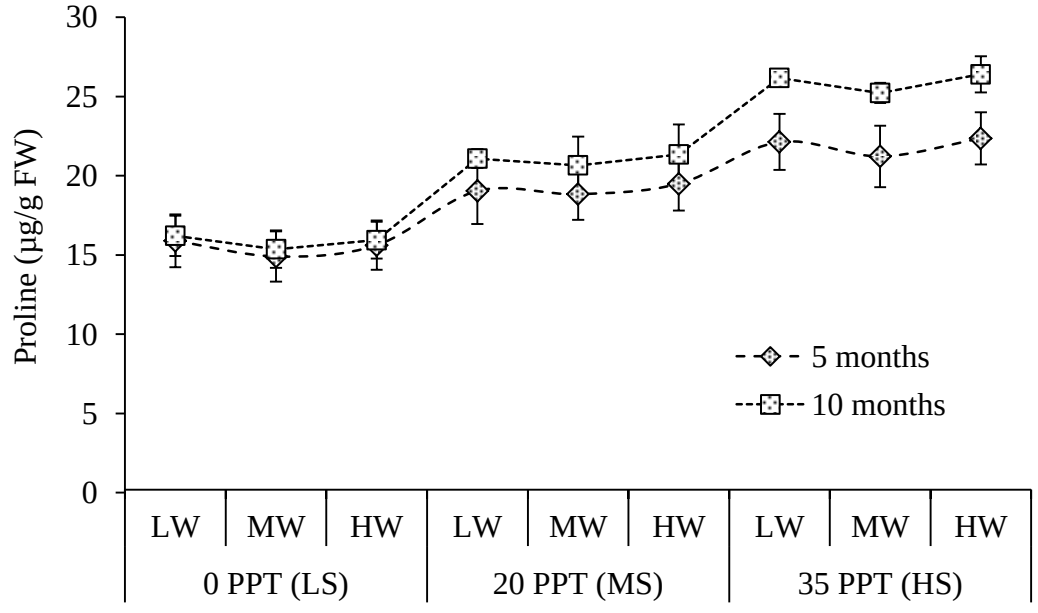


Figure 18. Proline content in the leaves of *R. stylosa* measured at 5 and 10 months (±s.d.).

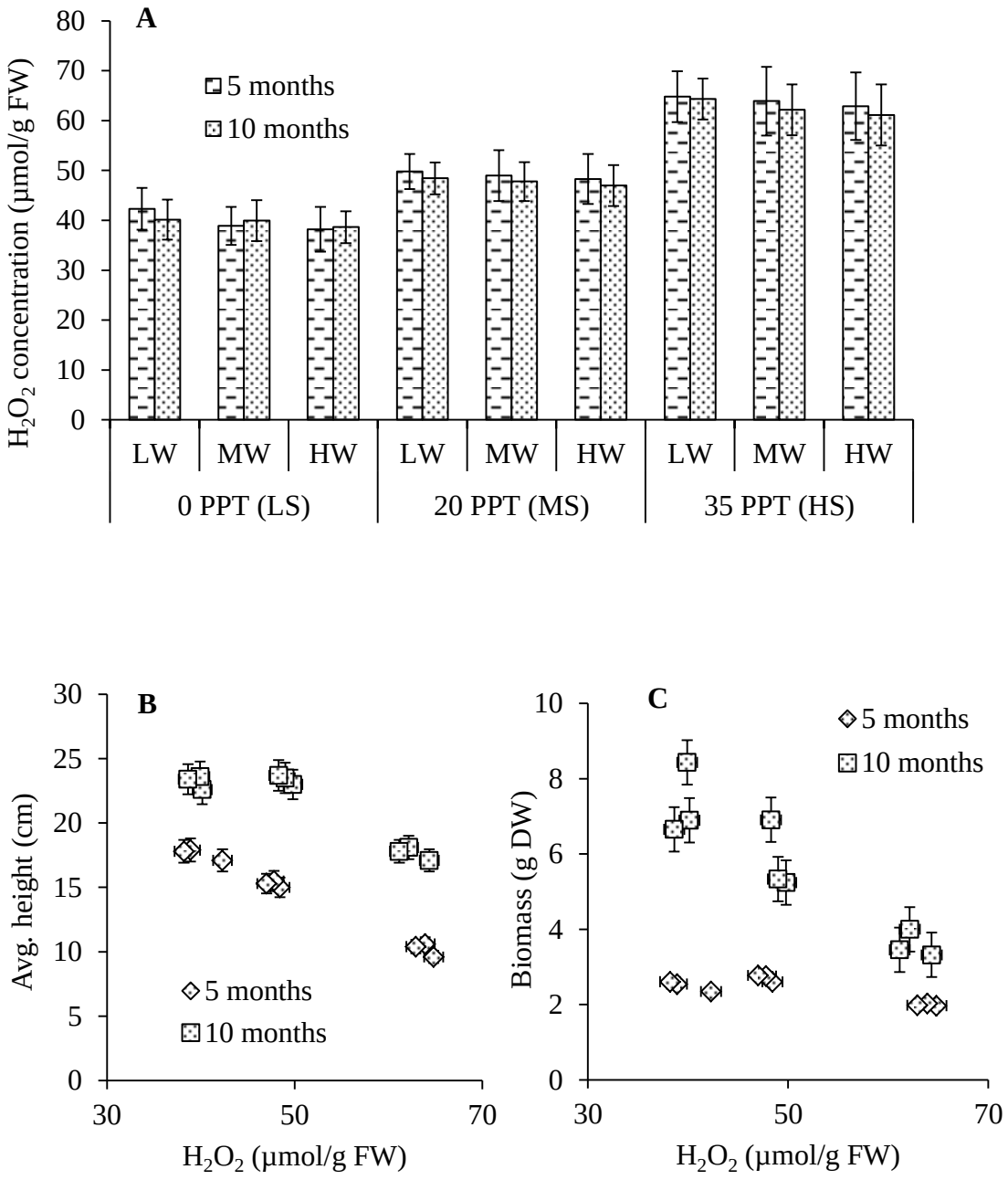


Figure 19. A) The H_2O_2 concentration in the leaves of *R. stylosa* measured at 5 and 10 months period and the relationship of H_2O_2 with growth and biomass production (±s.d.).

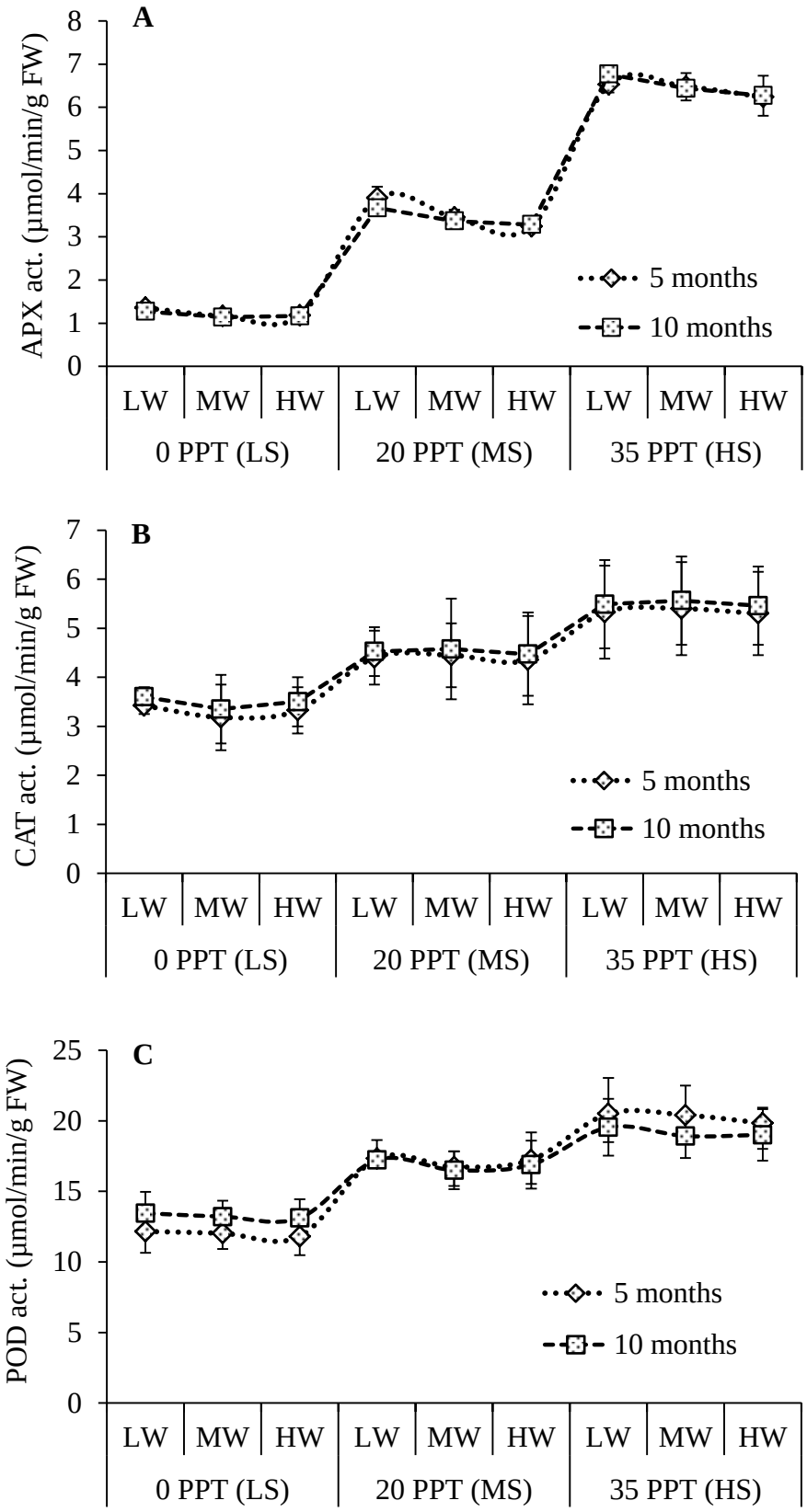


Figure 20. Activities of the antioxidant enzymes (APX, CAT, POD) measured for the 5 and 10 months cultured seedlings (±s.d.).

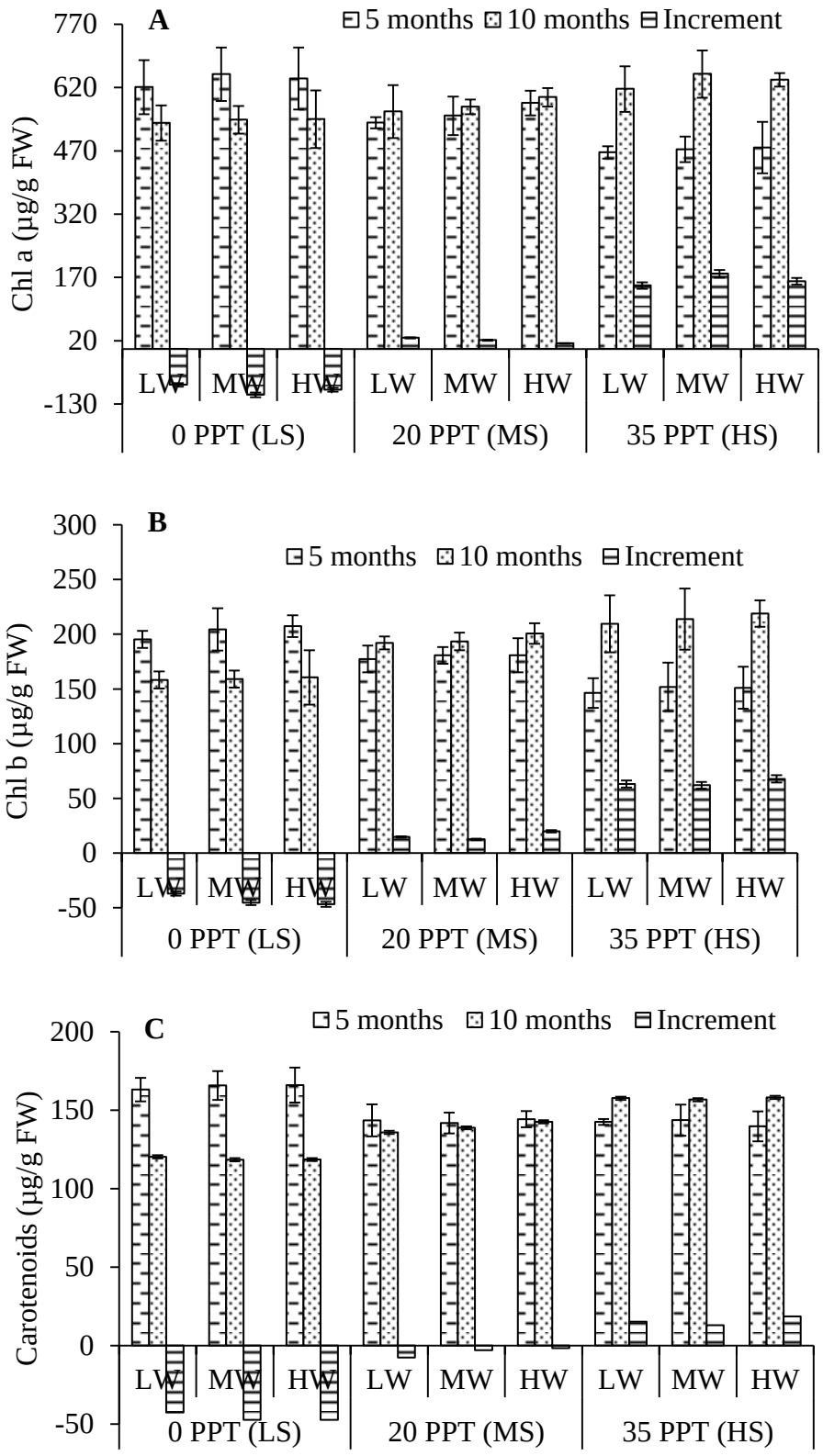


Figure 21. Pigments (chl a, b) and carotenoid content in the leaves of *R. stylosa* measured at 5 and 10 months cultured seedlings (±s.d.).

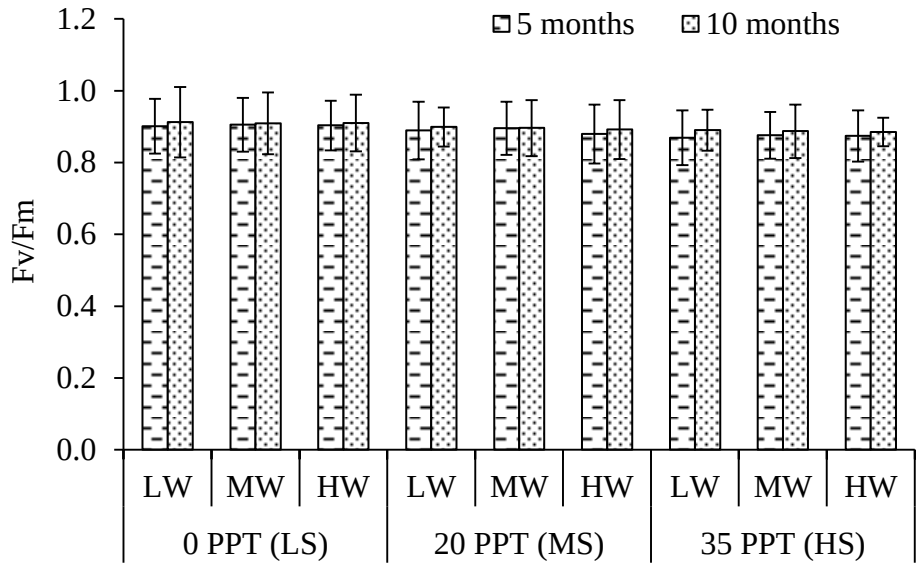


Figure 22. Fv/Fm ratio measured at 5 and 10 months cultured seedlings (±s.d.).

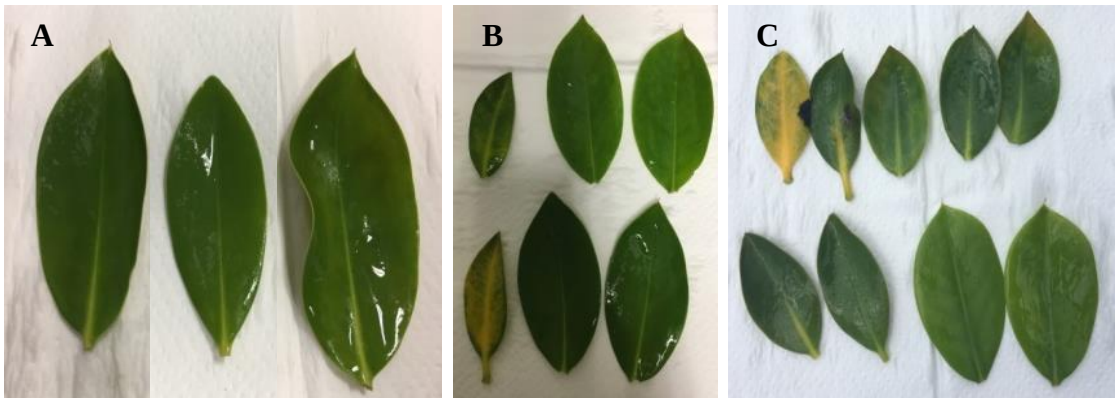


Figure 23. Photo of the leaves of *R. stylosa* subjected to permanent submersion (A – 0 ppt, B – 20 ppt, C – 35 ppt). The seedlings in 20 and 35 ppt exhibited sublethal damage as shown by the yellowing of the leaves.

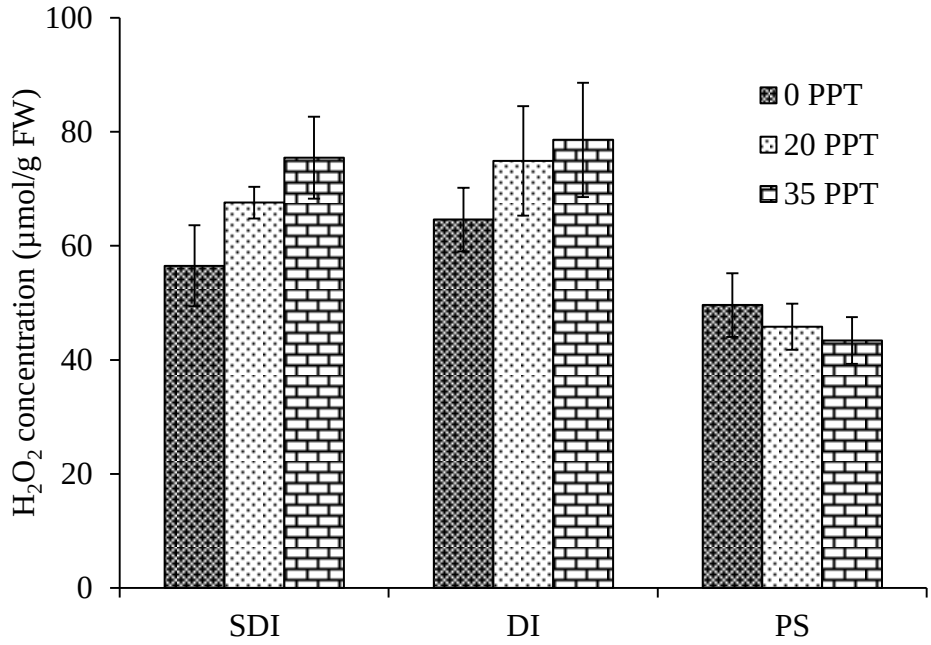


Figure 24. Variation of H_2O_2 concentration in the leaves of cultured *R. stylosa* subjected to semi-diurnal inundation (SDI), diurnal inundation (DI) and permanent submersion (±s.d.)

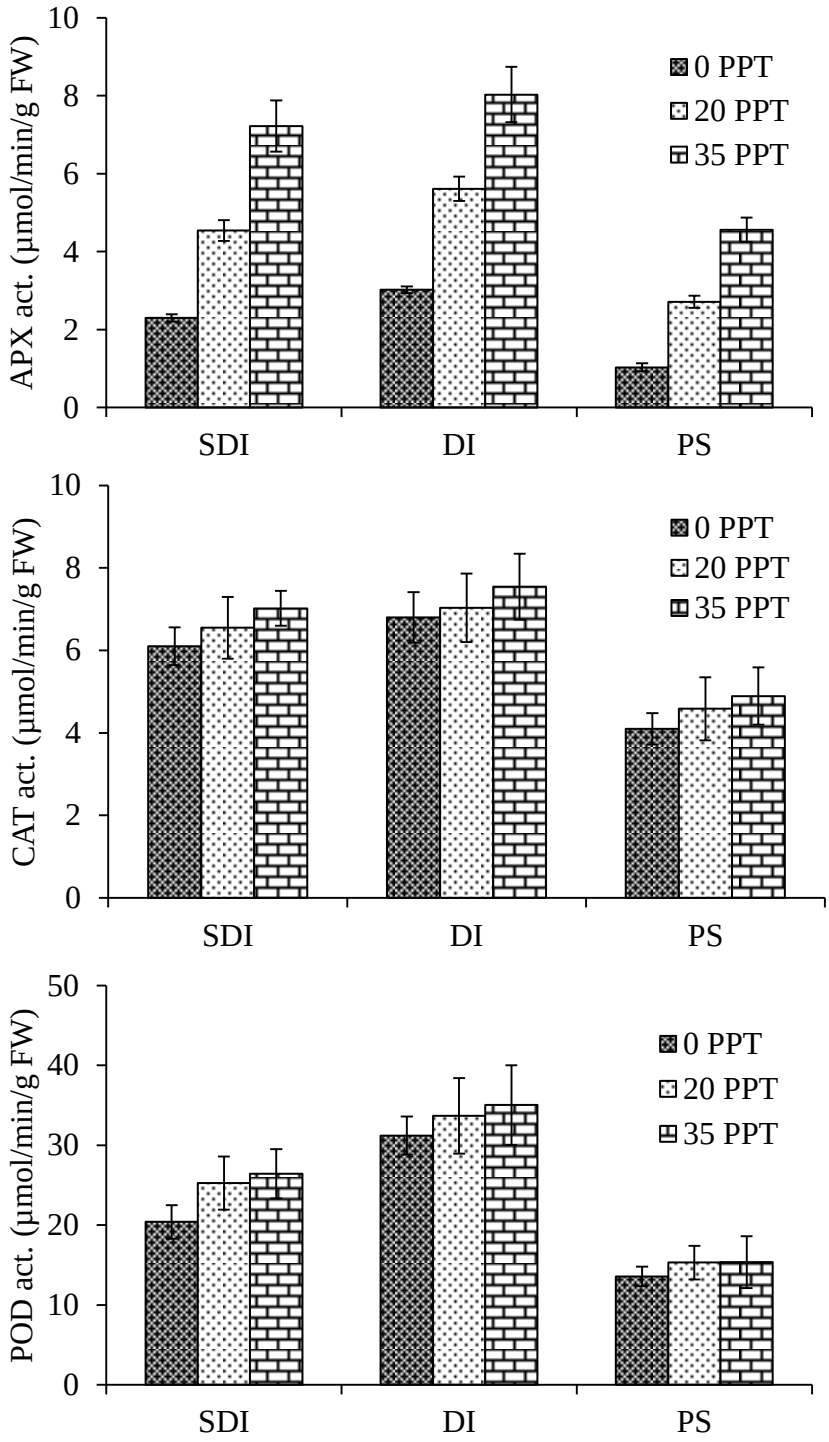


Figure 25. Variations in the activities of antioxidant enzymes (APX, CAT, POD) as a respond to different inundation regimes (±s.d.).

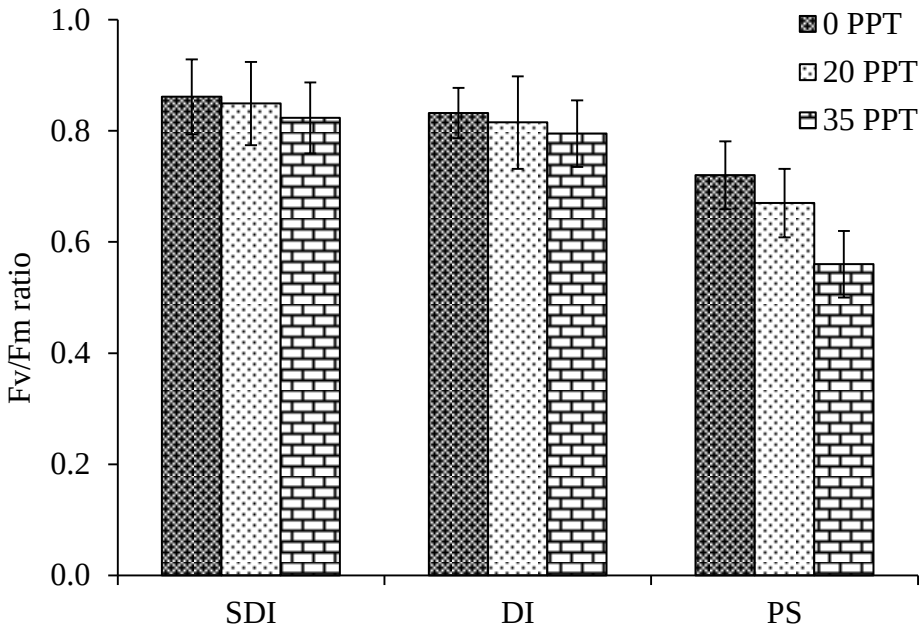


Figure 26. Variation of Fv/Fm ratio under different inundation hydroperiod regimes (±s.d.).

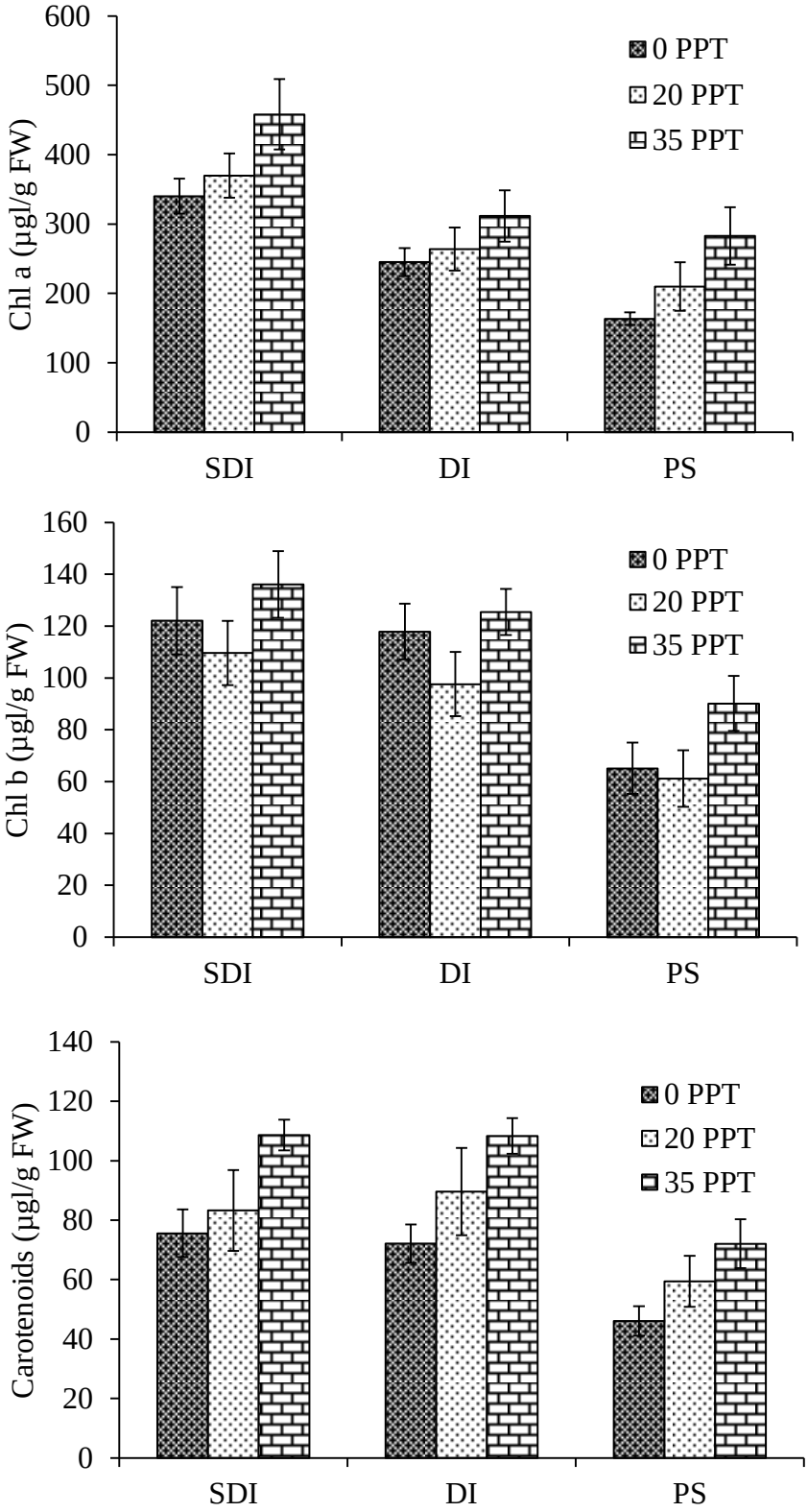


Figure 27. Variation of pigments (chl a and b) and carotenoids under different inundation hydroperiod regimes (±s.d.).

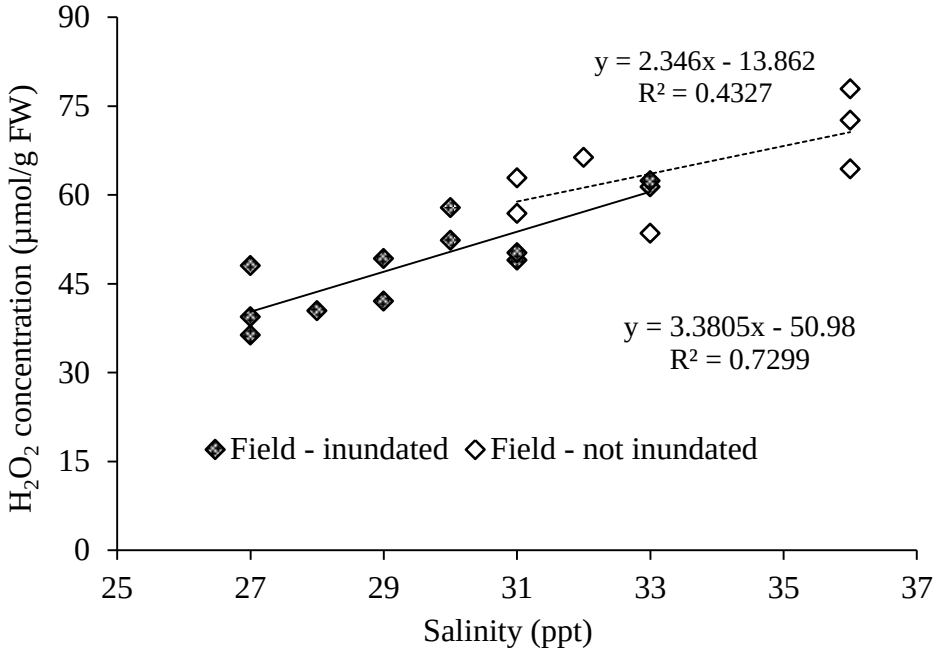


Figure 28. Effect of salinity on H_2O_2 concentration for *R. stylosa* field samples.

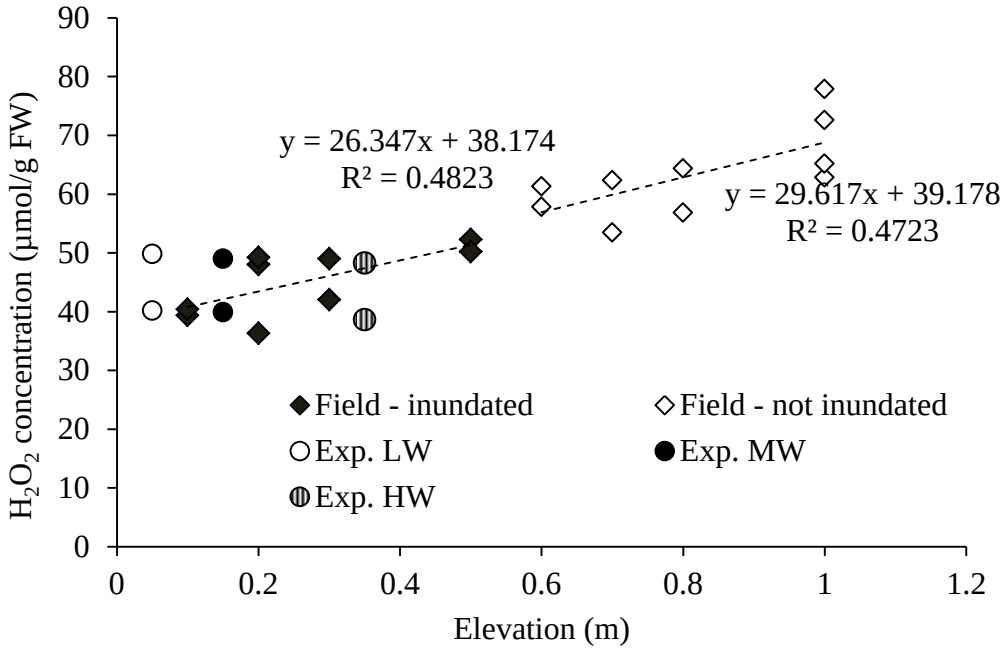


Figure 29. Effects of elevation on the H_2O_2 production in the greenhouse experiment and field conditions.

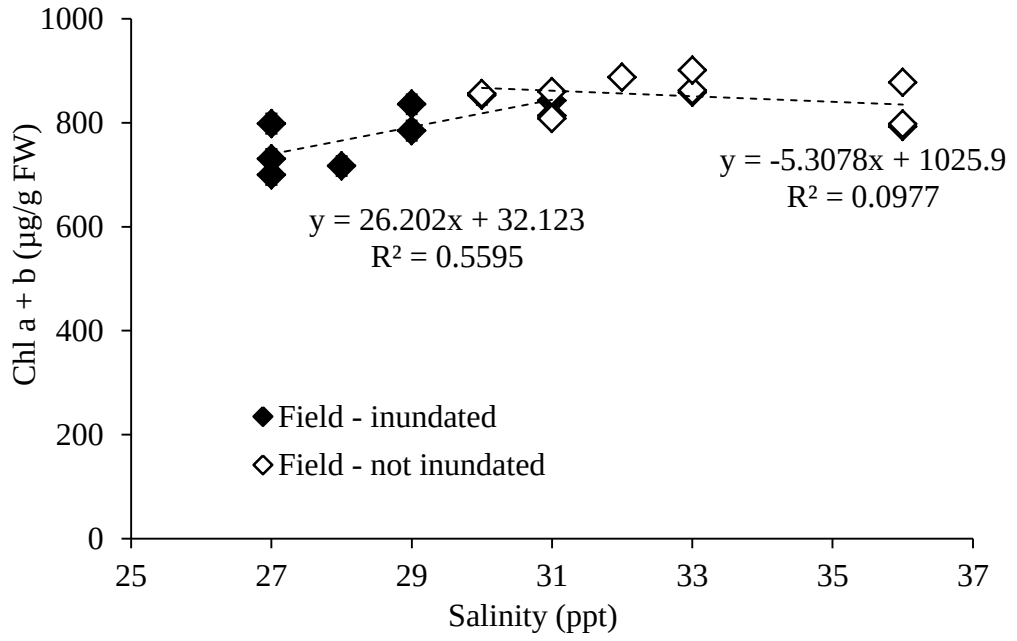


Figure 30. Effects of salinity on Chl a and b of greenhouse experimental plants and field samples.

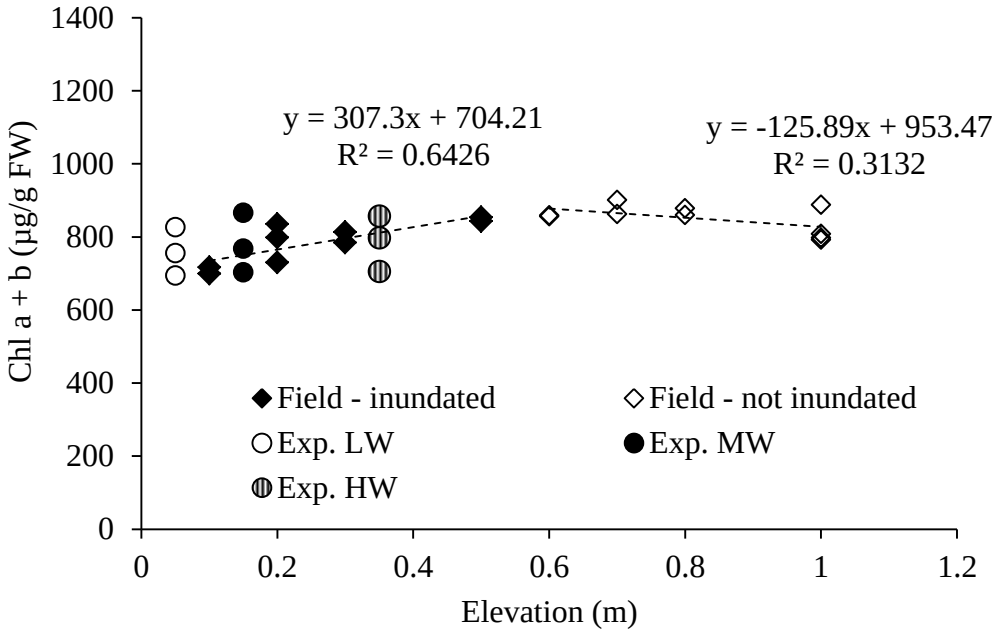


Figure 31. Effects of elevation on Chl a and b in the greenhouse experiment and field samples.

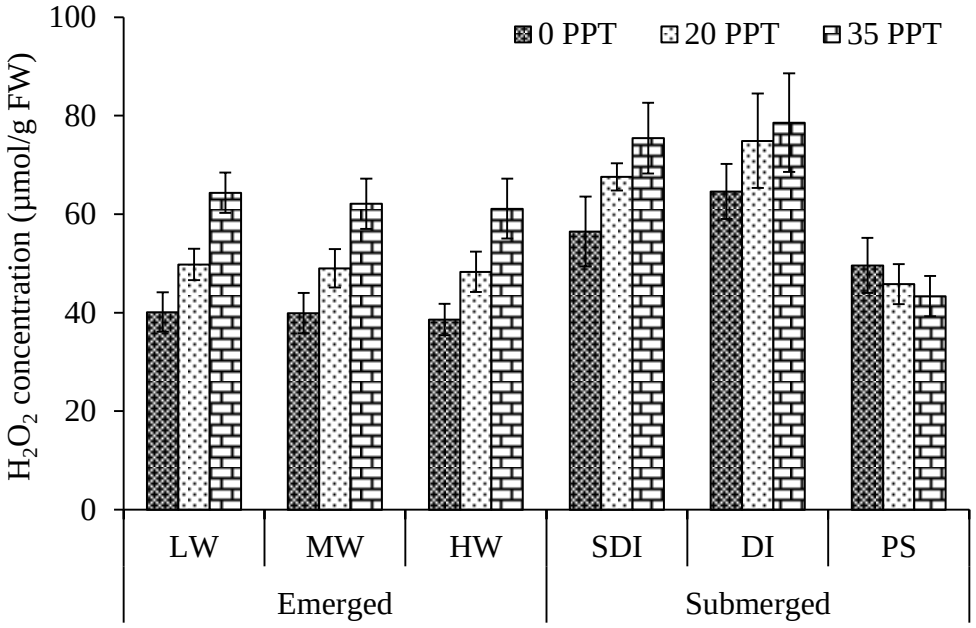


Figure 32. Comparison of H_2O_2 concentrarions in the leaves of *R. stylosa* in emerged and inundated conditions (±s.d.).

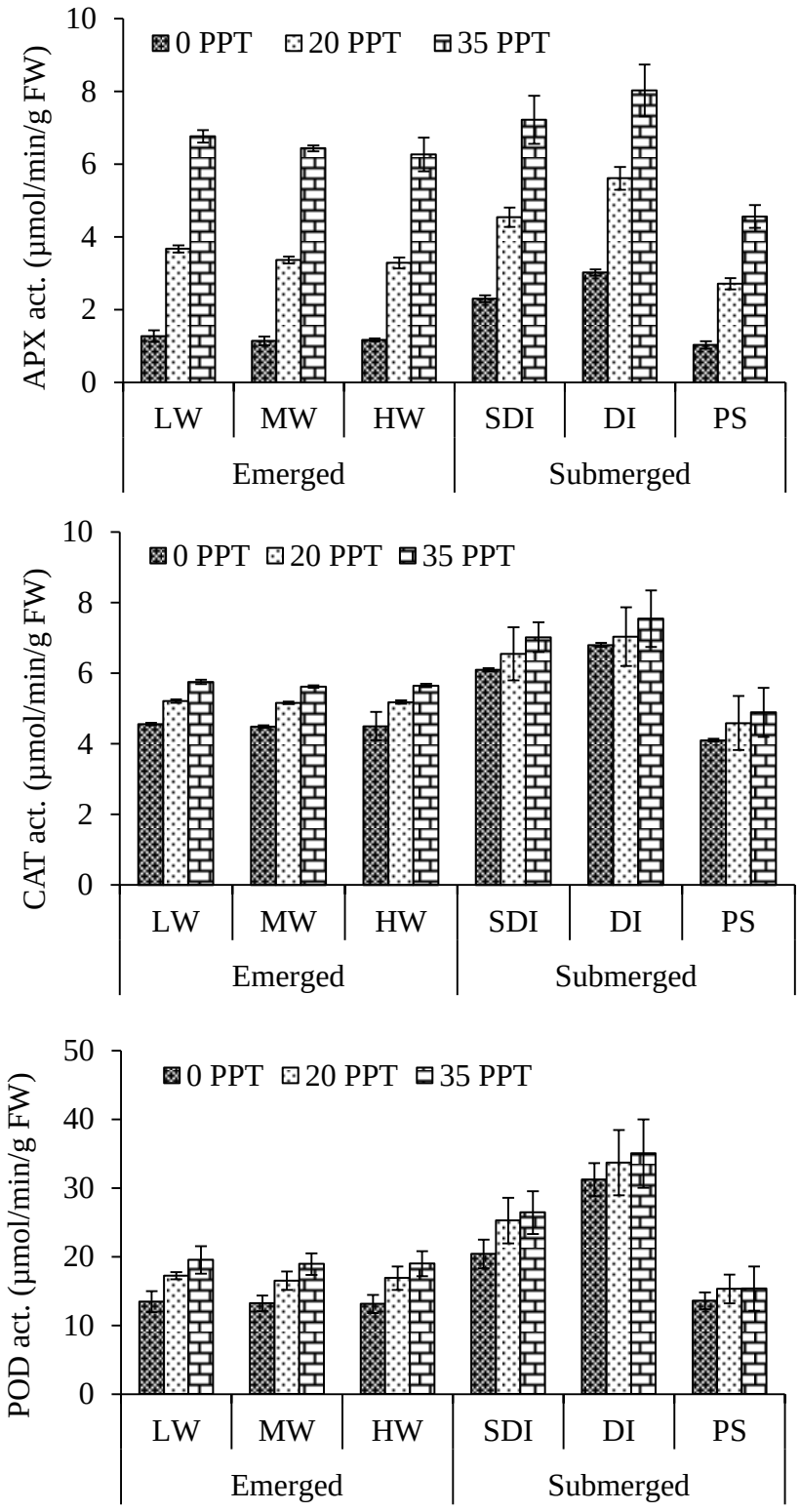


Figure 33. Comparison of antioxidant enzyme activities in the leaves of *R. stylosa* in emerged and submerged conditions (±s.d.).

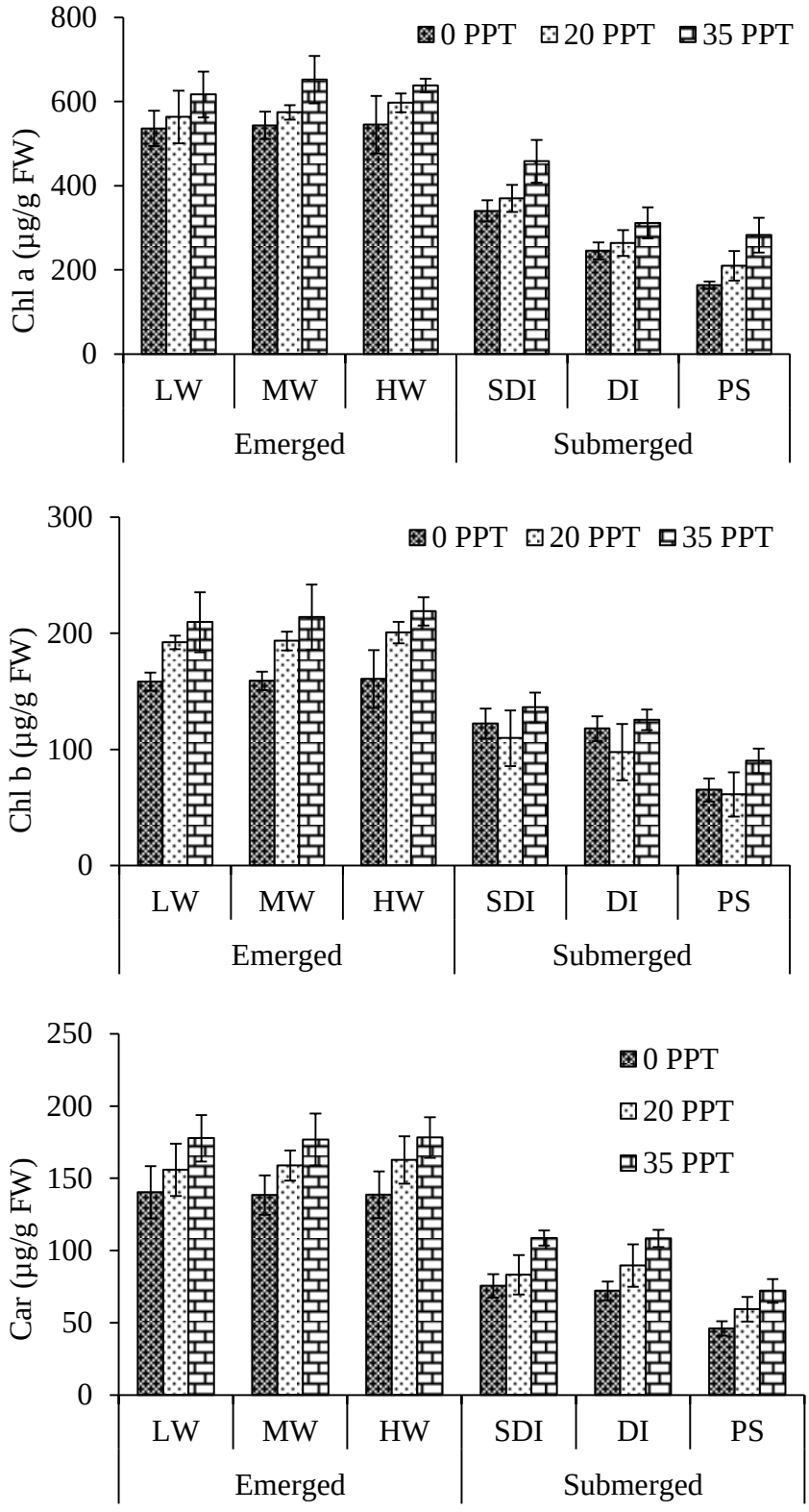


Figure 34. Comparison of pigments (Chl a and b) and carotenoids of *R. stylosa* in emerged and submerged conditions (±s.d.).

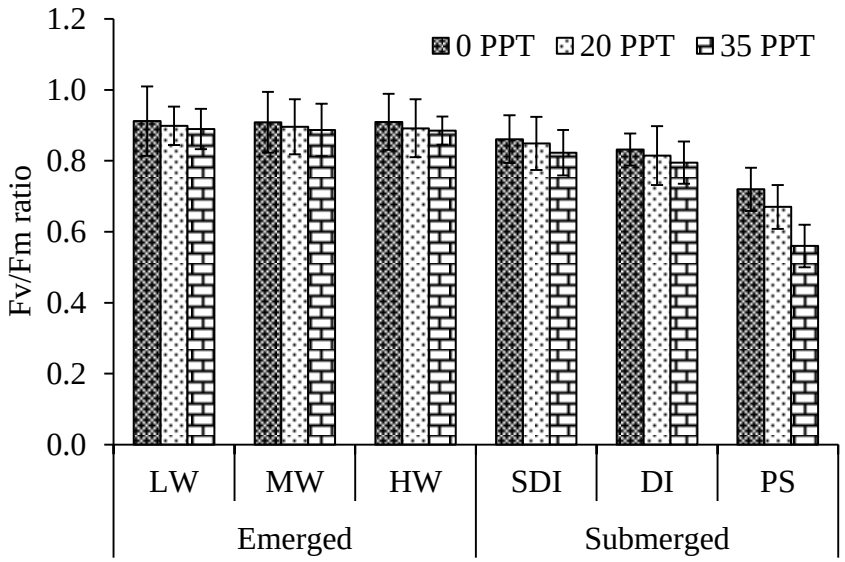


Figure 35. Variation of the Fv/Fm ratio in emerged and submerged conditions (±s.d.).

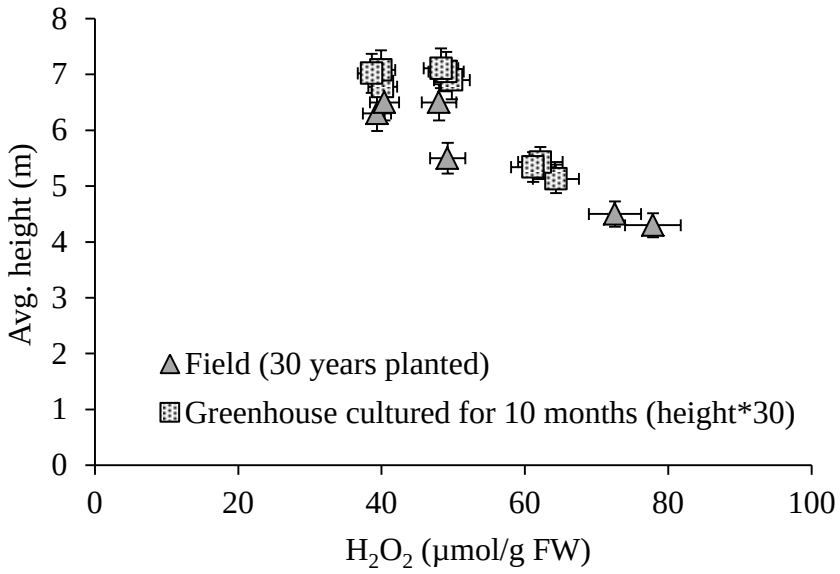


Figure 36. Relationship of H_2O_2 with growth and average height of greenhouse cultured *R. stylosa* and in the field condition.

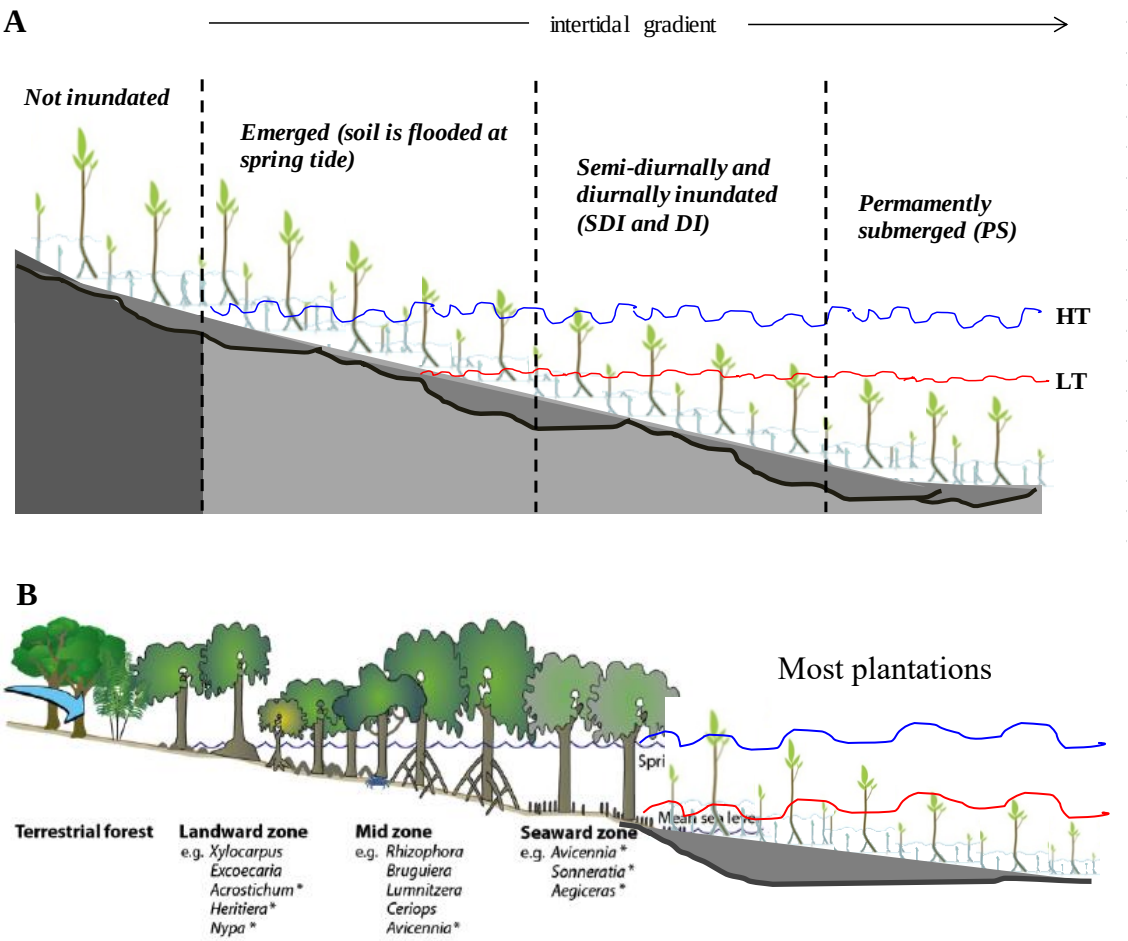


Figure 37. a) The niche-width preference of *R. stylosa* across the intertidal gradient. a) The species of *R. stylosa* showed a relatively lower stress when in emerged condition at the inundated areas. b) Most mangrove plantations that suffered high mortality were established at the lower intertidal areas which frequently inundated.

APPENDEX

Appendix A. Aerial photo of the a) planted mangrove forest and b) natural mangrove forest.

Appendix B. Photo of the greenhouse where the experiments were conducted.