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RESEARCH ARTICLE

Oxidative stress, leaf photosynthetic capacity and dry matter content in young mangrove plant *Rhizophora mucronata* Lam. under prolonged submergence and soil water stress

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Abstract Young plants of *Rhizophora mucronata* Lam. were tested for oxidative stress, photosynthetic capacity and dry matter accumulation under two abiotic stress conditions; prolonged submergence and soil water stress. The experiment of prolonged submergence was performed in field conditions with two treatment levels; 50% inundation (control) and 100% inundation levels. The experiment of soil water stress was conducted in a plant-house with four treatment levels, 100% water holding capacity (WHC) (control), 50% WHC, 25% WHC and high salinity (> 35 psu). The experimentation period was 18 months. According to the results, antioxidant activity was increased in the 100% inundation level in field conditions and in the 25% WHC, 50% WHC and high salinity levels in plant-house conditions. However, decreased radical scavenging capacity reflected by low 2,2-diphenyl-1-picrylhydrazyl (DPPH) and high IC_{50} values were only observed in the 25% and 50% WHCs. Plant cell membranes were highly damaged in the 25%, 50% WHCs and high salinity level and a significant decrease in photosynthetic capacity (~ 90% reduction) and in dry matter content of *Rhizophora* plants were also observed in the same treatment levels. It was recorded that a higher proportion of dry matter is allocated to the root system under the 100% inundation level and it may be an adaptation to keep up the standing stability. Although, the antioxidant and scavenging capacities of young *Rhizophora* plants have increased under abiotic stress conditions, oxidative stress and its associated impacts on leaf photosynthetic capacity and dry weight contents were unavoidable under persistence of the stress.

Keywords Mangroves · Reactive oxygen species · Antioxidants · Scavenging capacity · Dysfunction · Survivorship

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Introduction

Due to poor mangrove planting practices, mangrove seedlings are often planted out of the natural growing boundary i.e., the intertidal zone, area between low and high tide marks (Field 1998; IUCN 2009; Kodikara et al. 2017). Mangrove seedlings which are planted beyond the intertidal range (in the supra-littoral zone) suffer from soil water deficit as the soil water content is below the field capacity, particularly during the dry season of the dry, arid and intermediate climate zones in Sri Lanka (pers. obs.). Seedlings which are planted more out into the lagoon (in the infra-littoral zone) are subjected to permanent flooding (prolonged submergence) (Field 1998) causing severe

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stress to mangrove seedlings as they are naturally adapted to periodic inundation by tidal water (Hoppe-Speer et al. 2011) rather than to dried soil substrate or to a permanent flooding. In case of prolonged exposure to environmental stresses, production of free-radicals i.e., highly reactive harmful derivatives of oxygen or nitrogen in excess is a common biochemical response (Smirnoff 1993; Bartosz 1997). Some well-known free radicals [mainly Reactive Oxygen Species (ROS)] which are actively involved in plant biochemical changes are the superoxide anion radical O_2^-, hydrogen peroxide H_2O_2, hydroxyl radical ·OH and singlet oxygen $^1O_2$ which can result in oxidative damage (Hernandez et al. 1995). Stress tolerant plants have protective mechanisms such as antioxidant systems comprising superoxide dismutase (SOD), catalase (CAT) and a variety of peroxidase enzymes e.g., ascorbate peroxidase (APx), glutathione reductase (GR). Moreover antioxidant substrates such as ascorbic acid, glutathione, α-tocopherol, flavonoids, carotenoids (CAR) (Dionisio-Sese and Tobita 1998; Sairam et al. 2002) may also help in detoxification and free radical scavenging (McKersie et al. 1996, Noctor and Foyer 1998). Particularly, phenolics are largely distributed in plants and are considered to be the most abundant secondary metabolites of plants (Dai and Mumper 2010). These compounds, predominantly phenolic acids, flavonoids and tannins have received much attention due to their antioxidant capacity (Li et al. 2009). According to the current knowledge, about 8000 different phenolic structures have been discovered and they are in the range of simple molecules (e.g. phenolic acids) to highly polymerized substances (e.g. tannins) (Dai and Mumper 2010). Considering the oxidation state of the C ring, flavonoids are primarily categorized into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (D'Archivio et al. 2007). Phenolic acids have two major groups; derivatives of benzoic acid (e.g. gallic acid) and derivatives of cinnamic acid (e.g. caffeic acid) while tannins are subdivided into two groups, hydrolysable tannins and condensed tannins (Khanbabaee and van Ree 2001).

Many studies have reported that phenolic and polyphenolic compounds in plants, such as phenolic acids, flavonoids, tannins, ellagic acid, corilagin gallic acid and geraniin, directly contribute to their antioxidative defense system (Li et al. 2009; Thitilertdecha et al. 2010). High phenolic and polyphenolic content is considered as an indication of a high antioxidant capacity of a plants (Li et al. 2007), and this has been well-documented for mangroves (Banerjee et al. 2008; Asha et al. 2012; Thatoi et al. 2014). Moreover, it has been reported that the following mechanisms operate in inhibiting the activity of free radicals; (1) scavenging of radical species (ROS/Reactive Oxygen Nitrogen Species: RONS), (2) suppressing formation of ROS/RON, and (3) up-regulating or protecting antioxidant defense. When all these facts are taken into account, apparently, phenolic compounds play a crucial role in protecting plants against oxidative stress and therefore, phenolic compounds in plants are given priority in the study (Cotelle 2001).

A balance between, free radical production and scavenging by antioxidants keeps the plants away from oxidative damage (Ashraf and Foolad 2007; Ashraf 2009). However, we argue that overproduction of free radicals or decreased scavenging capacity under prolonged submergence, soil water deficit and hypersalinity indicates inadequate antioxidant defense which may disturb ROS balance. Despite increased antioxidant capacity, free radicals in excess may be phytotoxic as they often cause damage to cellular macro-molecules such as lipids, proteins and nucleic acids. Eventually such damage results in disruptive metabolism and either cause serious disorders or lead to cell, then plant death (Sairam et al. 2002; Valentao et al. 2002). Unsaturated fatty acids in membrane systems are also susceptible to be damaged by free radicals causing leakage (Smirnoff 1993). Therefore, electrolyte leakage is considered to be an indicator of membrane damage under stress conditions (Liu and Huang 2000). There are several reports on antioxidant activities in different mangrove species (*Avicennia alba* Bl., *Aegiceras corniculatum* (L.) Blanco, *Bruguiera gymnorrhiza* (L.) Lamk., *Ceriops decandra* (Griff.) Ding Hou, *Rhizophora apiculata* Bl.), assessed through FRAP (ferric reducing antioxidant potential), ORAC (oxygen radical absorption capacity) and DPPH (2,2-diphenyl-1-picrylhydrazyl assays) (Banerjee et al. 2008; Krishnamoorthy et al. 2011; Asha et al. 2012; Thatoi et al. 2014).

In addition to the oxidative damage, water stress and prolonged submergence cause numerous impacts on the leaves such as wilting, chlorosis, necrosis, reduced leaf area, thickening of cuticle, leaf bud emergence, leaf area expansion, leaf mass and extension growth (Hussain and Ali 2015). These multifaceted impacts lead to reduce photosynthetic capacity of a plant as leaves are primarily damaged. This may eventually disturb dry matter production in the plant (Shao et al. 2008). Thus, when assessing the impacts of water stress and prolonged submergence on plants, it is more accurate to take level of oxidative stress, photosynthetic capacity and dry matter accumulation of the plant into account. This study was, therefore, designed to understand the root causes of sudden leaf damages, growth retardation and physiological dysfunctionality of *R. mucronata* seedlings by observing of antioxidant activity, scavenging capacity (biochemical responses) for ROS, photosynthetic capacity and dry matter accumulation under abiotic stress conditions. We addressed the following questions in this study: (a) Is antioxidant activity increased under prolonged submergence and substrate drought (stress

conditions; hypersalinity and water deficit), (b) Is scavenging capacity reduced under the stress conditions, (c) How does the tradeoff between antioxidant and scavenging capacity affect cell membrane integrity, sudden leaf damages of *Rhizophora mucronata* plants under the stress conditions? (d) How are photosynthetic capacity and dry matter accumulation affected under the given stress conditions?

Methodology

Study site

The research was conducted both in field conditions and in a plant-house. For the field experiment, it required a lagoon which has a higher tidal amplitude within Sri Lanka's microtidal system as it was designed to use two inundation frequencies. Attention was paid only on the ground inundation; under the both inundation classes and even at the high tide, at least the upper part of the seedling remained exposed to air. However, the maximum tidal amplitude in Sri Lanka is less than 100 cm. Therefore, Maampuriya, Puttalam lagoon (08° 00′ N, 79° 44′ E) which is situated in Puttalam district, north western province, the only lagoon that has the maximum tidal amplitude, 40 cm in average (maximum 75 cm; station data: NARA) in Sri Lanka was selected for the purpose (Fig. 1). Rainfall data for the dry and wet seasons were collected from the Department of Meteorology, Sri Lanka. Soil pH and redox potential were recorded with a Multimeter (18.52.01. Eijkelkamp) and soil bulk density was calculated using the equation explained by NRCS, Department of Agriculture, USA (2014) (Supplementary data: Table S1). The experiment in the plant-house was carried out in the Department of Botany, University of Ruhuna, Matara. Sri Lanka.

Selection of species

All the brackish water bodies were surveyed between 2012 and February 2014 in order to evaluate mangrove species that were used for the planting projects in Sri Lanka. During that survey, it was observed that *Rhizophora mucronata* and *R. apiculata* (Rhizophoraceae) provided a large majority ($\sim$ 80%) of the total number of mangrove seedlings planted. Therefore, *Rhizophora mucronata* Lam. was selected for the experiments.

Experimental design

Seedlings of *R. mucronata* were maintained in a nursery established by the Small Fishers Federation of Lanka (SFFL), Pambala, Chilaw, Sri Lanka, under field conditions in the adjacent mangrove, up to the age of 3 months and then used for the field study. Inundation levels and soil water stress levels were determined based on the literature as well as on preliminary on-site observations made during the survey carried out along the Sri Lankan coastline. Accordingly, two treatment levels (a) 50% inundation level (henceforward the abbreviation 'OPT-INUN' was used for the 50% inundation level with the sense of 'optimum inundation') and (b) 100% inundation level (prolonged submergence) (the abbreviation 'HIGH-INUN' was used for 100% inundation level) (see supplementary data; Fig. S1) which were marked under the supervision of mangrove restoration practitioners in SFFL and based on the field data collected from the National Aquatic Resources Research and Development Agency (NARA) were used. According to the previous studies (Hoppe-Speer et al. 2011), 50% inundation level (OPT-INUN) was used as the control to compare to the data that were collected from the 100% inundation level (HIGH-INUN). Three alternative plots were used for each treatment level (in total, six plots for both) in which 200 seedlings per plot were assigned along the 500 m belt in the Maampuriya area. In a plot, 10 rows were arranged, where each included twenty (20) *R. mucronata* seedlings with about 1 m distance in between two successive plants. The average size of a plot was about 200 m^2 [$\sim$ 20 m (width) $\times$ $\sim$ 10 m (length)]. A total of 1200 seedlings (200 $\times$ 3: 600 plants for the 50% inundation level and 200 $\times$ 3: 600 for the 100% inundation level) were subjected to the field study (see supplementary data; Fig S1).

In the plant-house experiment, mature propagules of *R. mucronata* collected from the natural mangrove forest in Pambala (07° 31′ N–79° 49′ E) were kept floating in a low saline (i.e. 2–3 psu) water container for about a month. Later, the propagules were transferred and maintained in a nursery to be used as the planting material. The pot-filling soil mixture was prepared by mixing sieved loam soil with sand and organic matter (degraded mangrove litter) in a 1:1:1 (v/v) proportion. The seedlings were then planted in plastic pots (with 8 cm diameter and 20 cm height) filled with the prepared soil mixture and same size seedlings (aged 1 ½ months) and the first two unfurled leaves (i.e. same development stage) were selected for the experiment. The experiments were carried out for water stress namely soil drought and hypersaline conditions. For drought stress experiment, three treatments 25% of WHC (Water Holding Capacity) (hence after, the abbreviation 'HIGH-WS' was used for the 25% WHC level) 50% of WHC (the abbreviation 'LOW-WS was used for the 50% WHC level) and 100% of WHC (the abbreviation 'NO-WS' was used for the 100% WHC) were used while for hypersalinity, $<$ 35 psu level (the term 'SAL-WS' was used for the hypersalinity treatment) was used. The level of 100% WHC (NO-WS) was considered as the control since optimum soil water content is

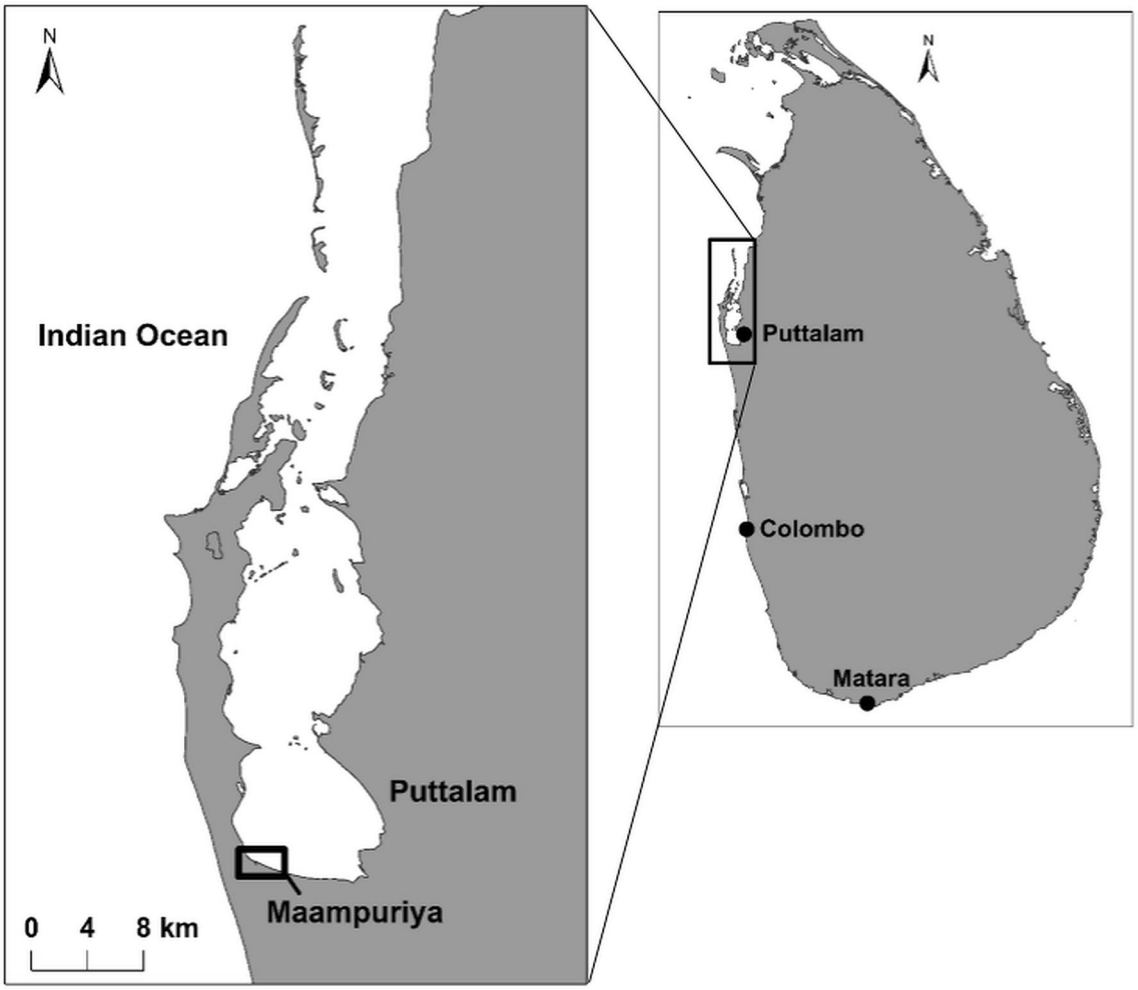

Fig. 1 Map of Puttalam lagoon (enlarged map), situated in the northwestern province in Sri Lanka and Maampuriya area is shown in black colour rectangle. Field experiments were carried in Maampuriya area and the plant-house experiments in University of Ruhuna, Matara as shown in the Sri Lankan map

known to be the field capacity (Thara et al. 2016). The water holding capacity was calculated based on the volume of the water held in the oven-dried, 100 g of soil sample when 100 ml water was added, the retained water volume was considered as the field capacity (100% WHC). Half and quarter of the field capacity volume were taken as 50% WHC (LOW-WS) and 25% WHC (HIGH-WS) of the soil respectively. The individual pots were treated with the respective water volume two times per day to keep the imposed stress levels. In addition, several soil samples, taken randomly from the pots, were tested for levels of water and salinity by using oven-dried method and refractometer (ATAGO S/Mill-E, Japan) for further confirmation (NRCAA 2010). Three replicates for each treatment level were used with 27 seedlings per replicate. A total of 324 seedlings (12 replicates $\times$ 27 seedlings) were subjected for the plant-house study.

Low saline water (i.e. 5 psu) that was prepared separately by mixing sea water and aged tap water, i.e. tap water kept in open containers for a few days before use to remove excess chlorine, was added to maintain the respective WHCs. Based on our preliminary findings, the salinity of water in the tanks was checked once every three days using a hand refractometer and adjusted when necessary. Commercially available fertilizer (blue crystals) was also applied once a month by providing the same amount per pot (Jayatissa et al. 2008; Dissanayake et al. 2014, 2018).

Determination of antioxidant capacity

Antioxidant assays

The plant leaves from different stress treatment levels were collected after 18 months of imposing stress conditions and were used for antioxidant assays.

Chemicals and equipment

Folin-Ciocalteu reagent, gallic acid, quercetin, 6-hydroxy-2-5-7-8-tetramethylchromane-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH), sodium fluorescein, 2,4,6-tripyridyl-s-triazine (TPTZ) and 4,4′-disulfonic acid sodium salt (ferrozine) were purchased from Sigma-Aldrich (USA). All the other chemicals used for the preparation of buffers and solvents were of analytical grade. Analyses were carried out using high-throughput 96-well micro-plate readers (SpectraMax Plus384, Molecular Devices, USA and SPECTRAmax-Gemini EM, Molecular Devices Inc, USA).

Preparation of samples

Leaves collected from the six different treatments (field and plant house) were air dried and extracted using methanol. Leaf samples were homogenized with methanol and extracted with 4-6 times methanol (w/v) at room temperature and sonicated for 3 to 4 h. The procedure was repeated thrice and all methanolic extracts were combined, filtered and concentrated to dryness. Dried extracts were dissolved in water, buffer or methanol with DMSO when required.

Total polyphenolic content (TPC) assay

The TPC of each extract was determined using Folin-Ciocalteu reagent method (Singleton et al. 1999) with modifications for 96-well micro-plates as described elsewhere (Ranasinghe et al. 2012). Leaf extracts dissolved in water at a concentration of 2 mg/ml were used in assay, five different concentrations of gallic acid (0.06, 0.12, 0.25 0.5 and 1.0 mg/ml) as the reference standards while distilled water was used as the blank. TPC of each extract was expressed as mg gallic acid equivalents per gram of extract. The curve was fitted as $Y = 4.377\ X + 0.003$ with R^2 value of 0.99.

Total flavonoid content (TFC) assay

The TFC of each extract was determined using the aluminium chloride method (Siddhuraju and Becker 2003) modified by Ranasinghe et al. (2012) using 96 well micro-plates. Leaf extracts dissolved in methanol at 5 mg/ml concentration were used in the assay, six different concentrations of quercetin (125, 62.5, 31.25, 15.62 and 7.81 μg ml^{-1}) as reference standards while methanol was used as the blank. TFC of each extract was expressed as mg quercetin equivalents per gram of extract. The curve was fitted as $Y = 0.032X + 0.024$ with R^2 value of 0.99.

DPPH radical scavenging assay

The DPPH (2, 2 diphenyl-2-picrylhydrazyl hydrate) scavenging activity was determined by a spectrophotometric method (Blois 1958) in 96 well micro-plates using Trolox as the standard. Reaction volumes of 200 μl containing 60 μl of freshly prepared DPPH radical and 50 μl of different concentrations of extracts were used. Trolox (3.12, 6.25, 12.5, 25.0 and 50.0 μg/ml) was used as the standard antioxidant while methanol was used as the blank.

Oxygen radical absorbance capacity (ORAC) assay

The ORAC radical scavenging assay was performed according to the method described by Ou et al. (2001) with some modification in 96-well micro-plates. The assay was conducted at 37 °C and pH 7.4 with a blank sample in parallel. Trolox standards (6.0 and 3.0 μM), fluorescein (4.8 μM), and 2, 2′-azobis (2-amidinopropane) dihydrochloride: AAPH (0.2 M) solutions were prepared prior to the use in phosphate buffer (75 mM, pH 7.4). Reaction volume of 200 μl, containing 100 μl of 4.8 μM fluorescein, 10 μl of each extract and 40 μl of phosphate buffer were pre-incubated at 37 °C for 10 min followed by addition of 50 μl of AAPH (0.2 M) to each well to initiate the reaction. The plate was then placed on the fluorescent microplate reader (SPECTRA max-Gemini EM, Molecular Devices Inc, USA) set with excitation and emission at 494 nm and 535 nm. The decay of fluorescein was recorded in 1 min intervals for 35 min. ORAC activities of the samples were calculated by comparing the net area under the curve of fluorescein decay between the blank and the samples. Results were expressed as ORAC values in mg of Trolox per gram of extract.

Ferric reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power assay was carried out according to the method of Benzie and Szeto et al. (1999) with some modifications in 96-well micro-plates. The working FRAP reagent was produced by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution and 20 mM $FeCl_3\ 6H_2O$ in a ratio of 10:1:1 just before use and heated to 37 °C. The TPTZ solution was prepared by making a solution of 10 mM TPTZ in 40 mM HCl. Trolox was used as the standard antioxidant and the readings were taken at 600 nm. Standard curve using Trolox (0.78, 1.56, 3.12, 6.25 and 12.5 μg/ml) was done with the equation $Y = 0.128X + 0.134$ where R^2 value was 0.99.

Assessment of membrane stability

Membrane stability was assessed indirectly by electrical conductivity measurements. The electrolyte leakage was measured on freshly harvested leaf samples from all treatments. Nine (9) leaves (each was selected from second mature leaf set) were sampled from each treatment level. The sampled leaves were weighed and wax was applied on the detached surfaces to avoid electrolyte leakage from the petiole surface. The leaves were put into a beaker of 100 ml distilled deionized water and shaken for 10 min to remove solutes from both leaf surfaces. Later on, the leaves were again put into a beaker of 100 ml deionized water and kept for 15 min to remove salt ions on leaf surfaces and electrolytes in the intercellular spaces. The electrical conductivity (EC_0) was measured by using Multimeter (18.52.01. Eijkelkamp). Afterwards, the container was covered with a lid and placed in a water bath maintained at a constant temperature of 35 °C for 2 h. The initial electrical conductivity (EC_1) of the solution was measured. Then the sample was autoclaved at 121 °C for 20 min to damage the cells and to release all electrolytes. Samples were then brought back to 30 °C and the final electrical conductivity (EC_2) was measured. The electrolyte leakage (EL) was expressed following the Dionisio-Sese and Tobita (1998) formula (1).

$$EL = \frac{EC_1}{EC_2} \times 100. \quad (1)$$

Determination of leaf photosynthetic capacity

Total average leaf area (TALA) per plant, stomatal conductance (SC), chlorophyll content (CC) were taken as the parameters to determine the photosynthetic capacity, In addition, leaf damage was also considered to study the reduction of leaf photosynthetic capacity. Total leaf area of the seedlings was also recorded using a millimeter paper model. Stomatal conductance was measured with a Steady State Porometer (SC-1; Decagon Devices, USA). The measurements were taken from late morning (10:00 h) to early afternoon (13:00 h). The youngest, fully expanded leaf exposed to full sunlight was selected and stomatal conductance was recorded on the abaxial surface. Chlorophyll content was determined by using CCM-200 plus (Chlorophyll content meter; Opti-Sciences, USA). After the initial calibration, each of the fully expanded leaves exposed to full sunlight was selected and an average of three readings on each side of the midrib was recorded. The damaged leaves (necrotic regions were taken into account) of the plants in the field plots as well as in the plant-house were studied and the damaged leaf area was estimated as a percentage reduction in comparison to the total leaf area of the plant. The data collection was done at monthly intervals. However, the data collected at the 18th month were used to determine the photosynthetic capacity.

Estimation of dry weight

Rhizophora mucronata saplings and the associated soil were removed with the help of a soil digging tool. The saplings were washed off carefully to remove any loose soil. The collected soil was also washed thoroughly to collect the broken root parts and adventitious roots. Sapling was separated into leaves, stem and roots. The plant parts were blotted and oven dried at 80 °C until constant weight was obtained. Shoot–root ratio was determined as the ratio of aboveground dry weight to root dry weight. Dry weights of plants were determined at the end of 18th month of the experiment.

Statistical analyses

The antioxidant content, scavenging capacity, total average leaf area, stomatal conductance, chlorophyll content, dry weight, shoot: root ratio were taken as continuous variables while electrolyte leakage as percentage data. For the aforementioned continuous variables, parametric assumptions were tested and all the conditions were met for the dependent variables, at 95% confidence level, parametric tests were performed taking the level of stress as fixed factor (e.g. level of inundation, salinity, and drought). The continuous variables were compared among the fixed factor levels using one-way ANOVA for the selected species *Rhizophora mucronata* and then, Tukey multiple comparison test was performed to check the pairwise comparison. A Generalized Linear Model (GLZ) was used to check the significance of percentage data on electrolyte leakage. Data were plotted using 95% confidence intervals, to allow visual inference of significant differences for all variables examined. All statistical analyses were performed using R-3.2.2 statistical software.

Results

Antioxidant capacity

Total phenolic content (TPC) was significantly different among all the treatments in the field as well as in plant-house conditions (Table 1a, b). It ranged between $100.00 \pm 0.54 - 187.53 \pm 8.75$ mg gallic acid equivalents g^{-1}. In general, total flavonoid content (TFC) was lower as compared to TPC that ranged between $13.34 \pm 0.49 - 20.95 \pm 0.77$ mg gallic acid equivalents g^{-1}. In field conditions, TPC and TFC of the *Rhizophora*

plants in the HIGH-INUN was significantly higher as compared to the plants grown in the OPT-INUN ($P < 0.05$). Similarly, significantly higher content of TPC was observed in HIGH-WS, LOW-WS and SAL-WS treatment levels in the plant-house condition as compared to the healthy seedlings in NO-WS ($P < 0.001$). The same trend was observed for TFC of the *Rhizophora* plants in the HIGH-WS, LOW-WS and SAL-WS treatment levels ($P < 0.001$). Antioxidant properties evaluated as FRAP, ORAC and DPPH also showed a greater variation among the treatments (Table 1a, b). In field conditions, FRAP and DPPH activities of the plants grown in the HIGH-INUN, showed significant increase as compared to the plants in the OPT-INUN while ORAC was significantly decreased ($P < 0.001$).

In the plant-house experiments, FRAP and ORAC activities of the *Rhizophora* plants in the HIGH-WS, LOW-WS and SAL-WS treatment levels were significantly higher in comparison with the plants in the NO-WS ($P < 0.001$). DPPH activity was significantly lower in the HIGH-WS and LOW-WS ($P < 0.001$) while a significant increase appeared in conditions of SAL-WS ($P < 0.001$). Dose response relationship was studied on the basis of IC_{50} value (Trolox IC_{50} value = 6.7 μg/ml as the reference value). The mangrove plants in the HIGH-INUN showed the lowest IC_{50} value (30.61 ± 0.08 μg/ml) in field conditions followed by the plants in the OPT-INUN(39.20 ± 0.95 μg/ml) (Table 2a). In the plant-house experiment, the *Rhizophora* plants in the NO-WS maintained better IC_{50} of free radical scavenging value though the lowest value was recorded in the SAL-WS treatment level. The highest IC_{50} of free radical scavenging value was observed in the HIGH-WS treatment, then in the LOW-WS (Table 2b).

Membrane stability

Membrane leakage as a damage marker was assessed by using the method of electrolyte leakage in *R. mucronata*

Table 1 Antioxidant properties that were obtained from different antioxidant assays for the field plants (1a) and the plant-house plants (1b)

Antioxidant properties	50% Inundation level (control)	100% Inundation level (prolonged submergence)
(1a)		
TPC (mg gallic acid equivalents/g of sample)	116.62 ± 7.70^{a}	161.16 ± 11.41^{b}
TFC (mg gallic acid equivalents/g of sample)	14.37 ± 0.66^{x}	23.21 ± 3.50^{y}
FRAP (mg Trolox equivalents/g of sample)	110.39 ± 2.38^{a}	161.22 ± 5.63^{b}
ORAC (mg Trolox equivalents/g of sample)	551.48 ± 22.75^{x}	534.56 ± 16.60^{x}
DPPH (mg Trolox equivalents/g of sample)	143.83 ± 3.51^{a}	199.20 ± 1.34^{b}

Antioxidant properties	100% WHC (control)	25% WHC	50% WHC	High salinity
(1b)				
TPC (mg gallic acid equivalents/g of sample)	100.00 ± 0.54^{a}	181.12 ± 5.61^{b}	161.24 ± 3.53^{c}	187.53 ± 8.75^{b}
TFC (mg gallic acid equivalents/g of sample)	14.06 ± 0.89^{x}	20.31 ± 1.31^{y}	13.34 ± 0.49^{x}	18.18 ± 0.99^{y}
FRAP (mg Trolox equivalents/g of sample)	86.21 ± 2.77^{a}	171.11 ± 10.50^{b}	131.84 ± 6.69^{c}	204.22 ± 16.53^{d}
ORAC (mg Trolox equivalents/g of sample)	243.49 ± 20.56^{x}	728.38 ± 34.18^{y}	724.07 ± 58.56^{y}	717.09 ± 56.34^{y}
DPPH (mg Trolox equivalents/g of sample)	176.14 ± 7.30^{a}	149.67 ± 6.62^{b}	136.41 ± 1.18^{c}	191.46 ± 3.17^{d}

Data represented as mean ± SE. TPC: Total polyphenolic content (n = 16); TFC: Total flavonoid content (n = 16); FRAP: Ferric reducing antioxidant power (n = 14); ORAC: Oxygen Radical Absorbance Capacity (n = 15), DPPH (n = 14). Values within each column with different superscripts are significantly different at $P < 0.05$. Significance level shows by using different letters and those were used separately for different parameters

plants. Electrolyte leakage varied among all treatments (Fig. 2). The level of electrolyte leakage (EL) of the *Rhizophora* plants grown in the HIGH-INUN level was similar to those of the plants in the OPT-INUN under field conditions and no significant difference observed among the treatment levels. In the plant-house experiment, EL of the mangrove plants in the HIGH-WS, LOW-WS and SAL-WS treatment levels was remarkably higher in comparison with the plants grown in the control treatment ($P < 0.001$). The highest EL was obtained in the HIGH-WS treatment level and the second highest in the LOW-WS level.

Leaf photosynthetic capacity

In the HIGH-INUN treatment level, total average leaf area and chlorophyll content of the mangrove seedlings were significantly low ($P < 0.05$) as compared to the OPT-INUN while there was no significant difference in stomatal conductance among the treatments. In the plant-house experiment, total average leaf area and stomatal conductance were significantly decreased ($P < 0.05$) in the mangrove plants grown in the HIGH-WS, LOW-WS and SAL-WS treatment levels as compared to the healthy ones in the NO-WS (Table 3a, b). The chlorophyll content of the plants was adversely affected in the HIGH-WS and LOW-WS treatment levels and showed significant reduction ($P < 0.001$) in comparison to the NO-WS. Interestingly, *Rhizophora mucronata* seedlings in the SAL-WS treatment maintained the highest chlorophyll content in the experiment and did not change significantly from the NO-WS.

Under prolonged submergence, total leaf area reduced by about 82% and the main cause for that was immature leaf senescence and leaf abscission (Fig. 3). In addition, about 50% chlorophyll degradation could also be seen in the HIGH-INUN level in comparison with the OPT-INUN level. When *Rhizophora* plants were subjected for soil water stress, total leaf area reduced markedly, by 98%, 97% and 88% in the HIGH-WS, LOW-WS and SAL-WS treatment levels respectively. Also, stomatal conductance of the stressed plants showed significant reduction as by 91%, 86% and 87% in the HIGH-WS, LOW-WS and SAL-WS treatment levels respectively in comparison to the NO-WS treatment. The existing photosynthetic area further decreased due to necrotic regions (Fig. 3) appeared on the leaves of the stressed *Rhizophora* plants. Also, 67% and 66% chlorosis could be observed in the HIGH-WS and LOW-WS in that order.

Dry weights

Dry weights in *Rhizophora* plants were quite differently affected by prolonged submergence and soil drought. Prolonged submergence significantly reduced ($P < 0.05$) the biomass of *Rhizophora* plants when compared to the

Table 2 Dose response relationship of field grown plant samples for DPPH radical scavenging activity (IC_{50} value)

Concentration (μg/ml)	50% Inundation level (control)	100% Inundation level (prolonged submergence)
(2a)		
3.125	8.10 ± 0.56	6.92 ± 0.19
6.25	9.42 ± 1.09	16.33 ± 0.71
12.5	15.06 ± 0.46	33.10 ± 0.41
25	34.18 ± 1.63	54.32 ± 0.67
50	63.13 ± 1.65	92.11 ± 0.91
100	–	113.21 ± 1.87
IC_{50}	39.20 ± 0.95	41.21 ± 0.11

Concentration (μg/ml)	100% WHC (control)	25% WHC	50% WHC	High salinity
(2b)				
3.125	3.95 ± 0.18	10.1 ± 0.61	–	6.30 ± 0.21
6.25	8.12 ± 0.59	13.2 ± 1.16	0.78 ± 0.07	4.40 ± 0.43
12.5	19.34 ± 0.59	17.6 ± 0.88	1.94 ± 0.29	18.61 ± 0.34
25	39.72 ± 0.63	45.1 ± 2.18	27.23 ± 2.54	42.91 ± 3.40
50	78.58 ± 4.85	82.7 ± 1.67	62.85 ± 0.32	87.18 ± 0.14
100	–	–	88.34 ± 0.15	–
IC_{50}	32.09 ± 1.31	50.12 ± 0.15	41.28 ± 0.35	29.43 ± 0.50

Data represent mean ± SE; n = 14. IC_{50} Trolox = 5.629 ± 0.119 μg/ml. IC_{50} = Concentration of green tea leaf extracts at 50% inhibition of DPPH radical

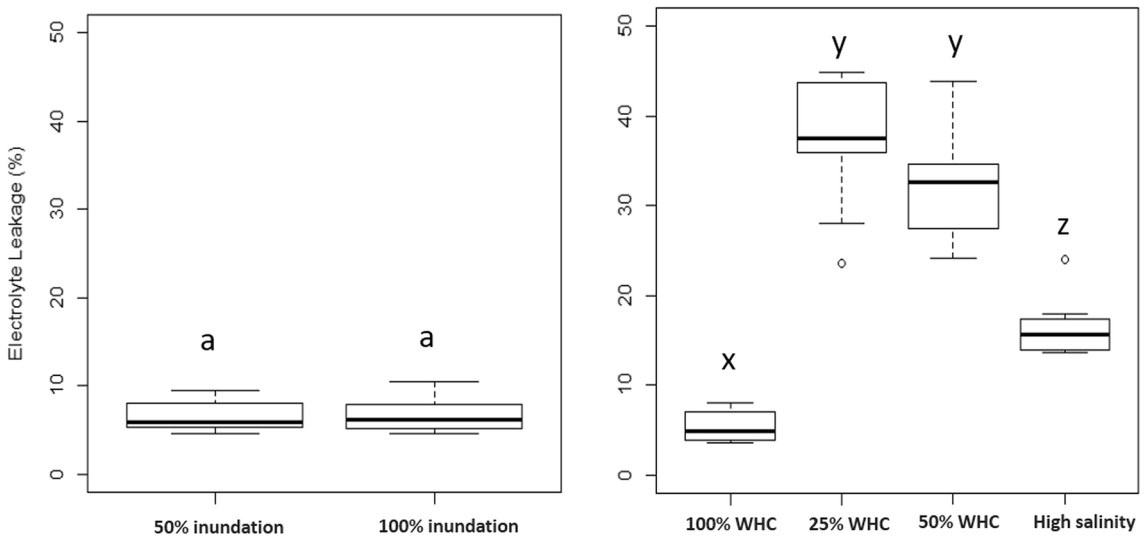

Fig. 2 The variation of electrolyte leakage (%) in cell membrane with respect to each treatment level in field and the plant-house conditions. 50% inundation as the control and 100% inundation as the treatment in field conditions. In plant-house experiment, 100% WHC as the control while 25% WHC, 50%WHC and high salinity as the water stress treatments. GLZ model results: For the field experiment; P value = 0.64 (df = 26) and for the plant-house experiment, 25% WHC, 50% WHC and high salinity treatment, P value < 0.001 (df = 39). Significance level shows by using different letters

Table 3 Leaf photosynthesis capacity parameters and dry matter accumulation of *R. mucronata* seedlings in response to two treatment levels in field conditions (2a) and four treatments in plant-house conditions (2b)

Leaf photosynthesis capacity parameters	50% Inundation level (control)	100% Inundation level (prolonged submergence)
(3a)		
Total average leaf area (cm^3) (n = 36)	804.1 ± 83.7^{a}	147.0 ± 37.0^{b}
Stomatal Conductance (mmol $m^{-2}s^{-1}$) (n = 114)	552.52 ± 57.28^{x}	544.10 ± 48.12^{x}
Chlorophyll content (CCI) (n = 124)	33.5 ± 4.7^{a}	16.6 ± 4.3^{b}
Dry matter accumulation (g) (n = 22)	189.3 ± 18.2^{x}	99.2 ± 13.6^{y}
Shoot: root ratio (n = 22)	4.3 ± 1.1^{a}	1.9 ± 0.9^{b}

Leaf photosynthesis capacity parameters	100% WHC (control)	25% WHC	50% WHC	High salinity
(3b)				
Total average leaf area (cm^3) (n = 38)	874.4 ± 109.2^{x}	13.7 ± 4.1^{y}	28.7 ± 3.9^{y}	104.4 ± 30.3^{z}
Stomatal Conductance (mmol m^{-2} s^{-1}) (n = 118)	532.02 ± 36.40^{a}	49.12 ± 9.77^{b}	76.78 ± 11.63^{c}	69.88 ± 12.05^{c}
Chlorophyll content (CCI) (n = 128)	101.7 ± 9.6^{x}	33.4 ± 2.0^{y}	35.0 ± 4.2^{y}	124.0 ± 19.8^{x}
Dry matter accumulation (n = 26)	141.32 ± 19.5^{a}	11.6 ± 1.8^{b}	27.5 ± 2.5^{c}	37.6 ± 5.3^{d}
Shoot: root ratio (n = 26)	5.1 ± 0.5^{x}	2.3 ± 0.4^{y}	2.5 ± 0.4^{y}	4.7 ± 1.2^{x}

Tukey multiple comparison; 95% confidence level, $P < 0.05$. Values within each column with different superscripts are significantly different at $P < 0.05$. Significance level shows by using different letters and those were used separately for different parameters

OPT-INUN level. *Rhizophora* plants allocated more biomass to root system under prolonged submergence, as indicated by significantly lower ($P < 0.05$) shoot: root ratio in comparison to the control plants in the OPT-INUN level. In the plant-house experiment, dry weights were significantly decreased ($P < 0.05$) in the mangrove plants grown in the HIGH-WS, LOW-WS and SAL-WS treatment levels in comparison to the mangrove plants in the NO-WS (Table 3a, b). Moreover, it could be observed a higher biomass allocation to the root system of *Rhizophora* plants, grown in the water deficit treatments, HIGH-WS and LOW-WS. However, the mangrove plants in the SAL-WS did not behave in the same way and shoot: root ratio was not significantly different from the control plants.

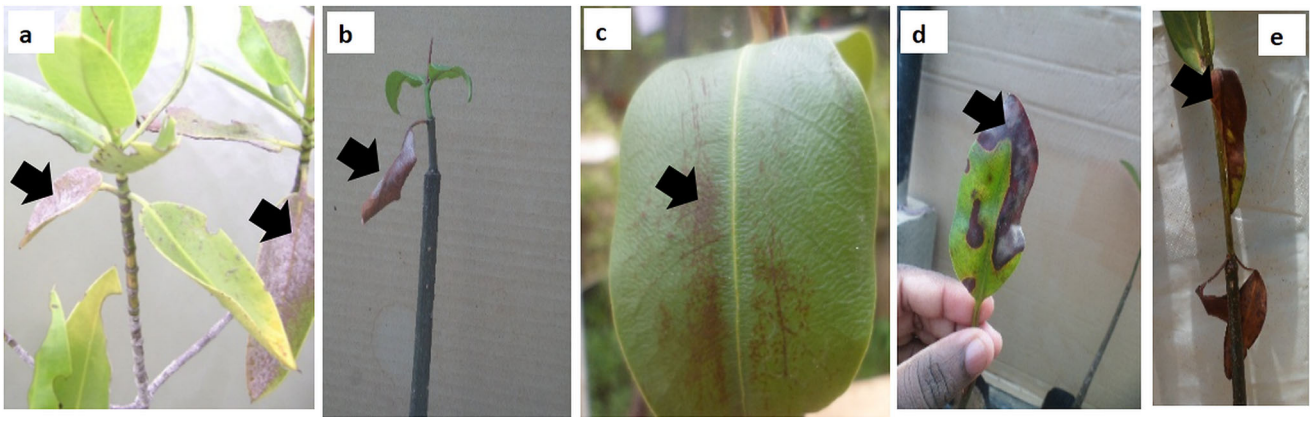

Fig. 3 The major symptoms which caused to reduce the leaf photosynthetic capacity of the *Rhizophora mucronata* plants. **a** Leaf yellowing and rust; **b** immature leaf senescence; **c** leaf tissue disintegration; d: severe leaf necrosis; e: leaf abscission (Photos: Kodikara KAS)

Discussion

Mangrove plants have evolved unique physiological traits which make them survive successfully under extreme abiotic stresses such as higher fluctuations in salinity, frequent inundation leading to hypoxia, low relative humidity and high temperature (Tomlinson 1994; Alongi 2002). However, mangrove seedlings appear sensitive during their initial development phases (Aziz and Khan 2001) hence under stressed environments various symptoms may develop leading to seedling damage and mortality. Apparently, abiotic stress conditions lead to generate excess reactive oxygen species in *Rhizophora* plants (Ali and Alqurainy 2006; Asada 2006) which causes to create unfavorable internal environment in plants. Particularly in this study, higher amount of toxic salts could be damaging to different sub-cellular compartments and could affect electron transport system leading to generate more ROS (Ali and Alqurainy 2006). Water deficit causes stomatal closure thereby resulting in reduced CO_2, supply despite high light intensity which directs electron transfer towards molecular oxygen. This electron transfer to molecular oxygen ultimately leads to ROS generation (Asada 2006). Free radicals in excess under stress may be harmful for metabolic processes, however, an efficient scavenging system may provide protection to the plants (Meloni et al. 2003). In such case, higher antioxidant activity may contribute to balance ROS. Leaf extracts of the mangrove seedlings grown in different stress conditions showed significantly higher antioxidant activity as indicated by total reducing power and oxygen radical absorption capacity compared to the *Rhizophora* plants in controlled conditions. Although higher antioxidant activities in mangroves have been reported in earlier studies (Ravindran et al. 2012a, b; Thatoi et al. 2014), their relationship with cellular functions and leaf photosynthetic capacity has not been reported. Lower scavenging capacity of free-radicals in plants usually results in damage of membrane fatty acids and other cellular components (Bajii et al. 2001; Sairam et al. 2002). Generally, biosynthesis of plant polyphenolics increases under stress conditions for cellular protection against oxidative damage (Ali and Alqurainy 2006; Krol et al. 2014).

It may be assumed that polyphenolics contribute to a higher antioxidant activity in *R. mucronata* as indicated by remarkably higher TPC and TFC values in HIGH-INUN. Substantial change in FRAP and ORAC values of the *Rhizophora* plants in HIGH-INUN as compared to the *Rhizophora* plants in the OPT-INUN and low IC_{50} values of the *Rhizophora* plants with increased DPPH in HIGH-INUN represent higher free radical scavenging capacity. Some other studies have reported that total phenolic contents (TPC) in mangroves ranged from 4.40 to 283.3 mg GAE/g dry material (Banerjee et al. 2008; Krishnamoorthy et al. 2011; Malik et al. 2017). Higher amount of phenolics were recorded in the genus *Ceriops* and the genus *Rhizophora* showed a moderate amount; 40.47 mg GAE/g dry material (Banerjee et al. 2008). In addition, total flavonoid content (TFC) ranged from 11.60 to 37.90 mg QE/g (Krishnamoorthy et al. 2011; Malik et al. 2017). The TPC contents obtained in our study are higher than the values recorded in the aforementioned studies and it could be due to the induced antioxidant activity under stress conditions (Ravindran et al. 2012a, b). However, the recorded TFC contents are congruent with the previous studies. Also, the IC_{50} values of *Rhizophora* plants grown in stress conditions (< 41 µg/ml) in our study are lower than those of the other studies (80-365.35. µg/ml) (Banerjee et al. 2008; Ravindran et al. 2012a, b) indicating the induced scavenging capacity under stress conditions. High membrane stability (lowest electrolyte leakage) of the surviving plants in HIGH-INUN indicates that the *Rhizophora* plants under prolonged submergence may have compromised growth at

the cost of survival and that they are well equipped to manage oxidative stress in the later stages by establishing a balance between ROS production and scavenging (Hameed et al. 2012).

Lowest IC_{50} values in saline conditions (SAL-WS) indicate an efficient radical scavenging capacity although growth is substantially reduced. However, the question that requires clarification is whether the scavenging capacity of seedlings is strong enough to overcome the oxidative damage that is caused by free radical accumulation in saline conditions? Poor growth in saline conditions may be related to the energetic cost of osmotic adjustment in plants (Aziz and Khan 2016) but further studies on antioxidant substrates and enzymes in field grown plants (HIGH-INUN) would give a clear answer to our question. In HIGH-WS, LOW-WS, TFC did not change but TPC, FRAP and ORAC increased which indicates higher antioxidant activity. However, lowest DPPH and highest IC_{50} values reflects poor radical scavenging capacity coupled with the lowest stomatal conductance and extremely poor growth. Increasing cellular damage with time also corresponds to an increased electrolyte leakage in the HIGH-WS, LOW-WS where visible symptoms of leaf damage, for example, leaf necrosis, and leaf fall were observed (Faoro and Iriti 2005). These findings indicate that *R. mucronata* cannot withstand oxidative stress caused by substrate dryness/water shortage. About 90% death and tissue damages suggest that elevated AO capacities of seedlings were not sufficient enough to neutralize the excess radicals generated in the HIGH-WS, LOW-WS.

In the field trials, significantly lower total average leaf area of the *Rhizophora* plants under submerged conditions (HIGH-INUN) is related to the fact that *R. mucronata* experienced immature leaf senescence and subsequent leaf abscission. This may be related to inadequate light interception or ABA signaling in the HIGH-INUN plants (Verslues and Zhu 2005). Also, reduced chlorophyll content in the HIGH-INUN plants may be due to chlorophyll disintegration under multiple stress conditions (Ashraf and Harris 2013). Therefore, these conditions, reduced total average leaf area and chlorophyll content collectively caused to decrease the photosynthetic area of the HIGH-INUN *Rhizophora* plants. This scenario is further proven by significantly reduced dry weight in the HIGH-INUN plants which resulted due to decreased photosynthetic area. This could further be linked towards immobilization of carbohydrates due to light insufficiency or energy shortage due to carbon dioxide limitation (Colmer and Voesenek 2009; Ashraf and Harris 2013). In this context, more biomass allocation to adventitious roots (Poorter and Nagel 2000) or any other root anatomical modifications (Pezeshki et al. 1990) may be an adaptation against concomitant tidal hits under prolonged submerged conditions; HIGH-INUN (Krauss et al. 2006). No significant difference in stomatal conductance is an indicator of abundant water availability (avg. salinity of the lagoon water in rainy season and immediate after about 4 psu) under submerged conditions; HIGH-INUN (Lauri et al. 2014).

In the plant-house experiment, all the *Rhizophora* plants in the HIGH-WS, LOW-WS and SAL-WS treatments were severely affected and total average leaf area, chlorophyll content, and stomatal conductance showed a considerable reduction by about 80-90% in comparison with the NO-WS plants. Reduced growth and physiological parameters in the HIGH-WS, LOW-WS and SAL-WS could be related to soil water deficit and sub-optimal substrate salinity owing to decreased soil water potential (Munns 2005; Munns and Tester 2008; Ashraf and Harris 2013; Thara et al. 2016). According to Naidoo (2006), such a growth decline can be due to drought induced turgor reduction and reduced cell expansion in plant cells. Also, it has been recorded that reallocation of nitrogenous organic resources for osmoregulation in plant cells could severely limit plant growth (Naidoo 1985). Plants usually respond to low soil water potential by decreasing net photosynthesis and subsequently growth (Aziz and Khan 2001). Apparently, under water stress conditions, the mangrove leaf reduces the transpiration rate by restricting stomatal conductance (Nandy and Ghose 2001). The causes of reduced photosynthesis could be due to either stomatal closure that may restrict CO_2 for carboxylation or by salt-induced damage to the photosynthetic machinery (Flexas et al. 2004). Stomatal conductance decreased in the HIGH-WS, LOW-WS and SAL-WS when compared to the NO-WS plants which may have caused reduced photosynthesis and growth despite higher chlorophyll content in SAL-WS treatment. Higher chlorophyll content in leaves could be related to fertilizer application in our experiments, therefore, it would be interesting to elucidate the role of leaf pigments with and without fertilizers in our future trials. In addition, severe necrosis and leaf abscission did matter to decrease the photosynthetic area of the *Rhizophora* plants. Therefore, highly reduced total leaf area, chlorophyll content (except in high salinity) and leaf damages caused to reduce the photosynthetic area of the *Rhizophora* plants. Proving this fact, this study has shown that dry weight is inhibited by soil drought and hypersaline conditions. Moreover, it showed that more dry weight in root system of the mangrove plants and it may be an adaption to develop deeper root system to acquire soil water.

In summary, we observed that *Rhizophora* seedlings increased their antioxidant capacity under the two abiotic stress conditions; prolonged submergence, and water stress. The plants grown in HIGH-INUN could be able to manage the effect of free-radical activities through the increased scavenging capacity. However, photosynthetic capacity of

the plants grown in HIGH-INUN was lower than that of OPT-INUN, due to pre-mature senescence and leaf abscission. We also recorded that the plants in HIGH-INUN allocated more dry matter to their root system. In contrast, the *Rhizophora* plants in the HIGH-WS and LOW-WS treatments failed to manage the antioxidant-free radical system resulting in oxidative damage. This was clearly observed through increased membrane damage and leaf necrosis in the stressed plants. This led to a remarkable reduction in photosynthetic area of the *Rhizophora* plants grown in the HIGH-WS and LOW-WS treatments. However, the plants in SAL-WS were able to handle the effect of free radicals through increased scavenging capacity. Nevertheless, the plants grown under the SAL-WS treatment showed lower growth and limited stomatal conductance. Therefore, oxidative stress and its associated impacts on leaf photosynthetic capacity and dry weights in young mangrove plants are unavoidable under the persistence of the stress condition. When mentioning the applied aspect of this study, it is highly recommended to select correct planting site with suitable hydrology to avoid such lethal damages in mangrove plants used in mangrove restoration projects.

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Author contributions KASK, PR, and IA designed the research; KASK and SKM performed the fieldwork; NK, FGD and, LPJ supervised the research; NK and SKM analyzed the data; KASK and PR wrote the paper; NK, LPJ, IA and FGD edited the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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