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Original Scientific Paper

Stress-induced carbon starvation in *Rhizophora mucronata* Lam. seedlings under conditions of prolonged submergence and water deficiency: survive or succumb

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ABSTRACT:
The behaviour of carbohydrate metabolism in a plant, particularly its total starch content, total soluble sugar (TSS) content and their utilisation, is of great importance in coping with abiotic stress conditions. With this in mind, we studied total starch and TSS contents, survival, growth, biomass accumulation and stomatal conductance in *Rhizophora mucronata* under conditions of prolonged submergence and water stress for a period of 11 months. The experiment was designed in such a way as to include three replicates per each treatment level, about 1600 young mangrove plants being subjected to study in the process. Under conditions of prolonged submergence and high levels of water stress, a small number of mangrove plants survived and they were promptly exhausted due to higher starch utilisation rates (0.75-1.05% dry mass/month). Although TSS content was increased under these intense stress conditions, it was not matched by increased seedling growth or biomass production; instead, a significant reduction in growth (i.e., ~78%) and dry matter content was observed in stressed seedlings as compared to young plants in the respective controls. It follows that the intense increase of TSS content might be due to the direct conversion of starch to soluble sugars in order to produce metabolic energy for tolerance mechanisms like osmoregulation and root anatomical adaptations under stress conditions. This indicates that more energy is allocated for plant maintenance than for growth and biomass production under stress conditions, which might be a good acclimatory strategy to rescue young mangrove plants at the early phase. However, stomatal closure under stress conditions may have caused restricted photosynthesis. Therefore, stress–induced starch degradation may upsurge, which in turn might lead in the long-run to carbon starvation, a condition lethal to mangrove seedlings.

Keywords:
acclimation, mangrove, plant maintenance, growth, starch, total soluble sugars

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INTRODUCTION

Mangrove forests are unique plant communities that grow in extreme environmental conditions such as high and changing salinity, frequent inundation with associated hypoxia, low air humidity and high temperatures (Mukherjee *et al*. 2014; Tomlinson 2016). Mangrove plants are adapted to grow in the intertidal zone, i.e., the area between low and high tide marks, where they can tolerate dynamics in soil characteristics and hydrology resulting from periodic inundation (Hoppe-Speer *et al*. 2011). However, the environmental conditions in areas out of the intertidal zone exceed the limits tolerable by mangrove plants. For this reason, selection of incorrect inundation elevation for mangrove planting often entails inappropriate environmental conditions (abiotic stress conditions) for mangroves, for example, hypersaline conditions, substrate drought in the supra-littoral zone or beyond [since soil water content is most of the time below the field´s capacity, particularly during the dry season in Sri Lanka (pers. obs.)] and prolonged submergence in the infra-littoral area (Field 1998; Kodikara *et al*. 2017a).

Several studies reported that abiotic stress conditions can affect the survival of plants (Shao *et al*. 2009; Anjum *et al*. 2011). However, stressed plants evidently make suitable modifications to their regulatory network of anatomical, biochemical, physiological and morphological processes to ensure their survival under stress conditions (Gupta & Kaur 2005; Duan *et al*. 2007; Sulpice *et al*. 2009). The ability of a plant to do so depends on several factors, among which the pool of both reserved and available carbohydrates (the level of energy) is considered to be one of the main factors (Rodrigues *et al*. 1995; Pinheiro *et al*. 2001; Sulpice *et al*. 2009), since many of these processes are energy-dependent (van der Werf *et al*. 1988; Poorter 1994). More particularly, starch is considered as a carbon and energy stock in many plants and starch metabolism can therefore moderate the adverse effects of stress-induced carbon depletion (de Block *et al*. 2005). Interestingly, starch can serve as a sugar-source when a plant needs carbon or as a sugar-sink when sugars are in excess (Dong & Beckles 2019). In general, starch is mobilised after converting to simple soluble sugars, which are the source of required metabolic energy and exists in the course of ATP production for various phyto-processes (Huner *et al*. 1998; de Block *et al*. 2005; Sulpice *et al*. 2009). Primary metabolites like glucose, sucrose, fructose and starch are known as non-structural carbohydrates (NSCs), which represent the bulk of plant carbon (Ravi *et al*. 2020).

Thus, we argue that available starch content and the rate of its utilisation, which is generally known as 'carbohydrate metabolism', under these stress conditions are crucial in determining seedling growth, development and survival. This aspect has become more important as the success of mangrove planting projects depends on the number of healthy mangrove plants growing and surviving in the planting sites (Kodikara *et al*. 2017a). As far as we know, the relationship between plant growth, survival of young mangrove plants and dynamics of the pool of reserve carbohydrates in propagule-dependent mangrove plants has not been well-studied under different stress conditions. A study of propagule dependency on the food reserve of *Rhizophora apicuata* Blume and *R. mucronata* Lam. was conducted by Dissanayake *et al*. (2014) reporting the starch variation in response to three contrasting salinities. The aim of the present study was to investigate the variation of total starch and soluble sugar contents under abiotic stress conditions, and its effect on growth, development and survival of young *R. mucronata* plants.

MATERIALS AND METHODS

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 high tidal amplitude within Sri Lanka's microtidal system, as it was designed to use two inundation frequencies. Attention was paid only to ground inundation; under both kinds of inundation and even at 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. The Mampuriya, Puttalam lagoon (08°00´N, 79°44´E), which is situated in Puttalam district, north western province, and is the only lagoon in Sri Lanka that has a tidal amplitude of 40 cm on average (maximum 75 cm; station data: NARA) was selected for the field experiment (Fig. 1). Rainfall data for the dry and wet seasons were obtained from the Department of Meteorology, Sri Lanka. Soil pH and redox potential values were recorded with an Eijkelkamp 18.52.01 Multimeter and soil bulk density was calculated using the equation explained by NRCS, Department of Agriculture, USA (2014) (Supplementary data: Table A1). The experiment in the plant-house was carried out in the Department of Botany, University of Ruhuna, Matara, Sri Lanka.

Selection of species. All brackish water bodies were surveyed between 2012 and February of 2014 in order to evaluate mangrove species for use in planting projects in Sri Lanka. During that survey, it was observed that *Rhizophora mucronata* and *R. apiculata* (Rhizophoraceae) accounted for a large majority (~80%) of the total number of mangrove seedlings planted (Kodikara *et al*. 2017a). Therefore, *R. mucronata* 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

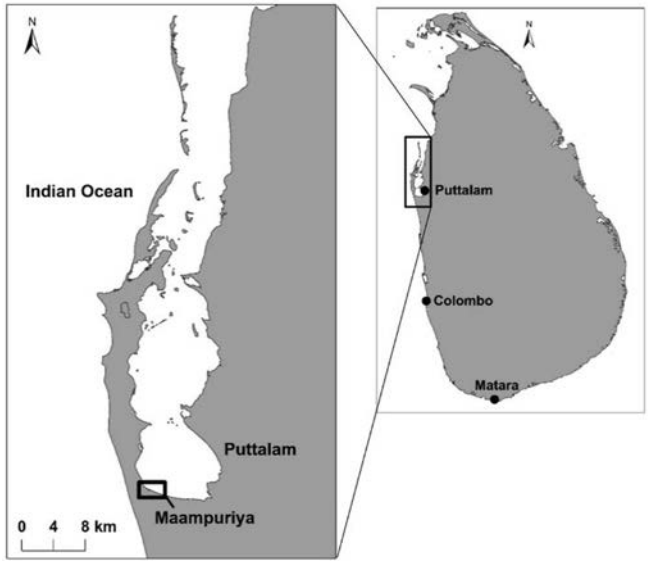

Fig. 1. Map of Puttalam lagoon (enlarged map), situated in the northwestern province of Sri Lanka and the Maampuriya area, enclosed in black rectangles. Field experiments were carried out in the Maampuriya area, plant-house experiments at the University of Ruhuna, Matara as shown on the Sri Lankan map.

Lanka, under field conditions in an 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 available literature as well as on preliminary on-site observations made during a survey carried out along the Sri Lankan coastline. During the survey, a considerable number of mangrove plantations were observed in the infra-littoral zone (farther out into the lagoon**'s** water), which resulted in permanent submergence of mangrove seedlings/samplings (Kodikara *et al*. 2017a). Accordingly, seedlings were planted either in positions where the ground was flooded approximately half of the day [(a) 50% inundation] or always covered with water [(b) 100% inundation] (see supplementary data; Fig. A1). Positions used for both treatment levels were marked under the supervision of mangrove restoration practitioners in SFFL and based on field data collected from the National Aquatic Resources Research and Development Agency (NARA). In accordance with the results of previous studies (Hoppe-Speer *et al*. 2011), a 50% inundation level was used as the control to compare to the data collected from the 100% inundation level. Three alternative plots were used for each treatment level (in total, six plots for both), in which 200 seedlings per plot were allotted along a 500-m belt in the Maampuriya area. In every plot, 10 rows were arranged, where each included 20 *R. mucronata* seedlings with a distance of about 1 m between two successive plants. The average size of a plot was about 200 m^2 (~20 × 10 m). A total of 1200 seedlings (200 × 3, 600 plants in total for the 50% inundation level and 200 × 3, 600 plants for the 100% inundation level) were subjected to the field study (see supplementary data; Fig. A1).

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 vessel containing low-saline (i.e. 2-3 psu) water for about a month. Later, the propagules were transferred to a nursery and maintained there 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 ratio of 1:1:1. The seedlings were then planted in plastic pots (measuring 8 cm in diameter and 20 cm in height) filled with the prepared soil mixture and same-size seedlings (aged 1½ months), and the first two unfurled leaves (i.e., ones in the same development stage) were selected for the experiment. Two experiments were carried out for soil water stress, namely a salt stress experiment, involving a situation in which water exists in the soil solution but plants cannot absorb it due to high salinity; and a drought stress experiment, where water supply to plant roots becomes limiting (Lisar *et al*. 2012).

Three treatments, viz., moderate salinity (15-17 psu), high salinity [i.e., 33-35 psu, a value selected on the basis of local conditions and the fact that too high salinities (< 40 psu) are uncommon in Sri Lankan water bodies)] and freshwater were used for salt stress, moderate salinity being considered as the control (Flowers & Colmer 2015). Different levels of water-holding capacity for the drought experiment were selected on the basis of measurements of soil water content performed in the study of Kodikara *et al*. (2017a). Three treatments were used, namely, 25% of WHC (water- holding capacity), 50% of WHC and 100% of WHC. The level of 100% WHC was considered as the control since optimum soil water content is known to be the field capacity (Thara Madhurangi *et al*. 2016). The water-holding capacity was calculated based on the volume of water held in an oven-dried, 100-g soil sample when 100 ml water was added, the retained water volume being considered as the field capacity (100% WHC). Half and a quarter of the field capacity volume were taken as 50% WHC and 25% WHC of the soil, respectively. The individual pots were treated with the respective water volume two times per day to keep up the imposed stress levels. In addition, several soil samples, taken randomly from the pots, were tested for levels of water and salinity using the oven-dried method and a refractometer (ATAGO S/Mill-E, Japan) for further confirmation (NRCCA 2010). Three replicates were used for each treatment level, with 27 seedlings per replicate. A total of 486 seedlings were used 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 WHC values. In the light of 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 fertiliser (blue crystals) was also applied once a month, adding the same amount per pot in the process (Jayatissa *et al*. 2008; Dissanayake *et al*. 2014, 2018).

Level of survival, plant growth, dry matter accumulation and stomatal conductance. The number of surviving young *R. mucronata* plants at each treatment level was counted and recorded during the study period (11 months). For growth parameters, cumulative shoot height (hereafter referred to as cumulative growth) of each seedling was measured on a monthly basis. Total leaf area of the seedlings was also recorded, using a millimetre paper. Stomatal conductance was measured with a Steady State Porometer (SC-1; Decagon Devices, USA). The measurements were taken from late morning (10:00 hrs) to early afternoon (13:00 hrs). The youngest, fully expanded leaf exposed to full sunlight was selected and stomatal conductance was recorded on its abaxial surface. In determining their dry weight, *R. mucronata* plants and the associated soil were removed with the help of a soil -digging tool. Young plants were carefully washed to remove any loose soil. The collected soil was also washed thoroughly to collect broken root parts and adventitious roots. For sampling purposes, plants were separated into leaves, stem and roots. The plant parts were blotted and oven dried at 80°C until constant weight was obtained. The shoot-root ratio was determined as the ratio of aboveground dry weight to root dry weight. This was conducted for all the treatment levels.

Reduction in growth parameters, dry matter accumulation and stomatal conductance in response to stress conditions was calculated relative to the performance of young mangrove plants in the control treatments, i.e., in this study, a 50% inundation level in field conditions, 100% WHC and moderate salinity in plant-house conditions.

The percentage of reduction (Re) was calculated according to the following equation (1):

$$\mathrm{Re} = \frac{(A1 - A2) \times 100}{A1} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1),$$

where *A*1 is the parameter´s measurement in the control treatment and *A2* is its measurement at the treatment level.

Measuring total soluble sugar and starch content. Three propagules (or seedlings) of *R. mucronata* from each treatment level were sampled and used for starch and sugar analysis. In the field experiment, samples were collected at 0 months (the leafless initial propagules used for the nursery) and at the 3rd (introduced to field conditions), 5th, 7th, 9th and 11th months. In the plant-house experiment, on the other hand, they were taken at 0 months (propagule with no leaves), after 1 ½ months, and at the 3rd, 5th, 7th, 9th and 11th months. The collected propagules/or seedlings were oven-dried at 68°C for 3 days. After 3 days of drying, propagules were ground with the aid of a grinder to pass through a 40-mesh sieve. The sieved samples were stored in air-tight plastic tubes in the dark until used in analysis.

From each propagule sample, three subsamples of 20 mg were taken and each was extracted with 5 ml of 80% ethanol by boiling the samples with capped glass tubes in a 95°C water bath for 10 min. After each extraction, the tubes were centrifuged at 2500 rpm for 5 min. The supernatants were collected and stored for sugar analysis. The residues were stored at -20°C for starch analysis. Sugar concentration of the extracts was determined by the phenol-sulphuric method using 2% phenol and a wavelength of 490 nm was applied as the optimized concentration, together with the absorbance wavelength adapted from Chow & Landhausser (2004). Further, the plant extracts of *R. mucronata* were subjected to sugar assay with and without phenol according to the methodology described by Chow & Landhausser (2004).

Afterwards, the sugar concentration [*Sugar*] of each extract was calculated according to the following equation:

$$[Sugar] = \frac{(A - A')}{(a - a')} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2),$$

where *A* is the absorption with phenol; *A'* is the absorption without phenol; *a* is the absorption coefficient of the GFG (glucose, fructose, galactose) standard; and *a'* is the is the absorption coefficient of the GFG standard without phenol.

Determination of starch was conducted by the acid hydrolysis method using a lower concentration (0.001 N) of sulphuric acid. In the extraction, 10 mg of the plant sample was refluxed for 1 hour with 5 ml of 0.001 N H_2SO_4 in a 95°C water bath, after which the contents were vacuum filtered through Whatman No. 40 filter paper. The filtrates were kept for glucose analysis. Glucose content was determined by the phenol–sulphuric acid method and concentrations determined against a glucose standard. Starch content of the samples was estimated by multiplying the glucose content by a glucose equivalent of 0.9 (Chow & Landhausser 2004). (For sample calculations, see supplementary Figs. A2 and A3).

Variations in total soluble sugars and total starch reduction. The rate of starch reduction (% dry mass/month) and rate of total soluble sugar variation (% dry mass/month) were calculated in relation to time. The following equations were used:

$$SRR = \frac{(St1 - St2)}{(t2 - t1)} \text{ \% drymass / month} \quad \ldots\ldots\ldots\ldots (3),$$

$$SVR = \frac{(SSt4 - SSt3)}{(t4 - t3)} \text{ \% drymass / month} \quad \ldots\ldots\ldots\ldots (4),$$

where *SRR* is the starch reduction rate; *St1* is total starch content at month *t1*; *St2* is total starch content at month *t2*; *SVR* is the sugar variation rate; *SSt4* is total soluble sugar content at month *t4*; *SSt3* is total soluble sugar content at month *t3*; *t2* > *t1* and *t4* > *t3*.

Statistical analysis. The variables cumulative shoot and branch length (height at the end of the study period), total leaf area, stomatal conductance and dry matter content were treated as continuous variables, while starch content and total soluble sugar content were treated as proportional data (given in relation to total dry matter of the plant). As all conditions were met for the dependent variables, parametric tests were performed taking the level of stress factors (level of inundation, salinity and drought) as a fixed factor. The continuous variables were compared among the fixed factor stress levels using one-way ANOVA at the 11th month (and some other selected months as well for certain variables). Following the one-way ANOVA test, a Tukey multiple comparison test was performed to check the pairwise comparisons. A generalized linear model (GLZ) was used to establish significance of the percentage reduction in values of the parameters used in

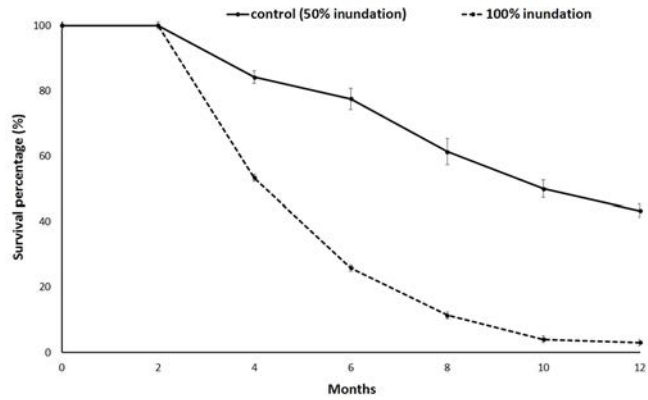

Fig. 2. Graph showing the percentage survival of *Rhizophora mucronata* seedlings in field conditions over the 11-month study period. Error bars indicate standard deviations.

Table 1. Comparison of growth parameters, dry matter accumulation and stomatal conductance of *R. mucronata* seedlings in response to two treatment levels, 50% inundation (control) and 100% inundation (prolonged submergence) in field conditions. Tukey multiple comparison; 95% confidence level, $P < 0.05$. Significance level shown by using different letters.

Parameters	50% inundation (control)	100% inundation
Cumulative growth (cm)	42.6 ± 1.8^{a}	22.5 ± 2.5^{b}
Total average leaf area (cm^3)	171.2 ± 14.7^{x}	77.6 ± 11.5^{y}
Stomatal Conductance (mmol $m^{-2}s^{-1}$)	565.5 ± 40.6^{a}	585.0 ± 19.5^{a}
Dry matter content (g)	49.3 ± 11.5^{x}	23.2 ± 13.6^{y}
Shoot:root ratio	3.7 ± 1.2^{a}	1.6 ± 0.7^{b}

Table 2. Comparison of growth parameters, dry matter accumulation and stomatal conductance of *R. mucronata* seedlings in response to three treatment levels for drought stress experiment and three treatment for salt stress experiment in plant-house conditions. Tukey multiple comparison; 95% confidence level, $P < 0.05$. Significance level shown by using different letters. Drought and salt stress experiments were treated separately in performing comparison tests.

Parameters	Drought stress experiment			Salt stress experiment		
	100% WHC	50% WHC	25% WHC	Moderate salinity	Freshwater	High salinity
Cumulative growth (cm)	59.8 ± 5.8^{a}	19.8 ± 3.9^{b}	All plant died after 4 months	58.1 ± 3.6^{p}	57.3 ± 4.2^{p}	17.8 ± 2.3^{q}
Total average leaf area (cm^3)	214.5 ± 11.6^{x}	52.7 ± 7.3^{y}		241 ± 13.8^{g}	236.5 ± 15.2^{g}	43.6 ± 6.3^{h}
Stomatal Conductance (mmol $m^{-2}s^{-1}$)	532 ± 36.4^{a}	76.8 ± 11.6^{b}		577 ± 42.7^{p}	598.7 ± 30.12^{p}	69.9 ± 12.05^{q}
Dry matter content (g)	54.5 ± 12.3^{x}	9.2 ± 2.5^{y}		58.6 ± 9.6^{g}	59.1 ± 8.1^{g}	7.6 ± 2.4^{h}
Shoot:root ratio	4.6 ± 0.8^{a}	2.1 ± 0.5^{b}		4.9 ± 0.5^{p}	5.0 ± 0.7^{p}	4.6 ± 0.8^{p}

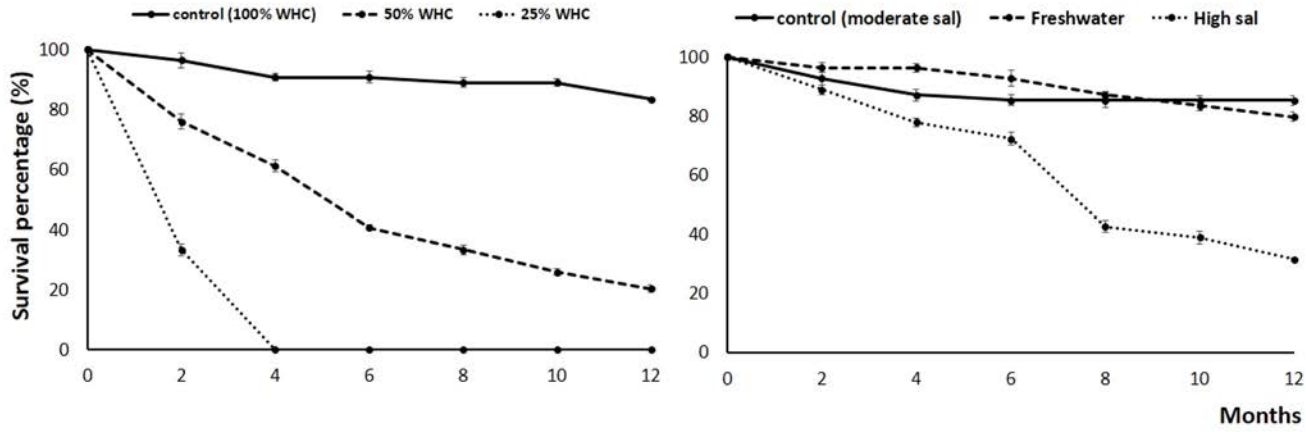

Fig. 3. Survival of *Rhizophora mucronata* seedlings over the 11-month study period in drought stress treatment (left side) and salt stress treatment (right side) in plant-house experiments. Error bars indicate standard deviations.

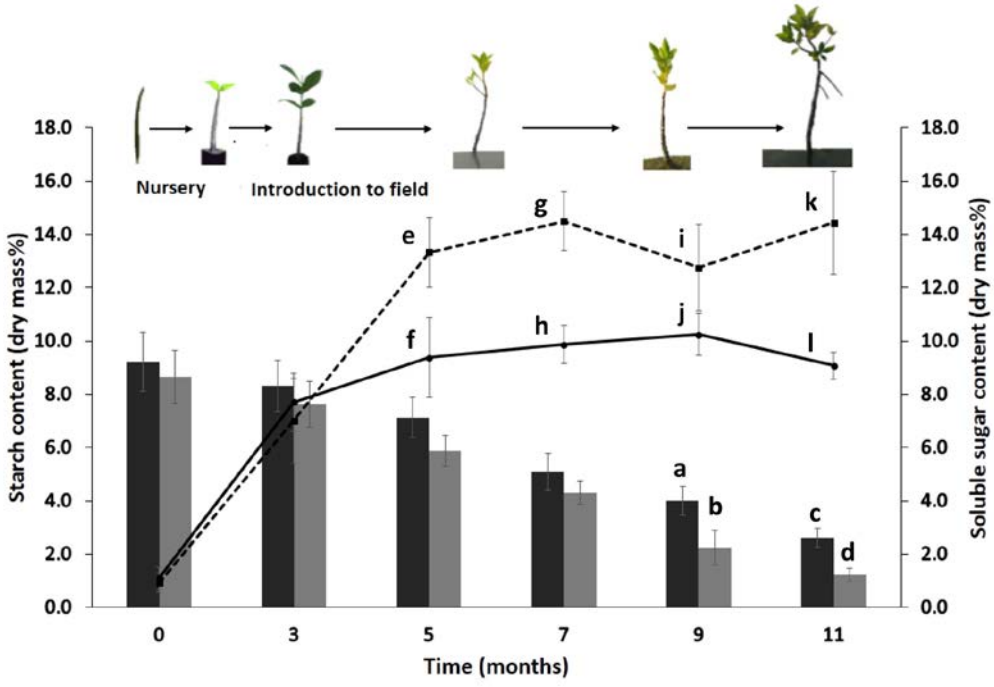

Fig. 4. Variation in total starch content (% dry mass) and total soluble sugar content (% dry mass) of *R. mucronata* seedlings at different ages in nursery and field conditions. Black bars: starch content under conditions of 50% inundation; grey bars: starch content under conditions of 100% inundation. The solid line: total soluble sugar content under the control treatment (50% inundation); the dashed line: total soluble sugar content under prolonged submergence (100% inundation). Error bars indicate standard deviations. Different superscripts show the level of significance at 0.05 (binomial proportion test).

this study. The binomial proportion test was performed to study significance of the proportional reduction in values of starch and total soluble contents. Data were plotted using 95% confidence intervals to allow visual inference of significant differences for all variables examined. Significant differences in the level of survival of *R. mucronata* plants (at the 11th month) under conditions of different treatment levels in the study were tested using the binomial proportion test [2-sample test for equality of proportions with chi-squared ($\chi 2$) values]. All statistical analyses were performed using R-3.2.2 statistical software.

RESULTS

Level of survival of young *Rhizophora mucronata* seedlings. The level of survival decreased at both inun-

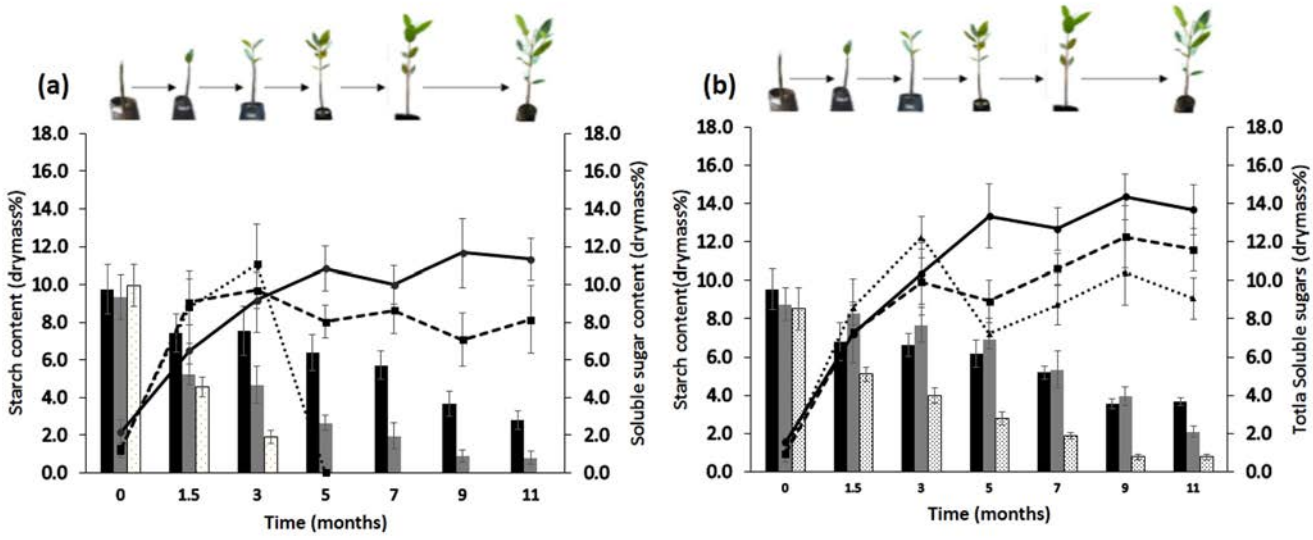

Fig. 5. Variation in total starch content (% dry mass) and total soluble sugar content (% dry mass) of *R. mucronata* seedlings grown in plant-house conditions. Drought stress experiment (left side) and salt stress experiment (right side). Bars represent total starch content, while lines stand for total soluble sugar content. On the graphs, the black bars and solid lines refer to healthy seedlings under conditions of 100% WHC (drought stress experiment) and moderate salinity (salt stress experiment), the grey bars and dashed lines refer to seedlings under conditions of 50% WHC (drought stress experiment) and freshwater conditions (salt stress experiment), while the white bars and dotted lines refer to affected seedlings under conditions of 25% WHC (drought stress experiment) and high salinity (salt stress experiment).

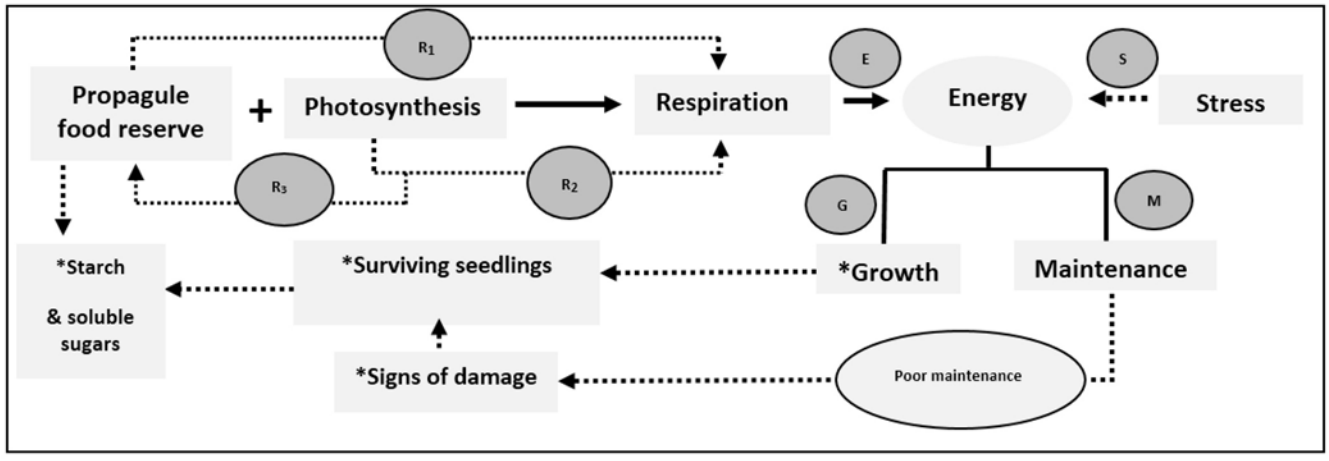

Fig. 6. Model proposed for energy use by *R. mucronata* seedlings in response to abiotic stresses. R1: rate of using propagule food reserve for respiration; R2: rate of using photosynthates for respiration; R3: rate of photosynthate contribution to the carbohydrate reserve pool; E: rate of ATP production; S: intensity of abiotic stress; G: rate of using energy for plant growth (growth respiration); M: rate of using energy for plant maintenance (maintenance respiration). The asterisk (*) indicates the variables for which data were collected.

dation levels in field conditions (Fig. 2). However, the level of survival was significantly lower ($P < 0.001$) at the 100% inundation level (prolonged submergence) after 11 months, and only 4% remained in the end at that level. In the plant-house experiment, the level of survival was about 85% for the 100% WHC treatment (control) after 11 months, whereas in the case of 25% WHC treatment all plants died within the first four months. It was observed that, only 22% of mangrove plants survived in the case of 50% WHC treatment, which was significantly lower ($P < 0.05$) than in the control (Fig. 3a). After the 11th month, the levels of survival after moderate salinity treatment (control) and freshwater treatment were not significantly different and comprised 85% and 83%, re-

spectively (Fig. 3b). After high salinity treatment, only 34% survival was observed at the end of the experiment, which was significantly lower ($P < 0.001$) than after the control treatment.

Growth, dry matter accumulation and stomatal conductance. In field conditions, almost all the *Rhizophora* plants were affected by prolonged submergence. Cumulative growth and total leaf area were significantly lower ($P < 0.05$) in plants grown in prolonged submerged conditions as compared to the control plants. However, there was no significant difference in stomatal conductance (Table 1). Dry matter accumulation in the *R. mucronata* plants was quite differently affected by prolonged submergence. Prolonged submergence significantly reduced ($P < 0.05$) the dry matter content of plants when compared to the plants subjected to a 50% inundation level. Moreover, *R. mucronata* plants allocated more biomass to their root system under conditions of prolonged submergence, as indicated by a significantly lower ($P < 0.05$) shoot: root ratio in comparison with the control plants under conditions of 50% inundation (Table 1).

None of the surviving seedlings were healthy in the variants with 25% (all died after 4 months) and 50% WHC. Cumulative growth of young mangrove plants in the variant with 50% WHC treatment was significantly lower ($P < 0.001$) in comparison with the control plants in the variant with a 100% WHC level (Table 2).The same trend was observed for total leaf area ($P < 0.001$) of mangrove plants in the variant with a 50% WHC level. The obtained results indicate that stomatal conductance of the *R. mucronata* plants was critically affected, being significantly lower ($P < 0.001$) in stressed plants in the variant with a 50% WHC level. In the plant-house experiment, dry matter accumulation was significantly reduced ($P < 0.05$) in mangrove plants grown under conditions of 50% WHC in comparison with 100% WHC. Moreover, it was observed that biomass allocation to the root system was higher in plants subjected to water deficit treatment in the form of a 50% WHC level (Table 2).

In the salt stress experiment, hypersaline conditions negatively affected young *R. mucronata* plants, significantly lowering cumulative growth ($P < 0.001$) and total leaf area ($P < 0.001$). In the same way, stomatal conductance of plants under conditions of high salinity was significantly lower ($P < 0.001$) than in the control plants under conditions of moderate salinity. More interestingly, young *R. mucronata* plants, grown in freshwater behaved in the same way as the control plants, and there was no significant difference of growth parameters and stomatal conductance between them. Significantly lowered dry matter content ($P < 0.05$) was observed in *R. mucronata* plants grown in the presence of a high salinity level as compared those subjected to moderate salinity treatment (Table 2).

Young *R. mucronata* plants showed significant reduction in cumulative growth, leaf area and dry matter content under conditions of prolonged submergence. The decrease in cumulative growth of submerged plants in relation to the control plants was 47% ($P < 0.001$), while in regard to total average leaf area and dry matter content this decrease comprised 55% ($P < 0.001$) and 53% ($P < 0.001$), respectively. In the same way, soil drought caused reduction in cumulative growth, leaf area, stomatal conductance and dry matter content in comparison with the control plants. Cumulative growth decreased by 67% ($P < 0.001$) in young mangrove plants grown under conditions of a50% WHC, while total average leaf area, stomatal conductance and dry matter content did so by 75% ($P < 0.001$), 86% ($P < 0.001$) and 83% ($P < 0.001$) in that order. Also, the cumulative growth of *R. mucronata* plants subjected to conditions of high salinity was reduced by 69% in comparison with the control plants under conditions of moderate salinity. Similarly, the total average leaf area, stomatal conductance and dry matter content of young mangrove plants in the high salinity variant were reduced by 82% ($P < 0.001$), 88% ($P < 0.001$) and 87% ($P < 0.001$), respectively. However, plants grown in the freshwater showed no significant difference in comparison with the control plants (Table 2).

Starch content and total soluble sugar content

Field conditions. Mean values of starch content in *R. mucronata* propagules at the time of collection (i.e., before being used for the nursery) and at the end of the 3-month nursery period (just before being used for the field experiment) were 8.85 ± 0.86 % and 6.25 ± 0.82 % of dry weight, respectively. In field conditions, the starch content of mangrove seedlings under all conditions decreased over time (Fig. 4). After introduction to field conditions (at the 3rd month), the proportional reduction of starch content (also called starch consumption) of *R. mucronata* seedlings at both inundation levels was notable. From the 7th month on, the starch content of seedlings in submerged conditions showed a significant reduction, viz., 81% ($P < 0.001$) as compared to seedlings in the control treatment. At the end of the 11th month (sapling stage), the stressed plants consumed more starch ($P < 0.05$) than did the control plants. In contrast to starch content, total soluble sugar content of the seedlings in both the control and the variant with submerged treatment increased with time, except for one drop at the 9th month in the latter case. The total soluble sugar content of young mangrove plants in submerged conditions was significantly higher (at the 5th month, 9th month and 11th month) ($P < 0.001$) than in the control plants. Seedlings in both control and submerged conditions showed a rapid increase of total soluble sugar content at the 3rd month, after which it increased continuously, but at a lower rate. After 11 months, total soluble sugar content of the *Rhizophora* samplings in submerged conditions was significantly lower ($P < 0.05$) than in the control plants. The rate of increase of total soluble sugar in young mangrove

plants was high under both control and submerged conditions, the highest rate of increase (i.e., 1.15% dry mass/month) being observed under submerged conditions.

Plant-house experiment. The starch content in seedlings used in the plant-house experiment (at 1.5 months) was 7.44 ± 0.56% of dry weight. The mean starch content decreased over time, which was common for both drought and salt stress experiments. The starch content of *R. mucronata* seedlings in the variants with 50% WHC and 25% WHC was significantly lower ($P < 0.05$) from 1.5 months onward than the control seedlings (Fig. 5). The highest rate of starch decrease was obtained in the variant with 25% WHC (1.05 % dry mass/month), followed by that in the variant with 50% WHC. Seedlings in the variant with 100% WHC maintained a high TSS content throughout the experiment. *Rhizophora* plants in the variants with 25% WHC and 50% WHC showed a prompt increase in TSS over a 2-3- month period. Maximum sugar content was obtained in 3-month-old propagules under conditions of 25% WHC (i.e., 5.01% of dry mass/month) before succumbing in the 5th month. However, significantly lower sugar content ($P < 0.05$) was recorded in plants of the 50% WHC variant after the first 3-month period when compared to healthy plants in the control. In the salt stress experiment, starch content showed a decreasing trend over the study period similar to that observed in the other two experiments, which was common for all treatment levels (Fig. 5). Young mangrove plants in moderate saline conditions and ones in freshwater conditions exhibited a similar decreasing trend. There was no significant difference in total starch content between seedlings in moderate conditions (control) and ones in freshwater conditions except in the 11th month ($P < 0.05$). The moderate saline plants showed the highest starch content at the end of the experiment. On the other hand, seedlings subjected to high saline treatment exhibited significantly lower starch content ($P < 0.001$) throughout the experiment, with a starch reduction of rate of 0.77% of dry mass/month. Affected seedlings in the variant with high salinity treatment showed the highest reduction rate at 1.5 months. Total soluble sugar content showed an increasing trend with time, young mangrove plants in moderate saline conditions maintaining the highest sugar content, followed by plants receiving freshwater treatment. In plants receiving high salinity treatment, total soluble sugar content showed a sudden increase during the period from 1.5 to 3 months, after which it decreased. That trend then continued for the whole remaining period. The highest increase of TSS content (1.26% dry mass/month) was obtained in moderate saline conditions and the lowest (0.98% dry mass/month) in a high salinity regime.

Proposed model for energy use by *R. mucronata* seedlings under stress conditions. The proposed model (Fig. 6) explains metabolic production of energy under abiotic conditions and its allocation for growth and maintenance. It is assumed that plants allocate more metabolic energy for plant maintenance processes, including defensive mechanisms and repair, than for plant growth under stress conditions. The rate of metabolic energy production and use are therefore supposed to be higher than under non-stress conditions. This causes reduction of food reserves and available photosynthates at a high rate, which eventually leads to their fast exhaustion, further intensified under conditions of minimal addition of photosynthates due to interrupted food production. Thus, plants run out of energy, which might ultimately cause seedling dysfunctionality and subsequent mortality.

DISCUSSION

Mangrove restoration practitioners still do not follow the technical guidelines introduced as a result of several studies, and that always leads to conditions where the effects of abiotic stress to mangrove seedlings prevail (Lewis & Brown 2014; Kodikara *et al*. 2017a). In coping with these intense conditions, strong defensive mechanisms are ineffective when plants suffer a loss of carbohydrates known to act as the biochemical source of energy for plant metabolism (Prado *et al*. 2000).

Under stress conditions, the optimal balance between carbon supply and utilisation may be disrupted because defence mechanisms and recovery activities impose a drain on stored energy (Thronley 2011). As a result, energy partitioning may be modified in such a way that more energy is allocated for plant maintenance. The level of reallocation determines plant growth, development and survival of mangrove seedlings (Dietze *et al*. 2014). The aspect of energy metabolism under conditions of stress has received little attention by investigators seeking to understand mangrove seedling dysfunctionality and early mortality at unsuitable mangrove planting sites. According to our results, prolonged submergence and water stress conditions cause reduced growth and survival of *R. mucronata* seedlings. To be specific, the trend of decrease in starch content over time (observed in all experiments) is not uncommon because starch utilisation is high in the early phase of propagules due to higher levels of phosphorolysis during morphogenesis, i.e., from the developing propagule to the seedling stage (Monma *et al*. 1991; Parida *et al*. 2002). In support of that assertion, von Fircks (1997) showed that starch content is higher during dormancy periods and lowest during growth. Decrease of starch content is generally attributed to conversion of starch to sugar used in growth and development of plants (Amthor 1984; Parida *et al*. 2002; Tomasella *et al*. 2017). Seedlings grown under conditions of 50% inundation, 100% WHC and moderate salinity showed higher growth, since mitochondrial phosphorylation was else-

where shown to be maximal in non-stressed conditions (de Vries 1975). However, except in freshwater conditions, the starch depletion of seedlings in stress conditions (i.e., prolonged submergence, 25% WHC, 50% WHC, high salinity) is more pronounced than that of their respective controls. Several studies reported significant starch degradation under abiotic stress conditions (Gonzalez-Cruz & Pastenes 2012; Stitt & Zeeman 2012). The significant reduction of starch content during stress treatments could be caused by extra starch utilisation (or a higher rate of starch use) by stressed mangrove seedlings. The energy can then be used for plant maintenance, development of cell structure and morphological, anatomical and physiological processes (de Vries 1975) such as increase of protein content, activities of various enzymes, alteration of the carbon metabolite profile, changes of pigment content, accumulation of proline-like biomolecules, defence mechanisms and cell repair (Parida *et al*. 2002; Reid & Rose 2011; Thronley 2011; Kumari & Parida 2018). Higher rates of starch use and rapid conversion of starch to sugar therefore continued in stressed seedlings. A number of studies reported that plants remobilise their starch reserve as a means of releasing metabolic energy, sugars and derived metabolites in order to moderate the effects of stress conditions such as water stress and salinity stress (Krasensky & Jonak 2012; Thalmann & Santelia 2017). More particularly, the released sugars and other derived metabolites function as osmoprotectants and compatible solutes to moderate impacts of the stress (Krasensky & Jonak 2012). It has further been reported that stress conditions (e.g., drought) can activate starch-hydrolysing enzymes leading to an increase in sugars (Dong & Beckles 2019). That is why low starch content (excessive use of starch) could be observed under severe stress conditions as compared to the control treatments in both the field and plant-house experiments in our study. Further, dramatic decrease in starch content and increase in total soluble sugar content at the 3rd month (the time of transfer from the nursery to field conditions) might be due to an immediate need for metabolic energy to cope with multiple simultaneously occurring factors in field conditions. The observed reduction of starch and dramatic increase of sugars could be due to reduced starch biosynthesis or higher starch degradation to sugars (Dong & Beckles 2019). Geigenberger *et al*. (1997) also reported that water deficits repressed starch biosynthesis and increased sugar content in some crop species. We propose that salinity-induced restriction of starch biosynthesis should also exist in the present study. In addition, the same change was observed from the 5th month onward under submerged conditions. This might be due to a large allocation for root growth, as the plants need robust anchorage to cope with frequent hydro-forces and to prevent root damage (rotting). That such is the case seems evident from the significantly higher allocation of biomass to the roots (the lowest shoot: root ratio) under conditions of prolonged submergence.

In plant-house conditions, the occurrence of maximum total sugar content at the 3rd month in 25% WHC before all the seedlings died might be caused by the need to produce maximum energy for rescuing metabolic processes. Similarly, several other studies also demonstrated that soluble sugars accumulate in response to stress (Damour *et al*. 2008; He *et al*. 2012). Those studies further explained that the type of sugar depends on the plant species and the stress treatment.

The conversion of starch to sugar is a necessary step for production of ATP, which is then used for plant metabolism, in particular for plant growth and development (Bhosale & Mulik 1992). However, increase in total soluble sugar content under stress conditions like submergence, 25% WHC, 50% WHC and high salinity does not promote growth performances in *R. mucronata* seedlings (where a ~70% reduction of growth and low dry matter content were observed), whereas growth was promoted in the control variants. It is therefore proposed that plant maintenance-related metabolic processes are prioritised over growth and development under stress conditions and that sugar molecules might play a significant role in a plant signaling process that includes a sugar-based signaling pathway (Moore *et al*. 1998; Stitt *et al*. 2007). Although plants have mechanisms to detect free sugar levels, it was previously reported that plants prioritise maintenance over growth and development by controlling the expression of growth-related genes under stress conditions (Gibson 2005). Therefore, as suggested by Thalmann & Santelia (2017), increased sugar content in stressed plants may be due to decreased demand, as a consequence of growth retardation. However, all the different processes involved in plant signaling and growth are not fully understood (Stitt *et al*. 2007).

Unlike seedlings grown in the variant with high salinity treatment, freshwater-grown seedlings did not show a stressed nature, the possible cause of which could be emergence of the "no salt stress" condition, although it should be noted that the salt requirement for mangrove growth is still debated (Kodikara *et al*. 2017b). However, it is clear that *R. mucronata* seedlings modified their metabolic energy allocation under abiotic stress conditions in such a way as to prioritise plant maintenance through the appearance of a number of defensive reactions and acclimatory responses, for example, development of shiny thickened leaves, leaf rolling and less secondary branching (not mentioned in the Results section). As indicated before, when a large fraction of total carbon is used for maintenance of a plant, that results in a significant reduction of its growth (Amthor 1984), something observed in highly stressful conditions like permanent submergence, 25% WHC, 50% WHC and high salinity (the proposed model clearly explains this scenario). If the stress condition prevails for a short period and/or if enough starch

is accumulated by photosynthesis, this modified regulatory system might not cause any great carbon shortfall in seedlings, thus securing their survival. This might be an acclimatory strategy until carbon is available. However, due to prolonged exposure to stress conditions, if enough starch is not gained from photosynthesis, propagules might solely depend on food reserves (Pirt 1965; Thronely 1970) and the pool of carbohydrates be depleted sooner in the long run. Further, stomatal closure (average reduction of stomatal conductance is 85% under conditions of 25% WHC, 50% WHC and high salinity) causes restricted CO_2 intake and hence reduces the net carbon gain by photosynthesis (Takemura *et al*. 2000; Blasing *et al*. 2005) and can easily lead to depressed carbon assimilation in the long run (Parida *et al*. 2002). As a result, a small amount of photosynthates ("none" in some cases, for example under conditions of 25% WHC) is added to the system and seedlings consequently run out of their energy source, creating an energy crisis (Smith & Stitt 2007; Gibon *et al*. 2009). This ultimately converts the carbon shortfall to acute carbon starvation, and any starch turnover is avoided in an attempt to prevent it (Sulpice *et al*. 2009; Thronley 2011). In the presence of acute carbon starvation, cell wall deterioration and lipid degradation take place as a result of using pectin and hemicellulose as additional carbon sources (Osuna 2007; Sulpice *et al*. 2009). Protein degradation is initiated, decreasing the activities of respiratory enzymes (de Vries 1975; Osuna 2007), inhibiting metabolism and cellular processes (Geigenbenger & Stitt 2000) and subsequently affecting organelle functioning, for example causing mitochondrial and chloroplast dysfunction (Yamamoto *et al*. 2001; Zhang & Xing 2008). This causes catabolic activities to be prioritised over anabolic processes (Dieuaide *et al*. 1993; Contento *et al*. 2004; Thompson & Vierstra 2005). Further, both auto-phagocytosis and excessive accumulation of free radicals due to accelerated energy production in the mitochondria (Schwanz & Polle 2001) may speed up cell disintegration through oxidative damage to DNA, membrane lipids and proteins, which in a previous study of ours was observed as developing necrosis on leaves and stems (Kodikara *et al*. 2020).

CONCLUSION

Abiotic stress conditions like permanent flooding and water deficit lead to depletion of starch content in *Rhizophora mucronata* seedlings at a higher rate than under non-stress conditions. Strong inhibition of mangrove seedling growth was observed, probably due to the establishment of a new balance of carbon-partitioning in which maintenance is prioritised over plant growth as an acclimatory response to ensure seedling survival under these highly stressful conditions. However, as a result of stomatal closure and physiological dysfunction under conditions of water deficit, photosynthesis of *R. mucronata* seedlings is hampered with submergence and it can be expected that more energy will be allocated for root growth. These long-persistenting extremely stressful conditions cause a temporary carbon shortage to be converted to acute carbon starvation, exacerbated in the long-run by minimal addition of photosynthates. This results in unhealthy seedlings with severe signs of damage like necrosis, leaf wilting and abscission, followed eventually by early seedling mortality.

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REZIME

Botanica SERBICA

Stres izazvan nedostatkom ugljenika u sadnicama *Rhizophora mucronata* Lam. usled dugotrajnog potapanja i nedostatka vode; preživeti ili podleći

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Metabolizam ugljenih hidrata u biljci, posebno ukupni sadržaj skroba, ukupni sadržaj rastvorljivog šećera (TSS) i njihova upotreba, važni su za suočavanje sa stanjima abiotičkog stresa. Zbog toga su tokom 11 meseci proučavani ukupni sadržaj skroba i TSS kod *Rhizophora mucronata*, zajedno sa nivoom preživljavanja, rasta i nakupljanja biomase i stomatalne provodljivosti, pod uslovima dugotrajnog potapanja i vodnog stresa. Eksperiment je dizajniran tako da se dobiju tri ponavljanja pri svakom tretmanu i oko 1600 mladih mangrova biljaka koje su proučavane u studiji. Pod dugotrajnim potapanjem i visokim nivoom stresa u vodi, mali broj biljaka mangrove mogao je da preživi i one su se brzo iscrpile zbog većih stopa iskorišćenja skroba (0,75-1,05% suve mase mesečno). Međutim, sadržaj TSS se povećao u uslovima intenzivnog stresa, što nije bio slučaj sa rastom klijanaca i proizvodnjom biomase; naime, uočena je značajna redukcija rasta (i.e., ~78%) i sadržaja suve materije kod biljaka pod stresom u odnosu na biljke u kontrolnim uslovima. Stoga, intenzivno povećanje sadržaja TSS može biti posledica direktnog pretvaranja skroba u rastvorljive šećere, kako bi se proizvela metabolička energija za mehanizme tolerancije, poput osmoregulacije i anatomskog prilagođavanja korena stresnim uslovima. Ovo ukazuje da se više energije izdvaja za održavanje biljaka nego za rast i proizvodnju biomase u stresnim uslovima, i može biti dobra aklimatorska strategija za spašavanje mladih biljaka mangrove u ranoj fazi. Međutim, zatvaranje stoma u stresnim uslovima možda je prouzrokovalo ograničenje fotosinteze. Stoga degradacija skroba izazvana stresom može porasti što zauzvrat može dovesti do nedostatka ugljenika dugoročno, što je stanje smrtonosno za sadnice.

Ključne reči: aklimatizacija, mangrove, održavanje biljaka, rastvor, skrob, ukupni rastvorljivi šećeri