

The role of carbohydrates in seed germination and seedling establishment of *Himatanthus sucuuba*, an Amazonian tree with populations adapted to flooded and non-flooded conditions

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- **Background and Aims** In the Amazonian floodplains plants withstand annual periods of flooding which can last 7 months. Under these conditions seedlings remain submerged in the dark for long periods since light penetration in the water is limited. *Himatanthus sucuuba* is a tree species found in the 'várzea' (VZ) floodplains and adjacent non-flooded 'terra-firme' (TF) forests. Biochemical traits which enhance flood tolerance and colonization success of *H. sucuuba* in periodically flooded environments were investigated.
- **Methods** Storage carbohydrates of seeds of VZ and TF populations were extracted and analysed by HPAEC/PAD. Starch was analysed by enzyme (glucoamylase) degradation followed by quantification of glucose oxidase. Carbohydrate composition of roots of VZ and TF seedlings was studied after experimental exposure to a 15-d period of submersion in light versus darkness.
- **Key Results** The endosperm contains a large proportion of the seed reserves, raffinose being the main non-structural carbohydrate. Around 93 % of the cell wall storage polysaccharides (percentage dry weight basis) in the endosperm of VZ seeds was composed of mannose, while soluble sugars accounted for 2.5%. In contrast, 74 % of the endosperm in TF seeds was composed of galactomannans, while 22 % of the endosperm was soluble sugars. This suggested a larger carbohydrate allocation to germination in TF populations whereas VZ populations allocate comparatively more to carbohydrates mobilized during seedling development. The concentration of root non-structural carbohydrates in non-flooded seedlings strongly decreased after a 15-d period of darkness, whereas flooded seedlings were less affected. These effects were more pronounced in TF seedlings, which showed significantly lower root non-structural carbohydrate concentrations.
- **Conclusions** There seem to be metabolic adjustments in VZ but not TF seedlings that lead to adaptation to the combined stresses of darkness and flooding. This seems to be important for the survival of the species in these contrasting environments, leading these populations to different directions during evolution.

Key words: Carbohydrate reserves, *Himatanthus sucuuba*, submergence tolerance, floodplains, galactomannans, raffinose, population differentiation, Amazon, storage.

INTRODUCTION

In central Amazonian white-water floodplains (várzea), the rivers carry large amounts of sediments in suspension and flood vast forested areas regularly every year. The physical characteristics of the water allow limited light penetration to <1 m in depth (Junk, 1984). In these environments, trees are waterlogged on average for 6 months with a water column that can reach about 10 m (Junk, 1989). Seedlings of these species survive long periods of flooding and even of prolonged submergence in darkness due to the extreme changes in water levels (Parolin, 2009). In addition to the oxygen deficit imposed by flooding, light can also be limiting, so that flooded plants have to deal with the lack of essential resources for respiration and photosynthesis which are critical for plant survival.

Oxygen deprivation interferes with root respiration at the level of electron transport by inducing anaerobic respiration. This leads to metabolic changes, which culminate in an increase in the glycolytic flow. The acceleration of the rate of anaerobic fermentation results in the depletion of the carbohydrate reserves. On the other hand, several plant species accumulate sugars in their tissues during the non-flooded period, which can be an important metabolic adaptation to overcome the anoxic conditions during the flooded period (Albrecht *et al.*, 2004; Piedade *et al.*, 2009). In fact, it has been postulated that the tolerance of plants to anoxia as a consequence of flooding is proportional to the level of available sugar reserves in the roots (Su *et al.*, 1998; Crawford, 1992; Scarano *et al.*, 1994; Schlüter and Crawford, 2001). Soluble sugars, especially sucrose, glucose and fructose, also appear to play an important role in the maintenance of the functions of all living cells. They are considered to be involved in the

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responses to several stresses and act as metabolic signals which activate specific hormone signal transduction pathways (Couée *et al.*, 2006).

Seed germination may be stimulated or inhibited by flooding, depending on flood duration and species considered (Kozłowski, 1997). Seeds of many terrestrial plants that display high germination rates in soil do not germinate in water, because they can quickly lose viability under such conditions (Hook, 1984; Parolin, 2001). The activation of the physiological processes necessary for germination requires an adequate oxygen supply which is inhibited by soil flooding. Thus, in many species, dormancy is imposed and germination is delayed (Ziburski, 1991; Kozłowski, 1997; Kozłowski and Pallardy, 1997).

In several species, the reserves stored in the endosperm of the seeds contain most of the necessary nutrients for the initial development of seedlings (Perata *et al.*, 1997). The type of reserves accumulated can be related to the establishment success and autotrophic developmental stage which guarantees initial seedling survival in each environment (Buckeridge *et al.*, 2004a). Such studies, however, must take into consideration that the germinative capacity and seedling development can vary within a genus (Hoffmann *et al.*, 2004) or species (Harbone, 1988; Ferreira *et al.*, 2005).

Despite the importance of understanding the metabolic adaptation in seed germination and seedling survival in response to flooding, information related to Amazonian floodplain trees is still scarce (Scarano *et al.*, 1994; Parolin, 2001; Ferreira *et al.*, 2007; Oliveira-Wittmann *et al.*, 2007). *Himatanthus sucuuba* (Apocynaceae) is one of a few tree species that occurs both in floodplains and non-flooded 'terra firme' ecosystems (Wittmann *et al.*, 2006). Different populations of the same species inhabiting such contrasting habitats are certainly subjected to different ecological requirements. In fact, 'várzea' (VZ) and 'terra firme' (TF) populations of *H. sucuuba* showed marked differences in root metabolism when seedlings are totally submerged. TF seedlings did not tolerate prolonged continuous submersion. In Ferreira *et al.* (2009), the anaerobic metabolism in both populations, TF and VZ, were evaluated by activity of the enzyme alcohol dehydrogenase. In TF seedlings, this activity increased until 15 d after submersion, followed by a continuous decrease and, subsequently, seedling death. In contrast, most VZ seedlings survived and kept alcohol dehydrogenase activity high even after 120 d of submersion. Thus, the differences in anaerobic respiration between submerged TF and VZ seedlings seems to reflect the greater storage capacity of root carbohydrates by VZ seedlings.

So far, one important stage for seedling establishment, namely seed germination, has not been studied in this species. The analysis of the storage compounds in seeds may be used as a tool to understand ecological aspects of germination and establishment of plants and their adaptations to flooded environments. Therefore, the main question of the present paper is whether the metabolic reserves of seeds in populations of *H. sucuuba* growing in floodplain and in non-flooded terra firme differ according to the diverse biotic and abiotic pressures imposed by their environments of origin. Here it is shown that these differences exist, with contrasting structural features of the storage polysaccharides and also

with differential metabolic changes in root carbohydrate reserves during the initial phase of seedling establishment.

MATERIALS AND METHODS

Study areas

The white-water rivers have their catchment area in the Andes and are loaded with nutrient-rich sediments. Ecosystems periodically inundated by these rivers are called várzea and occur on an area of approx. 200 000 km² (Prance, 1979; Junk, 1993). They are characterized by a monomodal, predictable flood-pulse. The yearly water level fluctuations can be up to 14 m and the period of inundation can last from 50 to 270 d (Junk, 1993; Fig. 1A, D). During the period of flooding, no light could be measured below 3 m in the turbid water of the várzea (Sioli, 1984; Parolin, 2009). Complete submergence in the dark occurs regularly at the seedling or sapling stage for many species that colonize low-lying positions in the flooding gradient (Fig. 1A, B). Here, hypoxic conditions prevail close to the water surface in moving water, while anaerobic conditions are common in stagnant pools (Parolin, 2009). Depending on the flood height and flood duration, different forest types are established (Fig. 1A): high-várzea forest (flooded <140 d year⁻¹), low-várzea forest (flooded 140–230 d year⁻¹) and chavascal (flooded >230 d year⁻¹) (Wittmann *et al.*, 2002). In contrast, upland terra firme forests (Fig. 1C, E) are those above the maximum flood level of Amazonian rivers. Consequently, these forests never flood. They represent the main forest type across the region.

Plant material

Himatanthus sucuuba (spruce) Woodson, Apocynaceae, is an arboreal latex tree ranging from 8 to 20 m in height (Plumel, 1991). In regions surrounding the city of Manaus *H. sucuuba* is frequently found in low-várzea (Fig. 1B, F, G) areas where the plants may remain under continuous inundation up to 5 months (Wittmann *et al.*, 2002), and in terra firme forests (Fig. 1E), where there is no waterlogging (Ribeiro *et al.*, 1999).

Experimental site and plant collection locations

Seeds of *Himatanthus sucuuba* were collected in várzea (VZ) and terra firme (TF), both in the vicinity of the city of Manaus, Brazil. The VZ location was on and around the island of Marchantaria (03°15'S, 60°00'W) and the TF location was in the forest reserve Adolpho Ducke (02°53'S, 59°58'W) along the Manaus–Itacoatiara road (AM 010). At each site, seeds were collected from three different populations (six populations in total). Seeds were mixed to obtain one single lot per environment.

The analyses of synchronicity and velocity of germination were performed in a greenhouse at the National Institute for Amazon Research (INPA) in Manaus. Biochemical analyses of seeds and seedlings were carried out in the Laboratory of Physiology and Biochemistry of Plants at the Institute of Botany of São Paulo, São Paulo, Brazil.

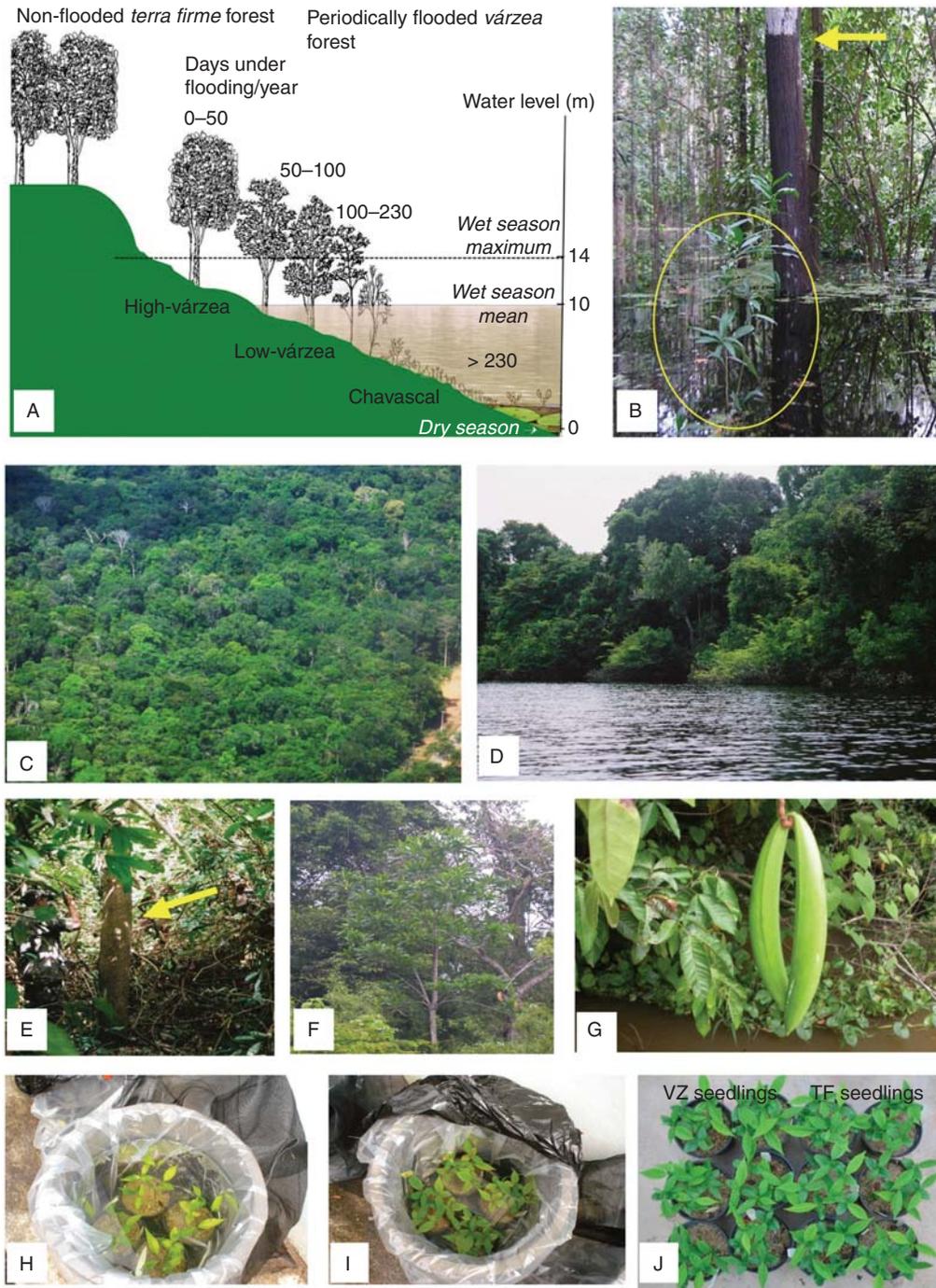


FIG. 1. (A) Schematic presentation of the major vegetation types along the flooding gradient. Flood amplitude, water levels and the duration of the terrestrial and aquatic phases vary considerably along the topographic gradient. (B) The interior of a periodically flooded várzea forest during the period of water retreat. The arrow indicates the maximum level reached by the water column during the flood period and in focus, a juvenile individual of *H. sucuuba*. (C) Non-flooded terra firme (TF) forest; (D) periodically flooded várzea (VZ) forest; (E) *H. sucuuba* within a TF forest; (F) tree in a VZ forest; (G) fruit of *H. sucuuba* during the flooding period in a VZ forest; (H) VZ and TF seedlings after 30 d of flooding under light conditions; (I) VZ and TF seedlings after 30 d of flooding under dark conditions; (J) VZ and TF seedlings after 30 d in the control treatment. (F) and (G) Pictures by Florian Wittmann.

Biochemical analyses of seeds

Non-structural carbohydrate reserves in whole seeds. Five lots of ten seeds each from the two populations (VZ and TF) were analysed with respect to the contents of non-structural carbohydrates,

i.e. content and composition of starch and soluble sugars. The seed coat was removed manually and the seeds were lyophilized for 48 h, weighed to determine dry weight and subsequently ground to a powder in a ball mill (Tecnal Ltda).

The analysis of starch was performed by the enzymatic method described by Amaral *et al.* (2007), and soluble sugars were extracted with 80 % ethanol at 80 °C (three times) and the carbohydrates determined by the phenol–sulfuric method of Dubois *et al.* (1956). The composition of sugars was determined by high-performance anion exchange chromatography with pulsed amperometric detector (HPAEC/PAD model DX500) on a CarboPak PA-1 column (Dionex Corporation, Sunnyvale, CA, USA) by elution with water, followed by a post column reaction using a flow of 1 mL min⁻¹: 0–15 min 200 mM NaOH (50 %) and H₂O (50 %), 15–20 min 200 mM NaOH (100 %), 20–25 min 200 mM NaOH (50 %) and H₂O (50 %). The areas of each peak were corrected in accordance with the sensitivity of the detector for each sugar (Santos and Buckeridge, 2004).

Composition of endosperm reserves. In seeds of *H. sucuuba*, the endosperm is not clearly visible and the seeds may easily be treated as being without endosperm (Ferreira *et al.*, 2005). However, visual observations during the first hour of imbibition of the seed revealed an endospermic structure adhering to the coat, with a viscous aspect. This endosperm decreased in volume with the emission of the radicle and disappeared completely after the stem became fully developed. To evaluate the importance of the endosperm as a supply of reserves for seedling development, the following procedure was adopted: for each seed group (VZ and TF) three lots of ten scarified seeds were incubated in water for 24 h. Thereafter, the coat was removed; the endosperm was exposed, and separated from the embryo. Both the embryo and endosperm were lyophilized for 48 h to determine dry weight. Storage carbohydrate content and proteins were determined in the endosperms.

The extraction of reserve carbohydrates was based on the method described by Buckeridge and Dietrich (1990), which consists of the extraction from powdered endosperm plus seed coat with hot water (80 °C, 0.1 g mL⁻¹) for 4 h. After filtration through cheesecloth, centrifugation was performed (10 000 g for 30 min at 5 °C) followed by precipitation with three volumes of ethanol. The precipitate was left overnight at 5 °C, collected by centrifugation and lyophilized. The residue was lyophilized for quantitative analyses of carbohydrates (Dubois *et al.*, 1956) and proteins (Bradford, 1976). The composition of the extracted carbohydrates was determined by acid hydrolysis, quantified through HPAEC/PAD as described in the methodology for the analyses of the reserves of the intact seeds. The profile of monosaccharides obtained was compared with the one of the following standard monosaccharides: fucose, arabinose, rhamnose, galactose, glucose, xylose and mannose.

Experiment 1: seed germination characteristics

Himatanthus sucuuba has quiescent seeds. A previous study (Ferreira *et al.*, 2007) showed that seeds of VZ and TF populations germinated in flooded as well as in non-flooded substrate, but they showed significant differences in germination rates and seedling emergence. Thus, in order to increase knowledge of the germinative strategies of the species,

average time, relative frequency and synchrony of germination of the populations in two treatments – non-flooded (washed sand; watered daily) and flooded (washed sand, submerged by a water column of 5 cm height) – were measured. The experiment was carried out as described in Ferreira *et al.* (2007): the seeds were allowed to germinate in trays (20 cm × 60 cm) at ambient temperature (average of 28 °C). Each tray was considered as a unit and there were four replicates with 50 seeds each. After germination, the seeds of the non-flooded treatment were transferred to polyethylene pots (2 L), filled with sand substrate and superficially covered with vermiculite, for the formation and the establishment of seedlings to be used in the flooding experiments.

Experiment 2: root carbohydrate reserves

During the period of flooding, light penetration in várzea is low and seedlings remain submerged in the dark for long periods (Sioli, 1984). In a greenhouse, the effect of flooding and light on the concentration of carbohydrate reserves during the initial phase of seedling establishment was evaluated. For the simulation of flooding and irradiance the experimental design was completely randomized, with four treatments and three replicates. Each replicate consisted of a pot with one seedling. The four treatments were performed in polyethylene tanks (Fig. 1H–J): (1) with light (daily irrigation and 70 % of full sunlight); (2) in the dark (daily irrigation and complete darkness); (3) flooded with light (total submersion of the plant, with a water column of 50 cm and 70 % of full sunlight); and (4) flooded in the dark (total submersion of the plant, with a water column of 50 cm and complete darkness). Complete darkness was achieved by covering the tank with two black plastic layers. Inside each tank two pots with seedlings from each of the ecosystems (VZ and TF) were randomly positioned. When the experiment started, the seedlings were 1 month old, with the third pair of leaves totally expanded. VZ seedlings had an average height of 11.5 cm while TF seedlings averaged 8 cm in height.

Starch and sugar concentrations were determined in the roots of seedlings from the two ecosystems (VZ and TF) immediately before the beginning of the seedling experiments (time zero) – and repeated after 15 d ($n = 3$, for each combination of time and place of origin). The total amount of non-structural carbohydrates was calculated by adding the concentrations of the reserves analysed.

For the analyses, three seedlings from each environment (VZ and TF) were collected, and separated into aerial part and roots, and kept in liquid nitrogen. The starch analysis was performed according to the methodology described by Amaral *et al.* (2007). Soluble sugars were measured by gas chromatography coupled with mass spectrometry (GC-MS Varian Saturn 2000R, DB-225 capillary column), using a derivatization procedure (trimethylsilylation). The resolution and the selectivity of this technique simultaneously allowed the determination of some carbohydrate classes (sucrose, glucose, fructose), using the same aliquot of extracted sample, in accordance with the methodology described by Roessner *et al.* (2000) and identified electron-impact mass spectrometry as described by Carpita and Shea (1989).

Statistical analyses

Calibration curves to determine the concentrations of soluble sugar, starch, carbohydrates and proteins present in the whole seed or in its endosperm were adjusted by means of regression analysis (r^2 between 0.98 and 0.99). The content of each compound was calculated in relation to the dry weight of the seed. The average time of germination (\bar{t}) was calculated in days, relative frequency (f) and synchrony index of germination (U) were evaluated as described by Labouriau (1983) for each treatment and origin. The following equations were used

$$\bar{t} = \frac{\sum_{i=1}^k n_i t}{\sum_{i=1}^k n_i}$$

and

$$U = - \sum_{i=1}^k f \log_2 f$$

where t = time of germination (in days); n_t = number of seeds germinated at the time t and k = the last time a seedling germinated.

A three-way analysis of variance was used to test for the effects of light, flood and plant origin on carbohydrate concentrations. To compare how much of the overall interspecific variance of a trait can be attributed to differences between light and darkness, flooded and non-flooded conditions or to differences between plant origin (VZ \times TF), the partial r^2 values for each trait were calculated. These were calculated from factorial ANOVAs, following Rosenthal and Rosnow (1985) where $r^2 = SS_{\text{factor}}/SS_{\text{total}}$ (SS_{factor} is the sum of squares for factor – irradiance, flooding or plant origin) and SS_{total} is the sum of squares).

RESULTS

Non-structural carbohydrate reserves in intact seeds

Seeds of *H. sucuuba* do not have starch as the main carbohydrate as only traces of this polysaccharide were detected. Soluble sugars are the accumulated carbohydrates and these correspond to approx. 15 % of total dry weight of the seeds in the two populations, which as a mean is equivalent to 140 mg g⁻¹ (± 20 s.d.) of seed dry weight for the VZ population and 135 mg.g⁻¹ (± 8.8 s.d.) for the TF population. VZ seeds of *H. sucuuba* showed the same composition of reserves as those from TF, although there were differences in the concentrations of these substances between the two populations. The highest concentrations of soluble sugars were raffinose (69–58 % for VZ and TF, respectively) and sucrose (27–40 % for VZ and TF, respectively). Among the monosaccharides, only glucose was present in lower concentrations (4–2 % for VZ and TF, respectively).

TABLE 1. Mean dry seed weight of *H. sucuuba*: mass of embryo, endosperm and entire seed

	VZ		TF	
	(mg ⁻¹ g MS)	(%)	(mg ⁻¹ g MS)	(%)
Embryo	338 \pm 14	45	399 \pm 4.2	45
Endosperm	413 \pm 21	55	488 \pm 5.2	55
Total	752 \pm 38	100	887 \pm 9.2	100

$n = 5$ lots of ten seeds each.

Composition of endosperm reserves

The seeds of *H. sucuuba* from the VZ and TF populations have an endosperm that alone contributes to more than half (55 %) of its total dry weight (Table 1). Seeds from the two ecosystems did not differ regarding the proportion among seed parts (Table 1). However, the endosperms from TF and VZ seeds contain cell wall storage polysaccharide (CWSP) and soluble sugars in different proportions. CWSP accounted for about 93 % of the endosperm of VZ seeds while soluble sugars accounted for only 2.5%. In contrast, 74 % of the endosperm in TF seeds was composed of CWSP, while 22 % of the endosperm was soluble sugars. The yield of proteins was similar between TF and VZ populations: 4 % and 4.5 %, respectively.

In addition to these differences in the proportion of CWSP and soluble sugars between the two populations, the acid hydrolysis of the CWSP revealed remarkable differences in the composition of the monosaccharides present in the endosperm of the two populations (Table 2). Mannans are usually classified as pure mannans, which contain over 90 % of mannose, or galactomannans and also contain higher proportions of galactose (>10 % of the polysaccharide). The results shown in Table 2 indicate that VZ seeds have pure mannan, whereas TF seeds probably have a galactomannan as the mannose:galactose ratio of the latter is approx. 3:1.

In Fig. 2, it can be seen that at the same time as mannose is lower in TF seeds, the higher proportion of galactose (9-fold in relation to VZ) concurs with 9-fold increases in arabinose and rhamnose. This suggests that the walls of seeds from TF might have relatively more pectin (i.e. arabinogalactan) in comparison with the mannan-bearing walls of VZ seeds. This does not change the suggestion that TF has a more water permeable endosperm, as pectins would play an analogous role to galactomannan.

TABLE 2. Composition (%) of monosaccharides in relation to total area of reserve monosaccharides of the wall in the endosperm of *H. sucuuba*

	Várzea (%)	Terra firme (%)
Fucose	Traces	0.5
Arabinose	1.5	9.7
Rhamnose	0.3	2
Galactose	3	17.7
Glucose	0.5	2.6
Xilose	0.4	2.9
Manose	94.3	64.6

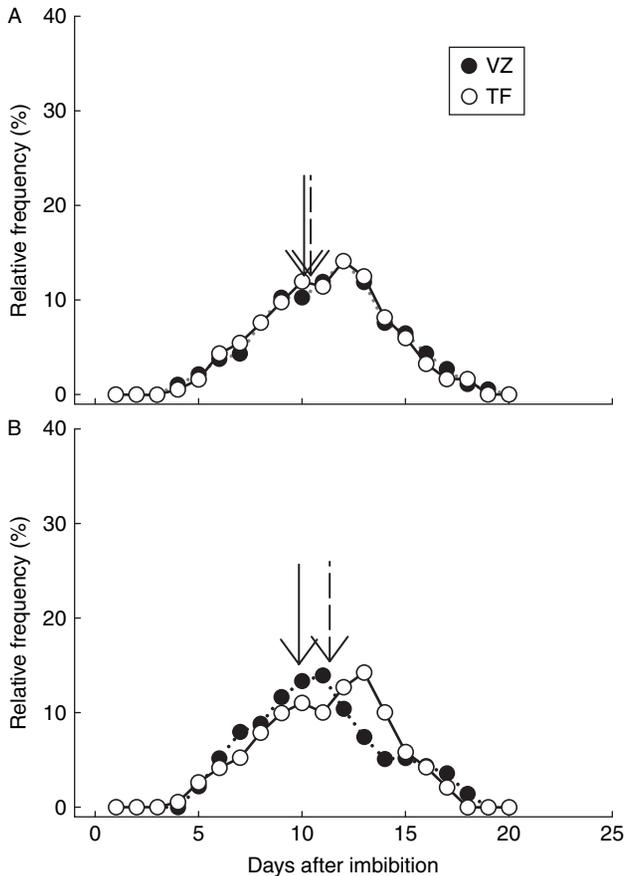


FIG. 2. Relative frequency of experimental seed germination in *H. sucuba* from VZ and TF populations, as indicated, in non-flooded (A) and flooded (B) conditions. Solid arrows indicate mean germination time of VZ populations; dashed arrows indicate mean germination time of TF populations ($n = 50$).

Germination characteristics of seeds

No differences were found in the germination characteristics of the seeds from the two populations (VZ and TF) which germinated in the non-flooded substratum. The percentage of germination for both VZ and TF was higher than 90%. In the flooded treatment, most seeds from VZ germinated faster, within an average of 10 d, compared with an average of 11 d in TF seeds, but the difference was not statistically significant

($P > 0.05$). On the other hand, VZ seeds, when flooded, showed a peak of germination earlier than TF (Fig. 2B). Seeds of VZ germinated well or even better when flooded (95%), whereas TF had a reduced percentage of germination (30%) as compared with the non-flooded treatment. The synchrony index ($P = 0.79$) was similar for both populations. However, a clear pattern was observed in seeds which germinated while flooded – it tended to be more synchronous than the ones in the non-flooded treatment ($P < 0.05$).

Analyses of non-structural carbohydrate reserves in seedling roots

Roots of seedlings from VZ and TF included starch, sucrose and the monosaccharides glucose and fructose in their composition (Table 3). The comparison of root carbohydrate reserves of light-exposed, non-flooded seedlings at time 0 (zero) and after 15 d showed that seedlings from VZ accumulated more carbohydrate reserves in their roots than those from TF ($P < 0.05$).

During 15 d in light, the sugar concentrations remained constant in both populations (VZ and TF) (Table 4). Starch decreased slightly, especially in TF population, whereas soluble sugars (sucrose, glucose and fructose) increased significantly. Root reserve concentrations decreased especially in the darkness, whereas flooding plus darkness had a significant effect on the concentrations of non-structural carbohydrate reserves accumulated in the roots. The roots of seedlings from VZ accumulated proportionally more non-structural carbohydrates than those from TF (Table 4). In an experiment in which light and flooding were combined, the level of non-structural carbohydrate was significantly reduced in both populations.

Starch decreased in both populations (VZ and TF), and at the same time there was an increase of the concentrations of most soluble sugars, especially sucrose (Table 4), denoting the fact that roots probably use stored starch when light is absent and also when flooding occurs. The combination of both factors also leads to a decrease in starch and concomitant disappearance of sucrose, glucose and fructose, indicating that the seedlings use their carbohydrates very intensely. An interesting difference between darkness with and without flooding is that in the former the level of sucrose is very low after 15 d, whereas in the latter case, i.e. with flooding, sucrose accumulates in the roots (Table 4).

TABLE 3. Significance values and fraction of total interspecific variance (r^2) as explained by light (light \times darkness), flooding (flooded \times non-flooded) or differences between origin of plants (várzea \times terra firme) in sugar concentrations

Trait	P light	P flood	P origin	P light \times flood	r^2 light	r^2 flood	r^2 origin	r^2 light \times flood
Starch	0.000	0.877	0.000	0.000	0.32	0.00	0.21	0.27
Sucrose	0.643	0.194	0.042	0.000	0.00	0.02	0.07	0.61
Glucose	0.307	0.463	0.000	0.004	0.01	0.00	0.34	0.17
Fructose	0.167	0.774	0.012	0.033	0.06	0.00	0.22	0.16
Total reserves	0.000	0.385	0.000	0.000	0.19	0.00	0.23	0.45

The r^2 values were calculated from ANOVA sums of squares as explained in Materials and methods.

TABLE 4. Reserves of carbohydrates (mg g⁻¹) in roots of seedlings of *H. sucuuba* before (0 d) and after 15 d of the experiment

	0 d	15 d			
		Light	Darkness	Submersion, light	Submersion, darkness
Varzea					
Starch	122.07 ± 12.88	119.85 ± 13.74	8.44 ± 3.08	74.47 ± 6.08	29.31 ± 3.56
Sucrose	13.78 ± 2.22	51.05 ± 8.22	1.88 ± 1.34	10.18 ± 2.05	56.32 ± 17.42
Glucose	7.67 ± 1.76	33.09 ± 8.28	28.63 ± 4.46	5.55 ± 0.78	35.75 ± 6.80
Fructose	2.23 ± 0.64	8.70 ± 1.30	8.16 ± 1.88	1.88 ± 0.75	11.47 ± 5.22
Total	145.76 ± 13.47	212.70 ± 21.89	47.11 ± 3.47	92.08 ± 4.10	132.85 ± 15
Terra firme					
Starch	91.07 ± 6.68	67.93 ± 12.24	12.57 ± 5.88	31.34 ± 10.64	24.71 ± 3.81
Sucrose	9.91 ± 2.61	27.77 ± 6.21	1.0 ± 0.55	11.99 ± 0.89	31.89 ± 6.34
Glucose	7.05 ± 1.98	12.39 ± 4.01	1.36 ± 0.58	11.77 ± 6.37	11.89 ± 1.96
Fructose	0.66 ± 0.15	2.99 ± 0.84	0.59 ± 0.44	3.23 ± 1.31	5.71 ± 2.06
Total	108.69 ± 4.67	111.09 ± 6.39	15.53 ± 7.15	58.34 ± 6.06	74.21 ± 13.77

Means and standard error; $n = 3$.

DISCUSSION

Storage compounds in seeds

Himatanthus sucuuba germinates in a short period of time. Under these conditions, storage compounds are quickly degraded and used as sources of energy for the protrusion of the radicle and further seedling development. Soluble sugars fill this role during germination whereas galactomannans come into play after germination (Buckeridge *et al.*, 2000). Soluble sugars in seeds are composed mostly of raffinose, followed by sucrose. In quiescent seeds, these are usually the most abundant reserve carbohydrates, which are normally associated with the raffinose-series oligosaccharides (raffinose, stachyose, verbascose) (Berna-Lugo and Leopold, 1992; Buckeridge *et al.*, 2004b) and in many seeds they are degraded during germination (Koops and Groeneveld, 1990; Buckeridge and Dietrich, 1996; Karner *et al.*, 2004). Besides the storage function, sucrose is also efficient in protecting membrane integrity in the systems which are exposed to drought. Raffinose is known to enhance the protective effect of sucrose, thus preventing the desiccation of the seed (Berna-Lugo and Leopold, 1992; Karner *et al.*, 2004). The concentrations of these compounds generally appear to have increased at the end of the period of seed maturation where they can play a role in protecting against desiccation. Later on, during germination, their main function is to serve as a carbon source for the seeds during the germinative process as they display features that confer advantages to germination, such as being fast-use reserves for energy production (Koops and Groeneveld, 1990; Buckeridge *et al.*, 2004b). Thus, the raffinose family oligosaccharides and sucrose can be considered to be dual function molecules.

The endosperm of *H. sucuuba* stores relatively large proportions of the reserves for the whole seed. This will be available for seedling development, i.e. after germination. In this case the storage compound is a cell wall polysaccharide. The monosaccharide composition found in the water-soluble polysaccharide of endosperms of VZ and TF populations suggests that the former has mannan and the latter a galactomannan (or a wall richer in arabinogalactan) as storage compounds. The biosynthesis and deposition of CWSP occur in the final

phase of seed maturation (Buckeridge *et al.*, 2000) and these substances are mobilized after germination, during seedling development. This is an especially sensitive stage of the life cycle of a plant, because growth and initial survival can be heavily influenced by the available reserves of the seed (Buckeridge and Dietrich, 1996, Santos and Buckeridge, 2004). At this stage, mortality is high and a small increase (due to a better strategy in using reserves) in the number of surviving individuals can have an important effect on the variability of the population. Another important aspect of having carbon reserves is the fact that seedlings can grow faster and higher. Rapid growth increases the time in which the cotyledons and young leaves can photosynthesize, thus producing a taller young plant and at the same time providing more investment by the plant to establish its roots more quickly. In *H. sucuuba*, these features also help young plants adapt to the rapid rise of water during the flooding of the Amazon river.

The difference in composition of the CWSPs in seeds of the two populations seems to be meaningful. In the case of VZ, the presence of a mannan would slow down water uptake, but at the same time, the presence of higher concentrations of soluble sugars would speed up germination. These features are consistent with a species that is more adapted to an environment where seeds are exposed to more water. Indeed, it has been assumed that more stripped galactomannan molecules (i.e. with higher mannose:galactose ratios) are usually (but not always) associated with environments with plenty of water (Buckeridge *et al.*, 2000). On the other hand, the more galactosylated galactomannans (i.e. with lower mannose:galactose ratios) absorb and lose water quickly and store proportionally more water in the seed tissues. Thus, environments with relatively less water (i.e. TF) may benefit from a more branched galactomannan.

Galactomannan is a more abundant CWSP in nature, being found in the seed endosperms of a large number of species (Reid *et al.*, 2003; Buckeridge *et al.*, 2004a). To date, the available data indicate that galactomannan has features compatible with bifunctional molecules, with an important role in the control of imbibition at the start of germination, and later serving as a carbon reserve for the initial growth of the seedling. This has been shown for *Sesbania virgata*

(Leguminosae; Faboideae), where the function of the galactomannan is more as a water supply to the seeds than as a carbon supply for the embryo (Potomati and Buckeridge, 2002). The control of the storage and mobilization of CWSP for some species of plants can improve the ecophysiological performance of seedlings in their natural environment (Tonini *et al.*, 2007). The results presented in this work strongly suggest that distinct ecological functions are played for galactomannan in this species during seed imbibition and seedling development.

This becomes evident when looking at the differences in the accumulated reserve types in seeds of the two environments. While in VZ CWSP make up for >90 % of the seed reserves, in TF the contribution of soluble sugars is of approx. 30%. Thus we suggest that VZ populations invest in carbohydrate production for seedling development, whereas the TF populations allocate comparatively more carbon for germination.

In the várzea, plants have only a short period of time (about 3 months) for germination and establishment to occur before the next flooding. This selective pressure is not imposed on the plants from a population that inhabits the non-flooded terra firme. Thus, it can be expected that in the germination process the seeds of the várzea would mobilize their reserves more quickly than those of the terra firme. This hypothesis has been confirmed by the results of synchronicity and by the differences in germination which is lower in the TF populations in a flooded substrate.

Storage compounds in seedlings in relation to flooding

The elevated starch concentrations found in the roots of seedlings of *H. succuba* indicate that this is the main reserve carbohydrate accumulated for the initial phase of establishment. These concentrations are similar to those measured on tree seedlings from tropical savannahs (Hoffmann *et al.*, 2004), where root carbohydrate reserves are essential for surviving fire and seasonal drought. These reserves should provide *H. succuba* seedlings with greater ability not only to survive long-term submersion, but also to replace their above-ground biomass following the retreat of the flood. Submerged seedlings of this species develop leaf chlorosis and premature leaf abscission in response to long-term flooding (Ferreira *et al.*, 2009). Moreover, the reduction in starch and the rise in concentration of other types of reserve carbohydrate (soluble sugars) in submerged seedlings exposed to a 15-d period of darkness, suggests that the reserves can quickly be used in response to flooding, probably to feed anaerobic respiration (Ferreira *et al.*, 2009).

Flooded plants need carbohydrates, which can become quickly available, to support an active fermentation metabolism (Guglielminetti *et al.*, 1995) as is the case in *H. succuba*. Although this occurred in seedlings of both ecosystems (VZ and TF), the concentrations of reserve carbohydrates in roots of VZ were significantly higher than in TF. Seedlings of *H. succuba* from populations of the TF do not survive under prolonged flooding, in contrast to high flooding tolerance in seedlings of the same species in várzea (Ferreira *et al.*, 2007). A reason for these discrepancies may be the differential capacity in accumulating and mobilizing reserves for anaerobic metabolism (Ferreira *et al.*, 2009).

Metabolic adjustments used as an adaptive strategy against the combined stress of flooding and absence of light, imposed on seedlings of the várzea but not of the terra firme, seem to be crucial for the survival of *H. succuba* in the white-water floodplains. The carbohydrate concentrations decreased as a response to flooding. However, the reduction was lower in the flooded treatment in darkness than in light. This suggests that under the typical conditions of the várzea, there is only a slow use of the reserves and growth is arrested (Ferreira *et al.*, 2007).

Although a connection between the events occurring in the endosperm and developing roots of *H. succuba* have not been specifically proven in this work, it is likely that there is a correlation between (galacto)mannan degradation in the endosperm and the accumulation of starch and soluble sugars in the roots. In the legume seeds studied, the CWSP is degraded and its products are transported to the growing seed parts (Buckeridge *et al.*, 2000). Thus, in the case of *H. succuba*, roots are these developing organs and a connection between CWSP degradation in the endosperm and synthesis of starch and sucrose metabolism in the roots is expected to exist, but this remains to be investigated.

Conclusions

Carbohydrates play several important roles in seeds and seedlings of *H. succuba*. The composition and distribution of soluble sugars and CWSPs appear to be related to differences in terra firme and várzea populations of the species to tolerate flooding. In roots, starch and soluble carbohydrates are metabolized differently in flooded and non-flooded seedlings. In the case of the Amazonian species *H. succuba*, carbohydrates appear to be one of the key features concerning seed germination and seedling survival in response to flooding. These findings denote the capacity of adapted species to respond in terms of carbohydrates to changes in environmental conditions, highlighting an interesting case in which two populations of an Amazonian species appear to be under different selective pressures, whose responses are related to the way they use their carbohydrate storage compounds. This seems to be important for the survival of the species in these contrasting environments and leading these populations to different directions during evolution.

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