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Parameters Affecting the Early Seedling Development of Four Neotropical Trees under Oxygen Deprivation Stress

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Some of the parameters that determine flooding resistance—and consequently habitat zonation—were investigated in four neotropical trees (*Schizolobium parahyba, Sebastiania commersoniana, Erythrina speciosa* and *Sesbania virgata*). The constitutive parameters of seeds (size, nature and amount of reserves) only partly influenced resistance to flooding, mainly through a high carbohydrate : size ratio. Parameters describing metabolic efficiency under stress conditions were more important. Among them, fermentation capacity and levels of ATP and of total adenylates played a key role. The highest resistance to anoxia was associated with increased availability of free sugars, elevated alcohol dehydrogenase activity and corresponding mRNA levels, more efficient removal of ethanol and lactate, and higher adenylate levels. Finally, as a lethal consequence of energy shortage, free fatty acids were released on a massive scale in the flooding-sensitive species *Schizolobium parahyba*, whereas lipid hydrolysis did not occur in the most resistant species *Sesbania virgata*.

Key words: Adenylate levels, energy shortage, fermentation capacity, flooding resistance, habitat zonation, lipid hydrolysis, metabolism, seed reserves, *Erythrina speciosa*, *Schizolobium parahyba*, *Sebastiania commersoniana*, *Sesbania virgata*.

INTRODUCTION

Flooding and a high water table frequently limit the distribution of plants (Crawford, 1992). This is shown by a distinct zonation of plants in wet habitats. Permanently flooded areas are occupied only by extremely flooding-resistant plants, and this resistance usually consists of morphological and physiological adaptations (Armstrong *et al*., 1994; Braendle and Crawford, 1999). Flooding can be considered primarily as hypoxic or anoxic stress. The most prominent morphological adaptation of plants is the formation of aerenchyma to facilitate oxygen transport, and the most important physiological adaptation is the switch from respiration to continuous fermentation. Morphological adaptations are related to plant development, whereas physiological adaptations start immediately after the onset of oxygen deprivation stress, both within their genetically fixed limits (Armstrong *et al*., 1994; Braendle and Crawford, 1999).

The fully grown neotropical trees *Sebastiania commersoniana* (Baillon) Smith & Downs (Gibbs and Leitão-Filho, 1978; Kolb *et al*., 1998), *Sesbania virgata* (Cav.) Pers. (Eisinger, 1984) and *Erythrina speciosa* Andrews (Lorenzi, 1992) show a preference for flooded and waterlogged areas, while *Schizolobium parahyba* (Vell.) Blake is unable to occupy such areas (Joly, 1994). *Schizolobium parahyba* (Leguminosae) is present in south, southeastern and in the southern part of northeastern Brazil in the Atlantic forest (Lorenzi, 1992), where it occupies well-drained soils (Joly, 1994). *Sebastiania commersoniana* (Euphorbiaceae) occurs in south and southeastern Brazil in riparian or gallery forests (Lorenzi, 1992). It is a dominant species and may account for 60–80 % of the arboreal cover (Reitz, 1988). Flooding takes place in the rainy season, when water rises 1–2 m above the soil surface. Inundation can continue for up to a few weeks. *Erythrina speciosa* (Leguminosae) is found in waterlogged areas in south and southeastern Brazil in the Atlantic forest (Lorenzi, 1992). *Sesbania virgata* (Leguminosae) occurs in the floodplains of rivers, lakes and dams (Eisinger, 1984), and is flooded in the rainy season. The height and duration of inundation are similar to those described for *Sebastiania commersoniana*.

For all flooded plants, including wetland plants, germination and early growth are periods of extreme sensitivity (Armstrong *et al*., 1994; Braendle and Crawford, 1999). It is not known whether differences already exist in the intrinsic properties of these seeds or among parameters of the early development of these species which could explain their relative resistance to oxygen deprivation and, consequently, their localization. Thus, our aim was to identify some of the characteristics that contribute to the distinct zonation of these neotropical trees and to distinguish between the role of the intrinsic properties of seeds and that of the stress-induced physiological and biochemical parameters. These parameters should help explain why, at the seedling stage, *Sesbania virgata* is the best adapted to withstand soil inundation, whereas the rather sensitive *Schizolobium*

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parahyba clearly favours dry sites. They might also explain the intermediate behaviour of the other two species, the seedlings of *E. speciosa* being better adapted to oxygen shortage than those of *Sebastiania commersoniana*.

MATERIALS AND METHODS

Seed collection

Sebastiania commersoniana seeds were collected in Parque Estadual Mata dos Godoy, Londrina, State of Paraná, Brazil. Seeds of the other three species were collected in Parque Ecológico Hermógenes de Freitas Leitão Filho, Campinas, State of São Paulo, Brazil. Seeds were collected from several trees of each species located in dry areas (for *Schizolobium parahyba*) and in drained and waterlogged areas (for the three other species). Seeds were stored at room temperature in glass containers. At 25 °C and with a photoperiod of 12 h, the germination capacities of *E. speciosa* and *Sesbania virgata* seeds were close to 100 %, whereas those of *Schizolobium parahyba* and *Sebastiania commersoniana* were close to 80 %.

Treatments

Seeds were scarified with sandpaper and allowed to germinate at 25 °C under a photoperiod of 12 h. After radicle protrusion (2 d for *Schizolobium parahyba*, 4 d for *E. speciosa* and *Sesbania virgata*, and 5–6 d for *Sebastiania commersoniana*), young seedlings were subjected to up to 48 h of anoxia and measurements were made on the apical part of the roots (0·5–1·0 cm for *Schizolobium parahyba*, 1·5–2·0 cm for *E. speciosa* and *Sebastiania commersoniana*, and 2·0–2·5 cm for *Sesbania virgata*). Anoxic treatments were carried out by incubating the plant material (stored in watered Petri dishes) in an anaerobic workbench (model 1029; Forma Scientific, Marietta, OH, USA) with an atmosphere of 90 % N_2 and 10 % H_2 and containing a palladium catalyst to scavenge residual O_2, as described by Rawyler *et al*. (1999). The O_2 level (monitored with a Toray O_2 analyser, model LF 700; Lippke GmbH, Neuwied, Germany) was always <10 ppm. For the flooding treatments, seedlings were planted in common garden soil and the water table was permanently maintained 1–2 cm above the soil surface. Seedlings were then kept under air with a 16/8 h light/dark regime at 25 °C.

Preparation of the biological material

Excised cotyledons of *Schizolobium parahyba*, *E. speciosa* and *Sesbania virgata*, as well as endosperm and cotyledons of *Sebastiania commersoniana* were ground to a powder using a pestle and mortar. The very hard endosperms of *Schizolobium parahyba* and *Sesbania virgata* were simply crushed into small pieces. In some instances, fresh roots were ground to a fine powder in the presence of liquid N_2 with a dismembrator (Mikro II; B. Braun, Melsungen, Germany) for 1 min. All powders were stored at –80 °C until use.

Extraction and analysis of seed reserves

Lipids were extracted from five replicates of approx. 100 mg powder each. The methods for extraction and quantitation of total lipids were as in Rawyler *et al*. (1999).

Carbohydrates were extracted from five replicates of 50–90 mg powder each. The fine powder was first defatted for 15 min with hexane : isopropanol (3 : 2, v/v) in a sonication bath at 80 °C. After centrifugation at 1000 *g* for 5 min and elimination of supernatants, ethanol : water (8 : 2, v/v) was added to the residue and the suspension was sonicated again for 20 min at 80 °C. After centrifugation, the pellet was re-extracted once as above. The pooled supernatants were used for free sugar determinations (Scott and Melvin, 1953). The pellet was incubated for 2 h in 30 % $HClO_4$. After centrifugation, the pellet was extracted again overnight. The pooled perchloric supernatants were used for starch determinations (Scott and Melvin, 1953). The values obtained were then multiplied by 0·9 to allow for conversion of hexose to starch (McCready *et al*., 1950).

Cell-wall storage polysaccharides were extracted in water from five replicates of endosperm pieces at 80 °C for 1 week. To the extracts obtained from *Sesbania virgata* were added three volumes of ethanol. After overnight incubation at 5 °C, the precipitated polysaccharides were collected by centrifugation, dried and weighed (Anderson, 1949). Total sugars in *Schizolobium parahyba* extracts were determined by the method of Dubois *et al*. (1956).

Measurements

Free sugars were determined in the apical part (0·5 cm) of fresh roots, using five or six replicates of three to five tips each. The material was homogenized in 2 ml of ethanol : water (8 : 2, v/v) with a Polytron (model PT 1200; Kinematica AG, Luzern, Switzerland) and the suspension was sonicated for 20 min at 80 °C. After spinning at 1000 *g* for 5 min, the pellet was re-extracted as above. The pooled supernatants were assayed (Scott and Melvin, 1953).

Root respiration was estimated by the O_2 uptake of fresh roots in Gilson respirometer flasks at 25 °C. Determinations were carried out in normoxic seedlings and after 48 h of incubation under anoxia (ten replicates for each treatment). Roots were placed in flasks containing 2·0–2·5 ml 50 mM phosphate buffer (pH 5·0) supplemented with 10 mM glucose. Readings were taken every 15 min for 1 h. Two or three flasks containing only buffer served as a control to account for thermo-barometer variations.

The fermentation end-products ethanol and lactic acid were extracted from six samples of frozen roots (250–500 mg) treated with 2–5 ml 6 % $HClO_4$. After Polytron homogenization and spinning for 15 min at 4000 *g* at 0 °C, supernatants were neutralized with cold 5 M K_2CO_3 in an ice bath. Potassium perchlorate was removed by centrifugation. Ethanol and lactic acid were determined enzymatically, using test kits (Boehringer Mannheim, Germany).

Alcohol dehydrogenase (ADH) activity in roots was determined in six replicates (200–400 mg). Fresh root samples were homogenized in 2·5–3·0 ml 0·1 M glycylgly-

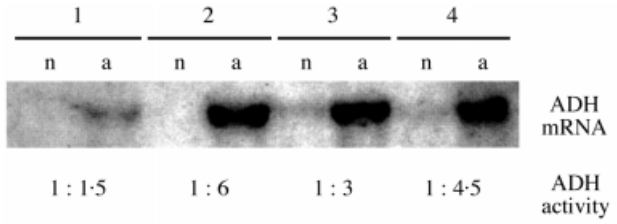

FIG. 1. ADH1 expression (northern blot analysis) and relative ADH activity increase in roots (normoxia control = 1) following 48 h anoxia treatment. Blots were hybridized at 65 °C with appropriately digested fragments of ^{32}P-labelled NtADH1 plasmids coding for tobacco ADH1. Lane 1, *Schizolobium parahyba*; lane 2, *Sebastiania commersoniana*; lane 3, *Erythrina speciosa*; lane 4, *Sesbania virgata*. n, Normoxia; a, anoxia.

cine, 20 mM Na_2-ethylenediamine tetraacetate, 20 mM diethyldithiocarbamate (pH 7·4) supplemented with 50 mg polyvinylpyrrolidone and 5 µl 2-mercaptoethanol. Homogenates were filtered and centrifuged at 4000 *g* for 15 min. All operations were carried out at 0–4 °C. The ADH activity of supernatants was assayed at 25 °C by monitoring NAD^+ reduction at 365 nm. The reaction medium (2 ml) contained 75 mM sodium pyrophosphate, 75 mM semicarbazide, 21 mM glycine (pH 8·7), 16 mM NAD^+ and extract. The reaction was started with 1·2 M ethanol. Protein was measured as in Bradford (1976). Since *Schizolobium parahyba* and *Sebastiania commersoniana* undergo a decrease in protein content under anoxia, the ADH activity for all species was expressed on the basis of fresh weight.

Levels of ADH mRNA were estimated by northern blot analysis. Total RNA was extracted from 100–200 mg of frozen root apex powder with phenol and quantified (Sambrook *et al*., 1989). Ten micrograms of total RNA was separated on a 1 % agarose gel. Northern blotting and hybridization followed standard procedures (Sambrook *et al*., 1989). Other details are given in the legend to Fig. 1.

Total adenylates were extracted from three replicates (25–50 mg) of frozen root powder by homogenization in 1·5 ml of 14 % $HClO_4$ with a Polytron. After spinning for 3 min at 15 000 *g* at 4 °C, supernatants were neutralized with 5 M K_2CO_3 in an ice bath. Potassium perchlorate was removed by centrifugation. Determination of adenylate levels was performed with the luciferin–luciferase system, using a biocounter (model 2500; Lumac) according to Sieber and Braendle (1991). Adenylate energy charge is the ratio ([ATP] + 0·5 [ADP])/([ATP] + [ADP] + [AMP]).

Total fatty acids and free fatty acids were determined on root powders from young (2- to 6-d-old) seedlings (three samples with 50–100 mg) maintained under normoxia and anoxia, and from older seedlings (three samples with 100–200 mg) that were permanently flooded from 3–5 weeks after radicle protrusion until 1–4 weeks later. In flooding treatments, the water level was maintained 1–2 cm above the soil surface. Extraction and quantification of total and free fatty acids were carried out by a combined thin-layer and gas chromatographic procedure (Rawyler *et al*., 1999).

Statistical analysis

Statistical analysis was carried out using SAS software. Significant differences between treatments were determined using one-way ANOVA followed by Duncan's test ($P < 0·05$). Data in Fig. 2 were analysed by two-way ANOVA and data in Table 1 by one-way ANOVA, using seed germination or leaf appearance rate as a weight variable.

RESULTS

Table 1 shows the intrinsic parameters of seeds; the species are arranged according to their capacity to survive/grow during anoxia/hypoxia treatments. Under normoxia, the more tolerant species developed faster than the less tolerant ones (Table 1). Furthermore, species with heavy seeds (e.g. *Schizolobium parahyba*) tend to develop more slowly than those with small ones (e.g. *Sesbania virgata*). Table 1 also provides information about the seed reserves. Seeds of *Sebastiania commersoniana* were richer in lipids while those of *Schizolobium parahyba* and *Sesbania virgata* were richer in carbohydrates. Seeds of *E. speciosa* contained about the same proportion of lipids and carbohydrates.

None of the species studied was able to germinate under anoxia. However, normoxically grown seedlings survived short periods of anoxia up to 48 h and resumed respiration at 65–79 % of their initial capacity, as shown by measurements made immediately after removing the seedlings from the anoxic environment (data not shown).

Table 2 compares levels of free fermentable sugars in normoxic young roots with those reached in these tissues after 48 h of anoxic treatment. Here, *Sesbania virgata* showed little difference as compared with the normoxic control. The other three species contained considerably smaller amounts of free sugars. The greatest decrease was exhibited by *Sebastiania commersoniana*, followed by *Schizolobium parahyba* and *E. speciosa* (Table 2).

We decided to measure only those end-products that enhance the glycolytic energy output *via* NADH reoxidation. Neither ethanol nor lactic acid were detectable in the roots of any of the four species under normoxic conditions. Under anoxia, however, free sugars were fermented, giving rise to high ethanol concentrations in *Schizolobium parahyba*, intermediate concentrations in *Sebastiania commersoniana* and low concentrations in *E. speciosa* and *Sesbania virgata*. In all cases, lactate production was much lower than that of ethanol, although more lactate was found in sensitive species than in *Sesbania virgata* (Table 3).

A common response of roots to anoxia is the synthesis of a subset of anaerobic proteins related to the glycolytic and fermentation pathways (Sachs *et al*., 1996). As expected, northern blots of ADH1 mRNA gave a much more pronounced signal in the root tips of *Sebastiania commersoniana*, *E. speciosa* and *Sesbania virgata* (Fig. 1). These results were corroborated by measurements of ADH activity. Under anoxia, these species showed a three- to six-fold increase in ADH activity, while in *Schizolobium parahyba* the increase was only 1·5-fold (Fig. 1).

We were therefore interested to know whether the energy status of these anoxic roots could meet the requirements of

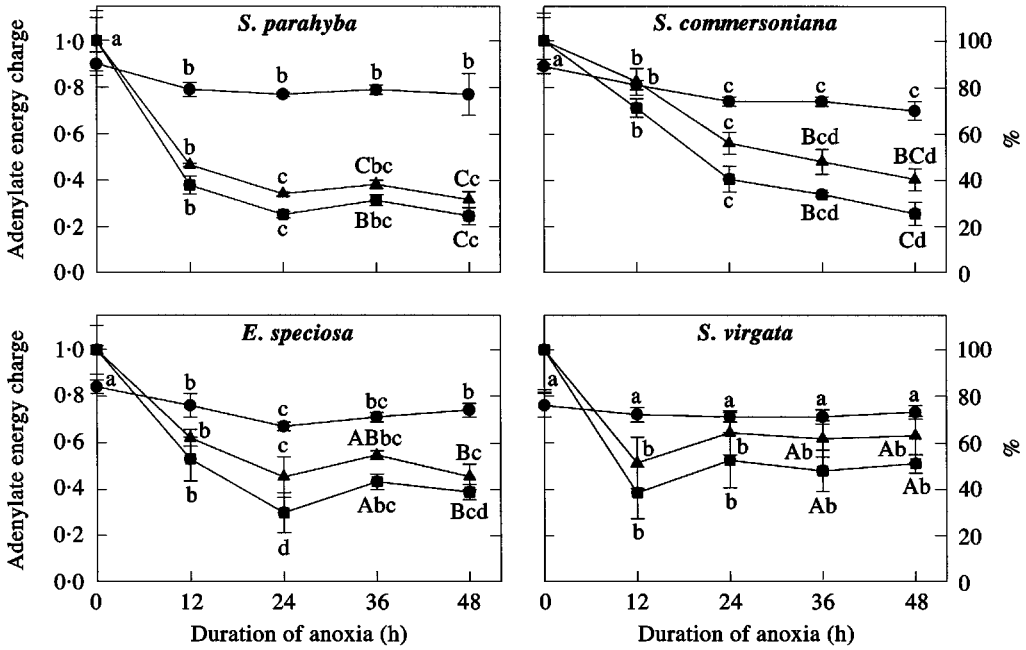

FIG. 2. Adenylate energy charge (circles, left scale), total adenylate (triangles) and ATP (squares) contents (right scale) of root tips over the course of a 48 h anoxia treatment (normoxia control = 100 %). Significant differences are indicated by different letters. Lower case letters compare values among treatment times for each response analysed for each species. Upper case letters compare values among species for ATP and adenylate levels for 36 and 48 h of anoxia. Two-way ANOVA and Duncan's test were performed on absolute values.

TABLE 1. *Intrinsic parameters of seeds under normoxic conditions*

Species	Time (d) required for		Seed mass (mg)	Reserves (mg g^{-1} seed)		
				Carbohydrates in:		
	Root protrusion*	Leaf appearance*		Cotyledon	Endosperm	Lipids
Schizolobium parahyba	4–7[a]	18–24[a]	1643 ± 103	126 ± 1	729 ± 67	86 ± 15
Sebastiania commersoniana	3–7[a]	20–24[a]	21·5 ± 1·5	16 ± 2[†]		432 ± 19
Erythrina speciosa	2–4[b]	11–17[b]	465 ± 25	109 ± 8	–	134 ± 9
Sesbania virgata	2–4[b]	11–15[b]	66·4 ± 3·8	84 ± 11	678 ± 22	100 ± 6

n = 5 experiments per parameter, each with ten seeds ± s.d. Species are listed in order of increasing tolerance to oxygen deficiency. Significant differences among species are indicated by different superscripts. Lipids are expressed here as total fatty acids, which include storage lipids (triacylglycerols), membrane lipids (phospho- and glycolipids) and free fatty acids.

* The start of the imbibition process is taken as time zero.

energy-demanding processes such as protein synthesis. As shown in Fig. 2, the adenylate energy charge decreased only slightly and stabilized at rather high values in all species. However, there was a steady decrease in the levels of total adenylates and of ATP in the less resistant species (*Schizolobium parahyba* and *Sebastiania commersoniana*). On the other hand, the initial decrease of these parameters in the more tolerant *E. speciosa* and *Sesbania virgata* was followed by a recovery (Fig. 2). For each of these three parameters, the statistical analysis reported in Table 4 shows that significant differences exist among the species, among anoxia treatments of different durations and in the interaction of these two factors.

To determine whether ultrastructural alterations would occur subsequently to these metabolic changes, the formation of free fatty acids, a reliable indicator of membrane degradation (Rawyler *et al*., 1999), was also examined. Under anoxic conditions, roots of *Schizolobium parahyba* showed a significant increase in the free fatty acid level; this was not observed in the other species (Fig. 3). Under flooded conditions, this increase in free fatty acids was again observed in *S. parahyba* (Fig. 3) and the seedling of this species did not survive the 8 d hypoxic treatment. Once again, the other species did not show any appreciable increase in free fatty acids. However, *Sebastiania commersoniana* shed its leaves when flooded whereas *E. speciosa*

TABLE 2. *Free-sugar levels in root tips of seedlings under normoxia and after 48 h of anoxic treatment*

Species	Normoxia (mg g^{-1} f. wt)	After 48 h of anoxia (mg g^{-1} f. wt)	Relative decrease (% of normoxic control)
Schizolobium parahyba	20·3 ± 5·0[a]	10·8 ± 1·6[b]	46·8 ± 7·8[A]
Sebastiania commersoniana	19·1 ± 5·1[a]	8·9 ± 2·7[b]	53·4 ± 14·3[A]
Erythrina speciosa	15·2 ± 2·8[a]	9·2 ± 3·6[b]	39·6 ± 23·8[AB]
Sesbania virgata	16·4 ± 2·0[a]	14·1 ± 2·9[a]	16·8 ± 13·5[B]

n = 5–6 ± s.d.

Significant differences between treatments are indicated with different superscripts (lowercase superscripts compare values within rows and upper-case letters compare values within columns). Percentages were transformed by taking the arcsin of their square root prior to statistical analysis.

TABLE 3. *Ethanol and lactic acid content of root tips of seedlings incubated for 48 h under normoxia and anoxia*

	Ethanol (μmol g^{-1} f. wt)		Lactic acid (μmol g^{-1} f. wt)	
Species	Normoxia	Anoxia	Normoxia	Anoxia
Schizolobium parahyba	n.d	29·5 ± 4·2[a]	n.d	1·8 ± 0·8[b]
Sebastiania commersoniana	n.d	15·2 ± 1·8[b]	n.d	1·6 ± 0·5[b]
Erythrina speciosa	n.d	6·7 ± 0·6[c]	n.d	2·7 ± 0·3[a]
Sesbania virgata	n.d	6·8 ± 0·7[c]	n.d	0·3 ± 0·1[c]

Values are given as mean ± s.d. of six replicates. n.d., Not detectable.

Significant differences among species are indicated by different superscripts.

TABLE 4. *Results of ANOVA for adenylate energy charge, ATP and total adenylate levels in four species subjected to anoxia treatment of different duration*

	Adenylate energy charge		ATP levels		Total adenylates	
Source of variation	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Species	13·69	<0·0001	8·50	0·0002	16·59	<0·0001
Time	25·08	<0·0001	148·67	<0·0001	102·04	<0·0001
Species × time	1·92	0·0610	5·38	<0·0001	4·81	<0·0001

and *Sesbania virgata* did not show any sign of stress under this condition.

DISCUSSION

The faster development of the more flood-tolerant species, *Sesbania virgata* and *E. speciosa* (Table 1), suggests that their metabolism survives better under the hypoxic stress generated within their seeds upon imbibition than does that of *Sebastiania commersoniana* and *Schizolobium parahyba*. This is mainly attributable to the marked decrease in energy-dependent transport activities under oxygen deficiency (Xia and Saglio, 1990). Carbohydrates are more efficient than lipids in sustaining fermentation processes (Table 1), in agreement with Alani *et al*. (1985). In the latter case, the most abundant compound of lipid degradation is acetate, the utilization of which by the tricarboxylic acid cycle would obviously be prevented under anoxia. It is therefore not surprising that seeds of *Schizolobium parahyba* and *Sebastiania commersoniana* are more sensitive to oxygen shortage than seeds of *Sesbania virgata*.

However, none of these species produced roots or shoots under the strict anoxic conditions provided by the anaerobic workbench. This suggests that in their natural habitat, such seeds would germinate only if the flooded ground was not fully deoxygenated (that is, at the very beginning of flooding), or when the water table falls below ground level. These species will therefore neither develop seedlings nor survive prolonged periods of anoxia. Nevertheless, they all survived the experimental period of 48 h of anoxia, as shown by the restored respiration of seedlings when returned to a normally aerated environment.

Fermentation processes are absolutely dependent on the availability of free sugars (e.g. hexoses) (Drew, 1997). The data of Table 2 reflect the balance between polysaccharide mobilization, transport capacity and sugar consumption during fermentation. The essentially stable level of fermentable sugars (as compared with the normoxic control) shown by *Sesbania virgata* suggests that mobilization,

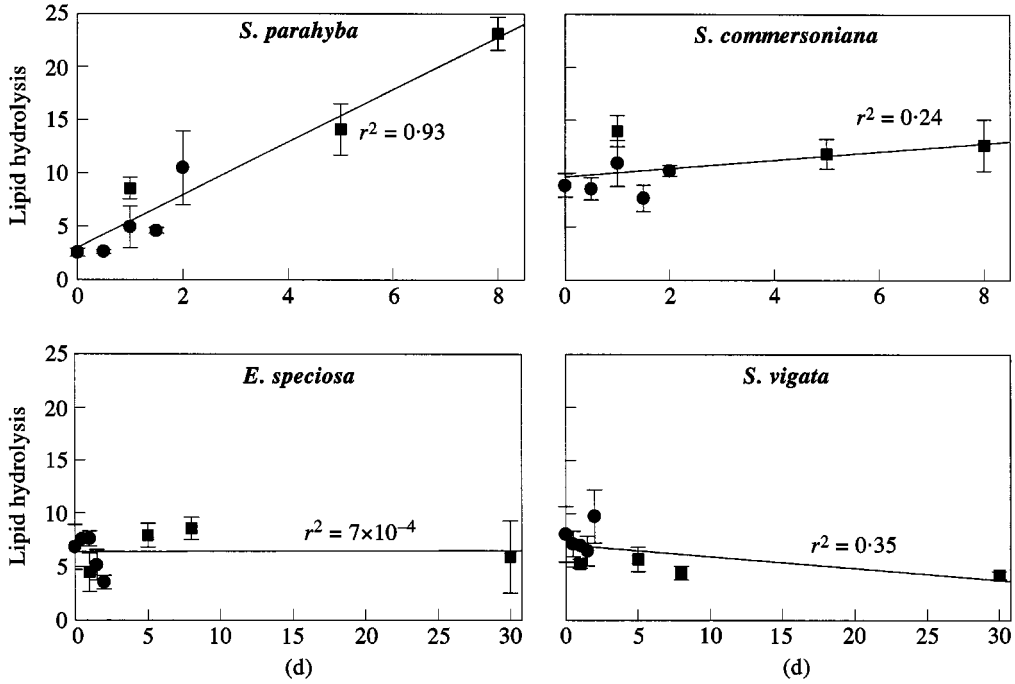

FIG. 3. Lipid hydrolysis, expressed as the amount of free fatty acids as a percentage of total fatty acids, occurring in roots of young seedlings (circles) during a 48 h anoxia treatment, and in 3–5-week-old seedlings (squares) flooded for up to 8 d (*Schizolobium parahyba* and *Sebastiania commersoniana*) and 30 d (*Erythrina speciosa* and *Sesbania virgata*). For each species, all data were used to calculate regression lines ($P < 0{\cdot}05$).

transport and consumption rates were still highly equilibrated in this particular species. The other three species contained much smaller amounts of free sugars, suggesting that their release from polysaccharides could no longer match the hexose utilization rate. In these latter cases, sugar starvation eventually occurs as a consequence of inefficient sugar mobilization during prolonged oxygen deficiency, leading to a severe restriction in energy supply (as ATP). Limitation in sugar availability has been attributed to a loss of α-amylase activity (Perata *et al*., 1997, 1998; Arpagaus and Braendle, 2000), and this could also be the case for *E. speciosa* and *Sebastiania commersoniana* (where starch accounts for 25 % of the total sugars). On the other hand, a similar sugar limitation in galactomannan-rich seeds (Anderson, 1949; McClendon *et al*., 1976), such as those of *Schizolobium parahyba* (Zawadski-Baggio *et al*., 1992), or in xyloglucan-rich seeds, such as those of the genus *Sesbania* (Buckeridge and Dietrich, 1996), would also necessitate the loss of other glycosidase activities.

Since the largest amounts of fermentation end-products in root tips were found in the most anoxia-sensitive species (Table 3), one may ask whether this can account for differences in resistance. An increased production of fermentation end-products is often negatively correlated with resistance of the tissue, although this effect is seen especially after reaeration (Pfister-Sieber and Braendle, 1994). On the other hand, high levels of such end-products do not necessarily indicate high rates of fermentation and ATP production (Kennedy *et al*., 1992; Joly, 1994), but may also reflect an impeded diffusion of catabolites (e.g. ethanol) out of the plant organ due to an unfavourable surface area to volume ratio. Under anoxia, ethanol concentrations were high in *Schizolobium parahyba*, intermediate in *Sebastiania commersoniana* and low in *E. speciosa* and *Sesbania virgata* (Table 3). Whether fermentation end-products are directly toxic is still disputed (Perata and Alpi, 1991; Joly, 1994). Since it is known that ethanol is easily released into the surroundings from most resistant species (Kennedy *et al*., 1992), this compound will certainly not reach a potentially noxious level in *Sesbania virgata* and *E. speciosa*. However, the back-conversion of ethanol to the much more cytotoxic acetaldehyde upon re-oxygenation of tissues (Perata *et al*., 1992; Crawford and Braendle, 1996) may constitute an additional hazard for those species having successfully overcome the anoxic step. Moreover, the increased amounts of lactic acid formed by less tolerant species (Table 3) could favour cytoplasmic acidosis and thus contribute to further metabolic dysfunctions (Drew, 1997).

Although fed by efficient polysaccharide mobilization processes, the glycolytic pathway cannot run optimally without a continuous supply of NAD^+ reoxidized via the ethanolic and lactic fermentation reactions. The higher signal given by ADH1 mRNA in northern blots of the more tolerant species (Fig. 1) suggests that the fermentation capacity of their roots is significantly enhanced. This is a well-established reaction of plants towards flooding stress (Bucher *et al*., 1996; Sachs *et al*., 1996). Our measurements of ADH activity levels (Fig. 1) supported this view, since the proportional increase was small in the most sensitive species *Schizolobium parahyba* but large in the other species. When subjected to flooding, tolerant neotropical trees can rely on very efficient fermentative energy

metabolism, which therefore appears to be one of the crucial determinants of prolonged survival under anoxia.

This is confirmed by the energy status of oxygen-deprived roots, depicted here in terms of adenylate energy charge, total adenylates and ATP levels (Fig. 2). A high adenylate energy charge means that the ATP production of the cell matches its instantaneous ATP needs. A limited decrease in adenylate energy charge, and its stabilization at intermediate values, is typical of living systems at the beginning of a period of oxygen deficiency (Pradet and Raymond, 1983; Joly and Braendle, 1995). The capacity of all four species to survive 48 h under anoxia suggests that their energy metabolism remains equilibrated during this period. An increased period of anoxia would merely have continued the trend for decreasing adenylate and ATP levels in the sensitive species, with a gradual slow-down in ATP production by fermentation and, consequently, in the ATP-consuming processes. In the resistant species, stabilization of ATP production at an intermediate level enables ATP-dependent metabolism to continue. It is likely that adenylate energy charge would decrease later to low, lethal levels (approx. 0·2–0·3) in sensitive species, whilst remaining at intermediate levels (approx. 0·5–0·6) in resistant species, as already reported in other species (Saglio *et al*., 1980; Raymond *et al*., 1985).

The data presented in Fig. 3 support our contention that the ultimate stage of cell survival under anoxia is reached when subcellular compartmentalization is disrupted. Membrane breakdown is the last event under ATP shortage before cell death occurs (Rawyler *et al*., 1999). The marked increase in free fatty acids observed in the roots of *Schizolobium parahyba* after 48 h of anoxia reflects an extensive hydrolysis of their membrane lipids, in contrast with the three other species. The rather high amounts of free fatty acids initially present in all species may be a remnant of former lipid mobilization processes. These findings are corroborated by the results obtained with normoxically pre-cultivated older seedlings (3–5 weeks) grown for 8 or 30 d on a permanently flooded soil, a condition that imposes at least a severe hypoxia (Fig. 3). The continuous increase of free fatty acids in roots of the most sensitive *Schizolobium parahyba* during flooding again indicates a progressive membrane damage, in line with the fact that the survival of flooded *Schizolobium parahyba* did not exceed 8 d. In spite of its longer survival, *Sebastiania commersoniana* continuously lost leaves that had become yellow upon flooding, suggesting that this increase in tolerance is rather marginal. On the other hand, having survived flooding for more than 30 d without any visible sign of damage, *E. speciosa* and *Sesbania virgata* show that they are able to develop into viable saplings with later formation of aerenchyma and of hypertrophied lenticels.

Thus, the observed habitat specialization towards flooding of the four species investigated (*Sesbania virgata* > *Erythrina speciosa* > *Sebastiania commersoniana* > *Schizolobium parahyba*) is already rooted in the juvenile stage through intrinsic properties of the seed, but exists to a greater extent in the events occurring during early development. Furthermore, habitat specialization is the result of several favourable factors leading to the high resistance in *Sesbania virgata*, or *vice versa* to the lower resistance of *Schizolobium parahyba* and *Sebastiania commersoniana*. Among the several steps of this adaptation process, one of the most important is the maintenance of an adequate fermentative energy metabolism in order to preserve cell homeostasis and compartmentalization during early development.

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