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Flooding tolerance of *Tabebuia cassinoides*: Metabolic, morphological and growth responses

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Abstract

Tabebuia cassinoides (Lam.) DC (Bignoniaceae) is a tree species that occurs in swampy areas of the coastal "restinga" in SE Brazil (a coastal sandy plains scrub and forest formation). To elucidate possible adaptive strategies that enable this species to occupy areas subjected to seasonal or perennial waterlogging, metabolic, morphological and growth responses of plants under flooding conditions were studied. The root system of *T. cassinoides* plants presented elevated amounts of ethanol (10.6 μmol g^{-1} fresh wt) only in the first 5 d of soil water saturation. The two-fold increase in ethanol production under flooding was corroborated by an increase in ADH activity in the same period. Lactic acid concentrations did not change significantly during four months of flooding treatment. The decrease of alcoholic fermentation under hypoxia was associated with the appearing of new roots. The induction of aerenchyma formation in roots developed under flooding conditions, allowed oxygen transport from the shoot to these organs, thus maintaining an aerobic respiration. We conclude that this characteristic and the capacity to oxidize the rhizosphere are probably responsible for the survival and growth of plants while flooded and for their success in an environment, which restricts the presence of the majority of competing tree species.

Keywords: *Tabebuia cassinoides*; Flooding tolerance; Fermentative metabolites; Aerenchyma; Hypoxia; Rhizosphere oxidation

Introduction

Although all higher plants require access to water, excess water in the root environment of land plants can be injurious or even lethal because it blocks the transfer of oxygen and other gases between soil and atmosphere (Crawford, 1992). With transient flooding followed by slow drainage, or in natural wetlands, plant roots can become oxygen deficient because of slow transfer of dissolved oxygen in the water-filled pore space of the soil (Drew, 1997). At higher temperatures microbial respiration is stimulated resulting in a complete depletion of oxygen in a few hours; and roots may then experience a fast transition from a fully aerobic to an anaerobic environment (Purvis and Williamson, 1972).

Oxygen shortage is one of the major abiotic stresses that determines the distribution of vascular plant species in many wetlands areas of the world (Crawford, 1992), including the Neotropical region (Joly, 1991). Plants that have their roots subjected to hypoxia or anoxia may avoid the stress by facilitating diffusion of oxygen from the aerial parts to the root system (Armstrong, 1979; Joly, 1996). Hypoxia and/or anoxia may also induce

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changes in the respiratory metabolism of the root system, as recently reviewed by Kreuzwieser et al. (2004), leading to an acceleration of glycolysis with the production of ethanol and lactate. Ethanol is the end-product of the alcoholic fermentation pathway, a two-step mechanism catalysed by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) while lactate is produced by a one-step reaction catalysed by lactate dehydrogenase (LDH).

Tabebuia cassinoides (Lam.) DC is an economically important species, because among the Brazilian taxa, it provides the best wood for pencil production, mainly for export. On a worldwide level, it is of highest quality and is only exceeded by the American cedar *Libocedrus decurrens* (Kuniyoshi, 1993). *T. cassinoides* is a small to medium size deciduous tree (4–18 m), with trumpet white with yellow in throat flowers (15–20 mm long, 8–12 mm wide) and linear-oblong fruits (13–15 cm long and 1–8 cm in diameter), seeds (0.6–0.7 cm long, 2.5–3 cm wide) with hyaline membranaceous wings and brownish veining, the wings sharply demarcated from gray-brown seed body (Flora Brasiliensis revisitada, 2008). The tree is restricted to freshwater coastal swamps from Southeastern Brazil to Panama, where it typically forms almost pure stands (Scarano et al., 1997).

The aim of this work was to study some adaptive strategies that allow *T. cassinoides* to occur in seasonally and/or permanently flooded areas of the Southeastern Brazilian coastal plain forests, locally named "restinga" forest (Assis, 1999; Lorenzi, 1992). In these areas, the water table, a mixture of fresh and seawater, is above or close to the soil surface for most of the whole year. These extreme conditions ensure that only few well-adapted species are able to survive in these areas (Assis, 1999).

Materials and methods

Plant growth conditions and treatments

T. cassinoides fruits were collected in Parque Estadual da Serra do Mar – Núcleo Picinguaba (23°31′–23°34′S and 45°02′–45°05′W). Seeds were germinated at 25 °C under a photoperiod of 12 h. After radicle protrusion, young seedlings were transplanted to 2 L bags, made of 1 mm mesh nylon nets, filled with coarse sand. The bags were used to ensure an initial good aeration of the root systems and to allow the growth of the roots through the bags in the flooded treatment. Plants were cultivated in a greenhouse, receiving water twice a day, and 10% (v/v) Hoagland nutritive solution (400 mL per plant) every 15 d for six months. The growth conditions in the greenhouse in clear days were of 800 $\mu mol\, m^{-2}\, s^{-1}$ of photosynthetically active radiation (PAR) and the temperatures were around 30 °C at midday. After this period, plants with 9 cm of height were separated in two groups: one group of 60 control plants was maintained under control conditions (normoxic plants) and watered twice a day, while another group of 135 plants was transferred to tanks, where the water level was raised to maintain a level 2–3 cm above soil surface (hypoxic plants). Every three weeks the water of the tanks was changed, to avoid algae development. During the time of the experiments, fertilization was abandoned to avoid differences in nutrient availability between control and flooded treatments.

Determination of ethanol and lactic acid in the roots of control and flooded plants

Ethanol and lactic acid were extracted from the roots of six plants flooded for 1, 5, 15, 30, 60 and 120 d intervals, respectively. Six control plants were measured after 30 and 60 d. Almost all apical parts (3–4 cm) of the lateral roots that were present in the plants were used for these measurements. Immediately after being removed from the sand bags, samples of 300–500 mg of roots were frozen with liquid nitrogen and then triturated with addition of 10 mL 6% $HClO_4$. The homogenates were centrifuged for 20 min at 3000*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).

Determination of ADH activity

Alcohol dehydrogenase activity in roots was determined in six replicates (250–500 mg) after 1, 3, 5, 10, 30, 60 and 120 d of flooding. Six control plants were measured after 30 and 60 d. Almost all apical parts (3–4 cm) of the lateral roots that were present in the plants were used for these measurements. Fresh root samples were homogenized in 8 mL 5 mM $MgCl_2$, 0.1 M Tris/HCl (pH 8.0) supplemented with 150 mg polyvinylpyrrolidone and 25 µL 2-mercaptoethanol. Homogenates were filtered and centrifuged at 3000*g* for 20 min. All operations were carried out at 0–4 °C. The ADH activity of supernatants was assayed at 25 °C by monitoring NADH oxidation at 340 nm. The reaction medium (3 mL) contained 5 mM $MgCl_2$, 0.1 M Tris/HCl (pH 8.0), 2.4 mM NADH and extract. The reaction was started with 0.3 M acetaldehyde. Protein was measured according to Bradford (1976), with BSA as standard.

Electron microscopy

For the microscopic analyses we used segments of lateral roots of similar length (5–6 cm) from controls

and from plants flooded for 30 d. Root segments of 0.5 cm (after removing the apical 0.5 cm) were fixed for 3 d in 2% paraformaldehyde and 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.0. Post-fixation was made in 1% osmium tetroxide overnight. After dehydration in ethanol, samples were dried by the carbon dioxide critical point drying technique, coated in gold and viewed with a Zeiss DSM 940A scanning electron microscope.

Oxygen diffusion

Radial oxygen loss from roots into the medium was detected by the methylene blue dye method (Armstrong and Armstrong, 1988). The experiment was carried out with three control plants and three plants flooded for 30 and 60 d. Sand was carefully washed from the roots that were then surface sterilized in 0.02% mercuric chloride, followed by rinsing in distilled water. The root systems were then put in beakers with 1.5% agar, methylene blue and sodium dithionate ($Na_2S_2O_4$). After addition of sodium dithionate the blue oxidized colour of the dye faded to leave a colourless reduced solution. The radial oxygen loss from roots can be visualized when the dye is re-oxidized around the roots. For a control of the effectiveness of the system, plants without shoots and with the cut-ends lanolin-sealed were utilized. Roots were submerged in sodium dithionate solution for 2 h before their transfer to agar medium, to remove any oxygen stored in these organs.

Growth evaluation

Growth was evaluated in five plants used for shoot and root dry mass determination. For this, the plants were dried for 2 d at 80 °C. Measurements were made after 30, 60 and 120 d of normoxia and hypoxia and in re-aerated plants (60 d under flooding condition followed by 60 d under control condition). The formation of new leaves during hypoxia and normoxia treatments was also recorded.

Statistical analysis

Significant differences between the treatments results of which shown by Fig. 1 and Table 1 were determined using one-way ANOVA followed by Tukey's test ($P<0.05$). Data in Figs. 4 and 5 were analysed by two-way ANOVA (dry mass data after 120 d of normoxia and hypoxia and in re-aerated plants were analysed by one-way ANOVA). Data were transformed when necessary to fit normal distribution.

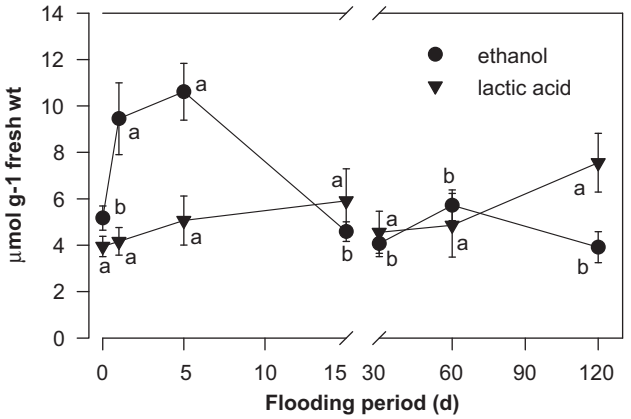

Fig. 1. Effect of flooding on fermentation end-products in roots of *T. cassinoides*. Because the content of root metabolites was similar in the two-normoxic treatments (30 and 60 d), the mean obtained for the two extractions is presented in the graphic (time zero). Values are given as mean ± s.e. of six replicates. Significant differences among treatments are indicated by different letters.

Table 1. Effect of flooding on ADH activity in the roots of *T. cassinoides*.

Flooding period (d)	ADH activity	
	µmol mg^{-1} protein min^{-1}	µmol g^{-1} fresh wt
0*	0.27 ± 0.05^{c}	0.70 ± 0.15^{b}
1	0.51 ± 0.08^{b}	1.09 ± 0.24^{b}
3	0.52 ± 0.11^{b}	1.19 ± 0.43^{b}
5	0.93 ± 0.09^{a}	2.67 ± 0.47^{a}
10	0.18 ± 0.02^{c}	0.64 ± 0.29^{b}
30	0.28 ± 0.02^{c}	0.58 ± 0.12^{b}
60	0.48 ± 0.02^{b}	1.25 ± 0.09^{b}
120	0.14 ± 0.04^{c}	0.37 ± 0.06^{b}

Values are given as mean ± s.e. of six replicates. Significant differences among treatments are indicated by different superscript letters.

*Because ADH activity in the roots was similar in the two-normoxic treatments (30 and 60 d), the mean obtained for the two extractions is presented in the table (time zero).

Results

Roots of plants under normoxic conditions showed ethanol amounts of 5.2 ± 0.5 µmol g^{-1} fresh wt, while 5 d flooded plants presented amounts of 10.6 ± 1.2 µmol g^{-1} fresh wt, indicating a significant increase by a factor of two. However, after 15 d of flooding, ethanol amounts decreased, remaining at the same values observed for normoxic plants (Fig. 1). Lactic acid amounts did not change significantly during the flooding treatment (Fig. 1).

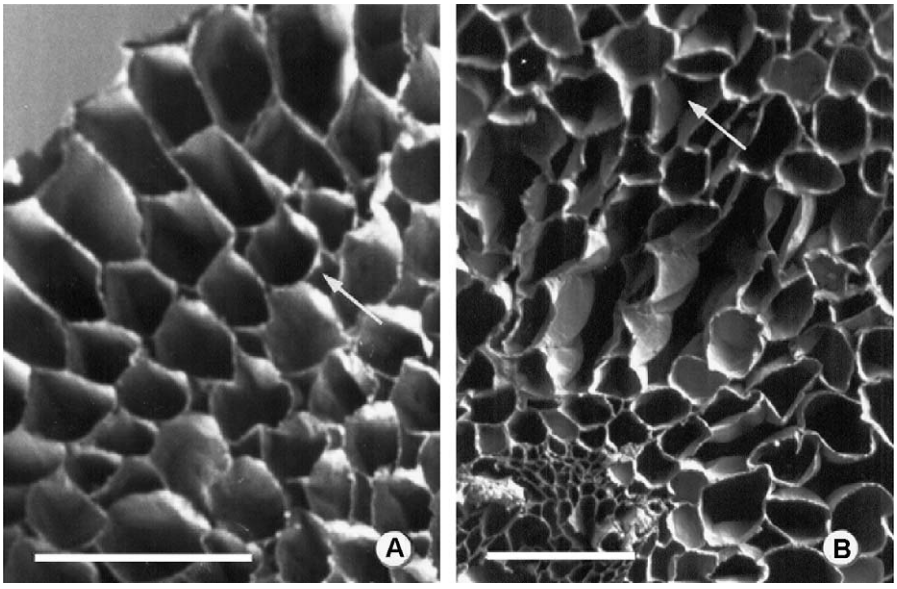

Fig. 2. Electron micrographs of transverse sections of lateral roots of *T. cassinoides* plants. A: 30 d of normoxia and B: flooding induced root after 30 d of treatment. The samples of 0.5 cm were taken after removing the apical 0.5 cm. Roots lengths were of 5–6 cm. Bar = 50 μm. Intercellular spaces are indicated by arrows.

The results of ethanol production in the roots under flooding were corroborated by measurements of ADH activity (Table 1). After 5 d of oxygen deficiency, *T. cassinoides* roots exhibited a significant three-fold increase in the enzyme activity. However, after 10 d of flooding, ADH activity decreased, remaining at the same levels observed for the control plants (Table 1).

At this time, *T. cassinoides* plants formed new roots that grew through the bags. These roots presented a modified morphology; they were rectilinear and less ramified. With 15 days, the roots growing outside the bags were 3–5 cm long and were formed continuously to replace dead roots. These roots were thicker and assumed a spongy aspect and were recognized as flooding induced roots. Still regarding to morphological alterations, a few plants produced adventitious roots (1–3 per plant) after 60 days of flooding. Hypertrophied lenticels appeared in some plants with 10–15 d of flooding and in others after 30–45 d of stress (unpublished data).

The anatomical study of the lateral roots of *T. cassinoides* indicated the presence of intercellular spaces in normoxic roots (Fig. 2A). However, after 30 d of flooding, it was possible to observe aerenchyma development by schizogeny, with lacunae formation, in flooding induced roots (Fig. 2B). These lacunae were absent from all normoxic roots analysed.

Results for radial oxygen loss suggested that the porous tissue observed for *T. cassinoides* roots was functional in the transport of oxygen from the aerial part of the plants to the roots. Plants flooded for 30 and 60 d, presented oxygen diffusion into the medium, mainly in the apical portions of the lateral roots (Fig. 3). Oxidizing sites around roots were not observed in plants without shoots and with the cut-ends lanolin-sealed confirming the effectiveness of the system.

Evaluation of the growth parameters revealed that *T. cassinoides* plants developed faster under flooding than under normoxic conditions. As shown in Fig. 4, there was a significantly higher investment in the production of new leaves in flooded plants when compared to the normoxic plants. This production was also influenced by treatments durations (Table 2).

In relation to the dry biomass increment the statistical analysis showed that significant differences exist between treatments, among treatments of different durations and in the interaction of these factors (Table 2). It was observed that shoots of flooded plants were larger than those of control plants (Fig. 5A) and this was a result of an increased stem length (data not shown) and of the larger number of new leaves produced by flooded plants (Fig. 4). The dry weight of the roots was also higher in flooded plants (Fig. 5C) and this occurs at least in part, because of the higher production of new lateral roots under flooding condition (data not shown). The shoot and root dry mass was significantly higher in flooded plants only after 60 and 120 d of treatment, respectively. *T. cassinoides* plants flooded for 60 d and re-aerated for more 60 d presented intermediary values of shoot and root dry mass when compared to plants after 120 d of normoxia and hypoxia (Fig. 5B, D), indicating

Fig. 3. Oxygen diffusion from *T. cassinoides* roots after 60 d of flooding, indicated by methylene blue colour formation. Bar = 4 cm.

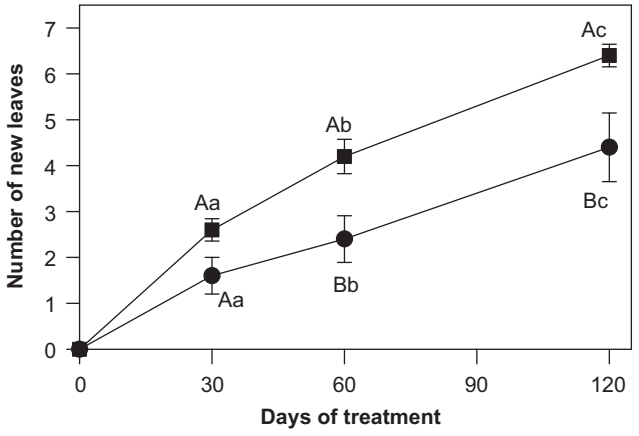

Fig. 4. Flooding effect on leaf initiation in *T. cassinoides* plants: normoxia (circles) and upon flooding (squares). Values are given as mean ± s.e. of five replicates. Significant differences are indicated by different letters. Lower case letters compare values among treatment times and upper case letters compare values between normoxia and hypoxia treatments.

Table 2

Source of variation	New leaves	Shoot dry mass	Root dry mass
Time	<0.0001	<0.0001	<0.0001
Treatment	<0.0003	<0.0001	<0.0001
Time X treatment	0.8443	0.0053	0.0044

P-values of ANOVA for new leaves, shoot and root dry mass in *T. cassinoides* subjected to normoxia and hypoxia treatments of different duration.

again that flooding favoured the development of *T. cassinoides* plants.

Discussion

The presence of ethanol and lactic acid in normoxic plants, albeit in low amounts, indicated that this fermentative pathway was functional. A possible explanation for this is that in certain regions of the roots, particularly the apical meristems, the cellular compactness makes oxygen access difficult. The roots could also be metabolically very active such that oxygen availability becomes insufficient to attend to demand, and so triggering the metabolism of these regions to anaerobic routes (Crawford, 1992).

Although ethanol was the main fermentative end-product produced under flooding, the amount decreased to normoxic levels after 15 d of treatment. This rise and fall of ethanol levels is supported by the data for ADH activity. Lactic acid concentrations did not accumulate during stress, thus avoiding a possible acidification of the cytoplasm and the death of the plant (Roberts et al., 1984). Because *T. cassinoides* roots did not present increased levels of fermentative metabolites under longer periods of flooding, we were interested to know whether the flooding tolerance of this species was related to morphological and anatomical responses to the stress.

The decrease in ethanol after 15 d of flooding was coincident with the appearing of new roots. These roots showed a spongy aspect and were recognized as flooding induced roots.

Roots induced by flooding showed a well-developed aerenchyma, with large lacunae formed by cell wall separation (schizogenous origin). The aerenchyma is composed of intercellular spaces and/or lacunae filled with air (Smirnoff and Crawford, 1983) and when functional, this tissue facilitates oxygen transport from the atmosphere and/or from the shoot into the roots and the rhizosphere (Drew et al., 1985).

Aerenchyma formation increases the chance of survival of many species that occur in waterlogged soils

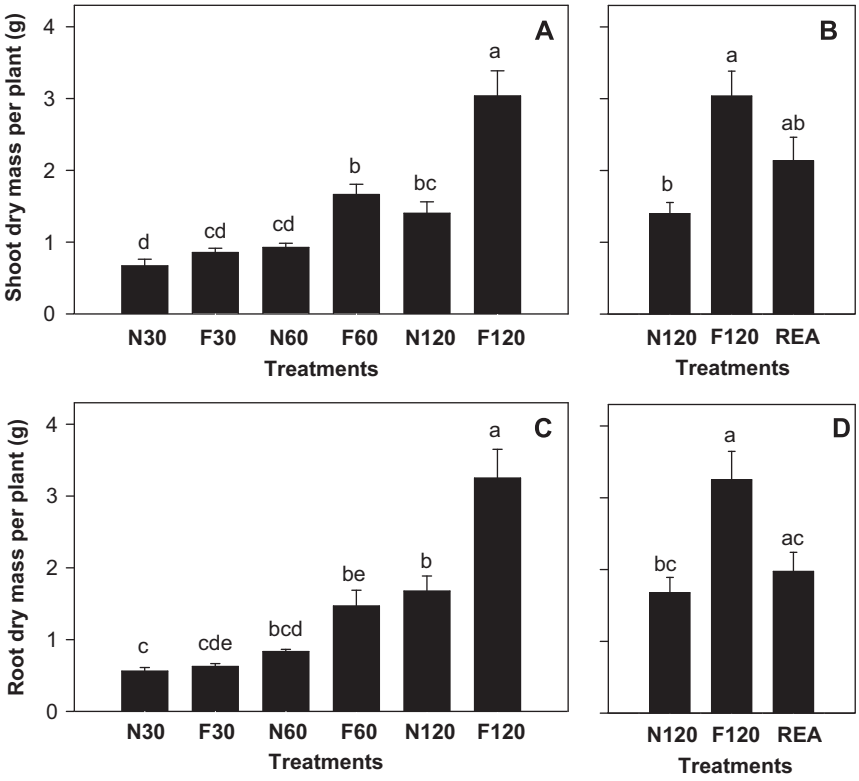

Fig. 5. Effect of flooding on shoot (A, B) and root (C, D) dry mass of *T. cassinoides* plants. N30, N60 and N120: 30, 60 and 120 d of normoxia; F30, F60 and F120: 30, 60 and 120 d of flooding; REA: re-aerated plants (60 d of flooding followed by 60 d under normoxia). Values are given as mean ± s.e. of five replicates. Significant differences among treatments are indicated by different letters.

(Kawase, 1981), and the presence of this tissue in roots of flooded species has been frequently observed (Aschi-Smiti et al., 2003; Justin and Armstrong, 1987; Medri et al., 2002).

Results for oxygen diffusion indicated the occurrence of oxygen loss into the medium for plants flooded for 30 and 60 d. This suggests that their porous tissues were effective in oxygen transport, allowing the occurrence of aerobic respiration and reducing the flooding effects.

The leaf initiation and the dry biomass of *T. cassinoides* flooded plants increased in relation to the control (normoxic) plants. Such an improved development of an arboreal species under flooding is uncommon. However, consistent with our findings, Davanso-Fabro et al. (1998) also observed an increase of the dry biomass of flooded plants of *Sesbania virgata*, a woody species that frequently occurs in inundated areas. The ability to increase root and stem mass under soil flooding conditions were verified in few other flood-tolerant tree species (Andrade et al., 1999; McKelvin et al., 1995; Mielke et al., 2005). A possible explanation is that flooded plants grew better because they were never under water stress. To further investigate this, it would be interesting to study the stomatal conductance and the photosynthetic capacity of these plants when flooded.

The ability of *T. cassinoides* plants to grow better when flooded, probably favours this species in competition with other ones and explains, at least in part, its large occurrence in swampy areas.

Re-exposure to oxygen after a period of deprivation can cause serious injury to plant tissues (Crawford, 1992). As a consequence, in the studies of plant susceptibility to low oxygen tension, it is necessary to consider both the oxygen deprivation period and the post-anoxic event (Blokhina et al., 2003). Re-admission of oxygen to tissues can generate superoxide radicals (Monk et al., 1989) and a rapid oxidation of anaerobically accumulated metabolites (Studer and Brändle, 1987). According to Asada and Takahashi (1987) and Larson (1988), oxidation of cellular components is prevented by the presence of natural antioxidants and by enzymatic protection against superoxide radicals. Re-aerated *T. cassinoides* plants did not show any visible injury symptom suggesting that this species has mechanisms to avoid any damage that could occur during this period.

As proposed by Joly (1991, 1994) flooding tolerance in *T. cassinoides* seems to depend on the interaction

between metabolic and morpho-anatomical responses. Our results indicate that the capacity to form a well-developed aerenchyma under flooding conditions increases oxygen diffusion from shoots into the roots. Consequently, aerobic respiration is maintained and this, together with the oxidation capacity of the rhizosphere, can maintain the growth of flooded plants, favouring the success of this species in areas where soil water saturation can last for months.

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