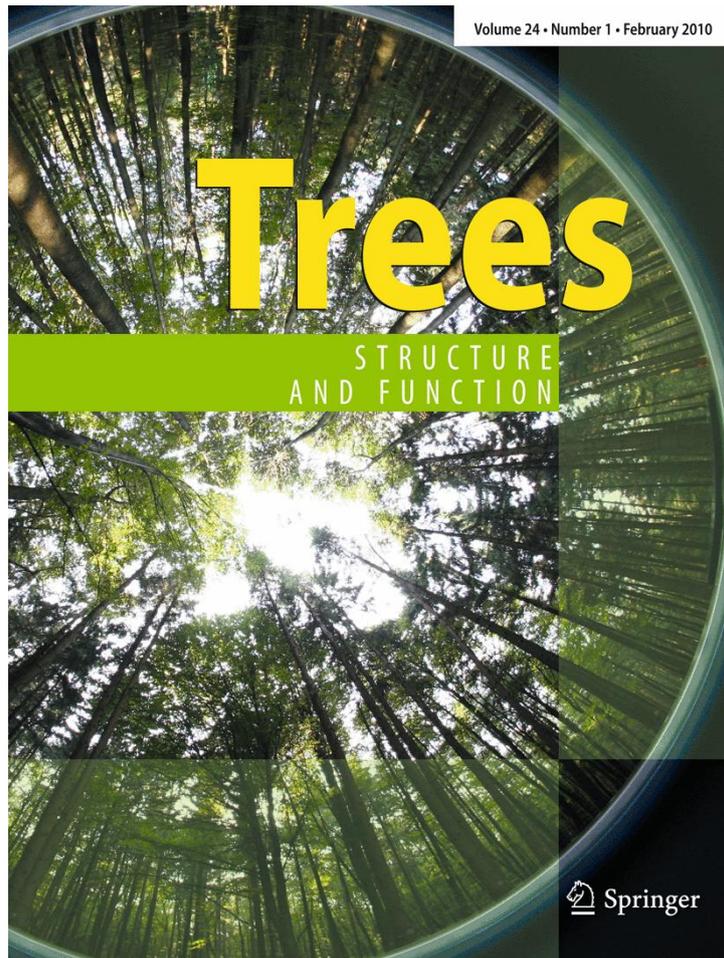


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Flooding tolerance of *Calophyllum brasiliense* Camb. (Clusiaceae): morphological, physiological and growth responses

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Abstract *Calophyllum brasiliense* Camb. (Clusiaceae) is a tree of swampy areas of the coastal “Restinga” in southeastern Brazil (a coastal sand-plain scrub and forest formation). To elucidate possible adaptive strategies that enable this species to occupy areas subjected to seasonal or perennial waterlogging, growth characters such as shoot height, biomass production, leaf expansion, new leaf development, stem diameter, carbon dioxide assimilation rate, stomatal conductance, chlorophyll concentration and fluorescence were studied in controls and plants flooded for up to 150 days. Although flooded plants kept incorporating carbon all through the experiment, their assimilation rate and growth rate were lower than control, non-flooded plants. Injuries such as leaf senescence and abscission were not observed but some flooded plants showed signs of leaf chlorosis. In view of its capacity to maintain carbon assimilation and growth during the treatment, *C. brasiliense* can be classified as flood-tolerant tree. Flooding induced hypertrophy of lenticels, increased stem diameter and development of adventitious roots. These characteristics of *C. brasiliense* are most probably responsible for its survival and success in naturally seasonally flooded areas, inhospitable environments for most tree species. Reduction in total chlorophyll concentration was probably the main cause of reduced carbon dioxide assimilation rate. Based on the results we recommend *C. brasiliense* for rehabilitation of native vegetation in flood-prone areas.

Keywords Clusiaceae · Flooding tolerance · Morphological adaptations · Photosynthetic CO₂ assimilation · Growth

Introduction

Flooding induces a series of physicochemical and biological changes in soil; one of the major changes is the rapid reduction in the amount of oxygen available for roots (Ponamperuma 1984; Drew 1997) thus restricting plant growth, development and productivity (Pezeshki 2001). However, sensitivity to flooding may vary with the type, duration and intensity of the stress, as well as the stage of development of the plant. Even considering only tropical species, there is a large diversity of responses in trees to periodic soil flooding (Joly 1991; Schlüter et al. 1993; Parolin 2000, 2001, 2003; Waldhoff and Furch 2002; Scarano 2002; Parolin et al. 2004; Duarte et al. 2005; Rengifo et al. 2005; Ferreira et al. 2007).

Calophyllum brasiliense Camb. (Clusiaceae) is one typical tree species of naturally waterlogged areas from Central America to the southern coast of Brazil, being present in the Amazon Forests, the Atlantic Forest, including the coastal Restingas (a type of forest on sandbanks of the coastal plains of south-eastern Brazil) and the swamp forests typical of water springs in the Cerrado (Brazilian Savannah) domain (Reitz et al. 1978; Oliveira-Filho and Ratter 1995; Scarano et al. 1997; Holl 1998; Assis 1999; Wittmann et al. 2004). This species is a specialist in flood-prone habitats and thrives in areas where extreme conditions, i.e. long waterlogging periods, ensure that only a few well-adapted species are able to survive (Marques and Joly 2000a, b). Fischer and Dos Santos (2001) found a much higher mortality of *C. brasiliense*

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seedlings in non-flooded than in flooded areas, in lowlands of the Atlantic Rain Forest. Besides commercial utilization principally for wood production, *C. brasiliense* may also be utilized for vegetation restoration programs (Torres et al. 1992).

Flooding tolerance of neotropical species has been explained by different adaptative mechanisms that may include morphological, anatomical and physiological changes. During evolution, different species have developed a variety of strategies that enabled them to occupy and thrive in flood-prone areas, and in most cases the successful strategy combines morphological, anatomical and physiological adjustments (Joly 1991; Parolin et al. 2004). Flooding tolerance is linked to the ability to survive and/or grow in soils naturally subjected to flooding (Rowe and Beardsell 1973), but plant responses to waterlogging may include premature leaf senescence, growth reductions of roots and shoots, development of adventitious roots, aerenchyma and hypertrophy of lenticels (Kozłowski 1997).

Stomatal closure, reduced photosynthesis and damage to photosystem II are common responses to soil oxygen deficiency caused by flooding (Kozłowski and Pallardy 1979; Pezeshki 1993). Both stomatal and non-stomatal limitations are responsible for decreases in photosynthesis after flooding (Kozłowski 1997). Despite unfavorable conditions, several studies show that flooding does not completely impede physiological processes, as some evergreen tree species are photosynthetically active while others flush new leaves during the flood period or produce flowers and fruits (Worbes 1997; Schöngart et al. 2002).

To address current concerns about preservation of natural areas and restoration of areas already degraded, it is essential to understand the physiological behavior and strategies that allow species to survive in environments under severe stress, to reintroduce native species and manage natural areas. In tropical areas there is still a large gap in data on growth, reproduction and dynamics of forests (Clark and Clark 1987; Heideman 1989; Scarano et al. 1997), and particularly in the Brazilian Atlantic Forest, considered a biodiversity hotspot (Myers et al. 2000). There is an urgent need for reliable studies to help preserve the few large remnants. Waterlogged areas, in the context of the Brazilian Atlantic Forest, are even more poorly studied and understood (Scarano et al. 1997).

The aim of this work was to study some adaptive strategies that allow *C. brasiliense* to occur in seasonally and/or permanently flooded areas as the Southeastern Brazilian Coastal Plain Forest, locally named Restinga Forest (Lorenzi 1992; Assis 1999). In this area, the water table, a mixture of fresh and seawater, is above or close to the soil surface for most of the whole year and this extreme condition affects the tree community structure and species

composition (Lacerda 2001). Thus, we evaluated the effect of flooding on the growth and photosynthetic CO₂ assimilation of young plants of *C. brasiliense*, as well as observed and described the development of morphological changes induced by flooding.

Materials and methods

Plant growth conditions and treatments

Fruits of *C. brasiliense* were collected from several trees at the Núcleo Picinguaba (23°21'S and 44°51'W), Serra do Mar State Park, Ubatuba, State of São Paulo, Brazil, on swamp areas of the Restinga Forest. The climate region is tropical and wet, with rains spread over the whole year, but more intense from December to March when the water table rises above soil surface, flooding the root system and part of the stem/trunk of the Restinga Forest plants. Topographic unevenness and variations in the organic layer deposited on the top of the sandy soil result in a mosaic of height and length of the flooding period.

In the laboratory the seeds of *C. brasiliense* were placed in 2-L bags, made of 1-mm mesh nylon nets, and filled with coarse sand. The bags were used to ensure initial good aeration of root systems and to allow the growth of the roots through the mesh of the bags in the flooded treatment. Plants were cultivated in a greenhouse and received water twice a day, and 10% (v/v) Hoagland nutrient solution (400 mL per plant) every 15 days for 6 months. The growth conditions in the greenhouse on clear days were 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR) and the temperatures around 30°C at midday. After this period, plants were separated into two groups: one was maintained under control conditions, i.e. non-flooded plants and watered twice a day, while the other group was transferred to tanks, where the water level was raised to maintain a level 2–3 cm above the soil surface, i.e. flooded plants, as described by Joly and Crawford (1982) and Kolb and Joly (2009). Every 3 weeks the water of the tanks was changed to avoid algae development and during the treatments, nutrient solution was not used to avoid differences in nutrient availability between non-flooded and flooded treatments. Light and temperature were also the same for flooded and non-flooded plants.

Growth performance and morphological changes

Plant growth was evaluated by total leaf area, number of leaves, shoot height, stem basal diameter, and dry masses of leaves, stems and roots. At the start of the experiments five plants were used for growth performance determination (reference values) and new measurements were made

after 30, 60, 90, 120 and 150 days of normoxia and hypoxia in five plants per treatment (different plants measured each time). Leaf area was obtained from scanned images converted by the AREA 2.1 program (©1992 by Carlos Bravo) and the total number of leaves was counted. Shoot height was measured from the base of the stem, just above soil surface, to its apex with a millimeter rule. Stem basal diameter was measured 2 cm above soil level using a caliper rule. For dry mass determination the plants were dried for 2 days at 80°C and weighed in an analytical balance. The original and presumably dying root system was included in the measurements of the root dry mass and total dry mass of the plants. Additionally, every week, possible morphological changes, such as the development of hypertrophic lenticels and adventitious roots, were observed and recorded.

Carbon assimilation, chlorophyll fluorescence and chlorophyll concentration

The carbon dioxide assimilation rate (A) and stomatal conductance (g_s) were measured initially after 15 days of flooding and monthly thereafter for 5 months on flooded and non-flooded plants, always at 9:00–10:00 a.m., with PAR >700 $\mu\text{mol m}^{-2} \text{s}^{-1}$, using a portable photosynthesis system (LCA-4, ADC, Hoddesdon, UK), as described by Kolb and Joly (2009). Light-adapted chlorophyll fluorescence was measured with a pulse-amplitude-modulated portable fluorometer (PAM 2100, Walz GmbH, Effeltrich, Germany). The initial (F_0), maximum (F_m) and variable ($F_v = F_m - F_0$) fluorescence and maximum quantum efficiency of photosystem II (F_v/F_m) were measured and calculated according to Maxuell and Johnson (2000). The electron transport rates (ETR), photochemical quenching

(qP) and non-photochemical quenching (qN) were calculated as described by White and Critchley (1999). The qP refers to photosynthetic electron transport and qN to thermal dissipation of excitation energy as heat. The measurements of A , g_s and chlorophyll fluorescence were always taken on two mature and fully expanded leaves of apices from five plants per treatment. These leaves were used to extract pigments after 15 days of treatments, according to Hiscox and Israelstam (1979) and the concentrations of chlorophyll a , chlorophyll b and total chlorophyll were calculated (Arnon 1949).

Statistical analysis

Significant differences between treatments were determined using two-way ANOVA followed by Tukey's test ($P < 0.05$) as described by Sokal and Rohlf (1995) and Zar (1999).

Results

Morphological changes

All plants survived the flooding period, although there were some stress symptoms, such as reduced shoot height, leaf expansion, new leaf production (Table 1), and leaf chlorosis (Table 3). During the first week of flooding there was conspicuous development of hypertrophic lenticels on submerged parts of stems (Fig. 1). Adventitious roots were rare and appeared in only a few flooded plants on the base of the stem. Compared to controls, after 90 days, the stems of flooded plants were very different, with increased basal diameter (Table 1), large number of hypertrophic lenticels

Table 1 Shoot height, number of leaves, stem diameter and total leaf area of flooded and non-flooded seedlings of *C. brasiliense*

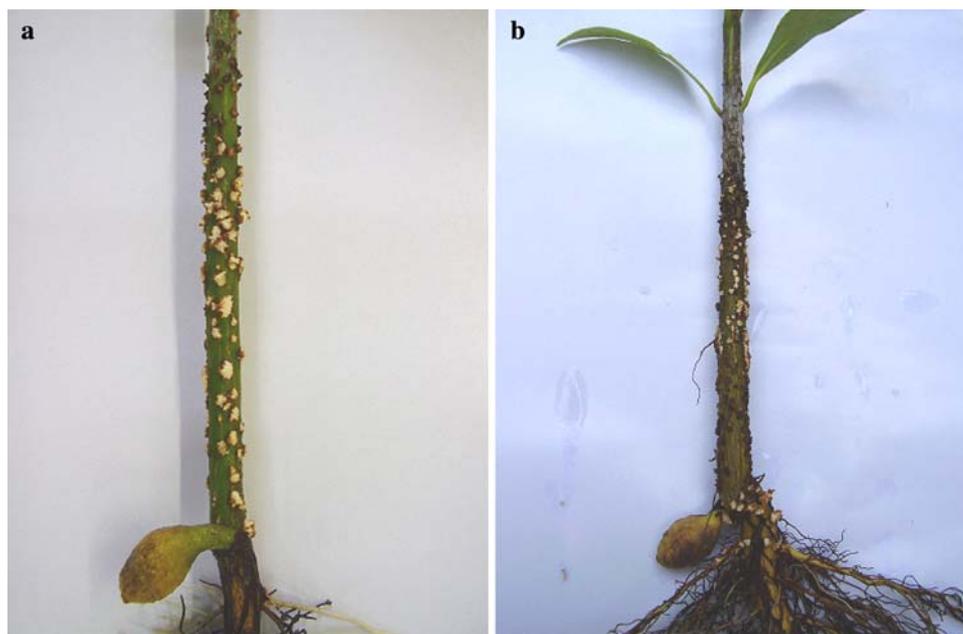
Days	Treatments	Height (cm)	Number of leaves	Stem diameter (mm)	Total leaf area (cm ²)
0	–	22.60 ± 3.05	8.0 ± 1.41	4.65 ± 0.31	86.66 ± 15.84
30	Non-flooded	18.52 ± 2.85	7.6 ± 0.89	4.73 ± 0.22	88.43 ± 11.11
	Flooded	19.06 ± 1.77 NS	6.4 ± 0.89 NS	4.77 ± 0.31 NS	72.09 ± 6.44 NS
60	Non-flooded	23.96 ± 2.21	11.6 ± 1.67	5.54 ± 0.51	88.43 ± 11.11
	Flooded	21.58 ± 1.67 NS	8.2 ± 1.09*	6.53 ± 0.67 NS	140.19 ± 33.89 NS
90	Non-flooded	27.02 ± 1.48	12.0 ± 0	5.70 ± 0.27	173.27 ± 28.15
	Flooded	20.96 ± 3.56 NS	9.0 ± 1.73 NS	7.60 ± 0.23*	124.69 ± 27.06 NS
120	Non-flooded	27.74 ± 2.46	12.2 ± 1.48	5.86 ± 0.33	213.49 ± 38.58
	Flooded	20.68 ± 3.47*	8.6 ± 1.94*	7.31 ± 0.86*	89.80 ± 17.07*
150	Non-flooded	28.78 ± 2.04	13.2 ± 0.83	7.07 ± 0.50	257.0 ± 27.27
	Flooded	21.82 ± 0.66*	7.60 ± 1.14*	8.35 ± 0.38*	102.06 ± 0.08*

Data are mean ± SE ($n = 5$)

NS not significant

* $P < 0.05$. Tukey's test

Fig. 1 Hypertrophied lenticels at the base of the stem of *C. brasiliense* seedlings after 30 days (a) and 120 days (b) of flooding



and some with adventitious roots. Flooding induced browning and death of the lateral roots developed before of the stress imposition. Within 2 months of flooding, new lateral roots developed from the remaining initials of the original root system. These new roots were shorter and thicker, growing horizontally in relation to the water surface.

Biomass production

Flooding reduced the height of plants and the production and expansion of leaves. Flooded plants had thicker stems

probably due to hypertrophy of lenticels and aerenchyma development (Table 1).

Flooding reduced the production of root dry mass at the beginning of experiment, but after 120 days of waterlogging leaf dry mass was lower than in control non-flooded plants, while significant differences in stem dry mass appeared only after 150 days of flooding. Consequently there were significant differences in total dry mass of flooded plants compared to non-flooded plants (Table 2). At the beginning of the experiment the root/shoot ratio was lower in flooded plants, but later on, due to changes in biomass allocation, observed in leaf, stem and root dry

Table 2 Stem, leaf and root dry mass, total dry mass and root/shoot ratio of flooded and non-flooded seedlings of *C. brasiliense*

Days	Treatment	Stem (g)	Leaves (g)	Roots (g)	Total dry mass (g)	Root/shoot ratio
0	–	0.631 ± 0.10	0.645 ± 0.10	0.896 ± 0.14	2.17 ± 0.31	0.71 ± 0.11
30	Non-flooded	0.655 ± 0.05	0.682 ± 0.05	0.971 ± 0.13	2.25 ± 0.22	0.76 ± 0.13
	Flooded	0.601 ± 0.14 NS	0.729 ± 0.08 NS	0.734 ± 0.09*	2.21 ± 0.20 NS	0.53 ± 0.05*
60	Non-flooded	1.021 ± 0.21	0.717 ± 0.06	1.582 ± 0.16	3.32 ± 0.59	0.90 ± 0.15
	Flooded	1.229 ± 0.24 NS	0.896 ± 0.27 NS	1.269 ± 0.22*	3.39 ± 0.56 NS	0.58 ± 0.08*
90	Non-flooded	1.421 ± 0.13	1.561 ± 0.31	1.962 ± 0.39	4.94 ± 0.79	0.65 ± 0.07
	Flooded	1.760 ± 0.49 NS	1.707 ± 0.49*	1.938 ± 0.23 NS	5.40 ± 0.96 NS	0.59 ± 0.16 NS
120	Non-flooded	1.520 ± 0.21	2.188 ± 0.32	2.142 ± 0.58	5.85 ± 0.81	0.58 ± 0.17
	Flooded	1.570 ± 0.41 NS	1.180 ± 0.26*	1.678 ± 0.23*	4.48 ± 0.93 NS	0.62 ± 0.09 NS
150	Non-flooded	2.844 ± 0.29	3.389 ± 0.54	3.694 ± 0.58	9.92 ± 1.15	0.59 ± 0.08
	Flooded	2.270 ± 0.21*	1.506 ± 1.94*	2.528 ± 0.43*	6.30 ± 1.94*	0.67 ± 0.06 NS

Data are mean ± SE ($n = 5$)

NS not significant

* $P < 0.05$. Tukey's test

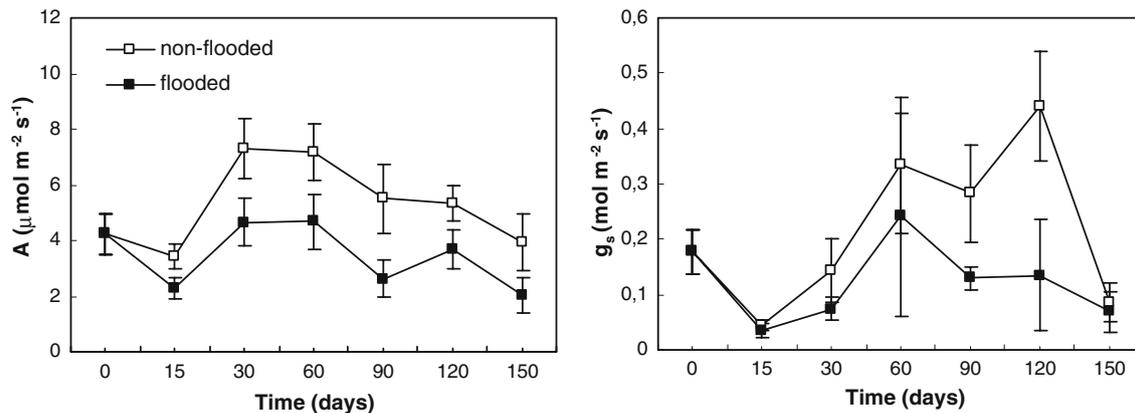


Fig. 2 Photosynthesis (a) and stomatal conductance (g_s) of flooded and non-flooded seedlings of *C. brasiliense*. Symbols represent mean \pm SE ($n = 5$)

mass, the root/shoot ratio was similar in control and flooded plants (Table 2).

Carbon assimilation

From 15 days of flooding onwards, plants had reduced A , but g_s showed no statistically significant decrease until between 60 and 90 days of flooding treatment when values became depressed to below those of controls until 150 days. At this time, conductances of non-flooded plants dropped sharply to match those of the flooded plants (Fig. 2). After 15 days of flooding, the F_v/F_m and ETR also dropped (Fig. 3), together with a significant drop in concentrations of total chlorophyll and chlorophylls a and b (Table 3). Quenching analysis also revealed a significant increase in qN and decrease in qP after 15 days of flooding (Fig. 3).

Discussion and conclusions

Waterlogging is harmful to most terrestrial plants, affecting growth and if longer than several weeks usually results in death (Bailey-Serres and Voesenek 2008). Generally flooding reduces plant growth because root systems are unable to survive in an oxygen-poor environment, losing the capacity to absorb water and nutrients, and to synthesize hormones like cytokinins (Jackson 1993). Under hypoxia and anoxia, the root system metabolism shifts from aerobic to anaerobic, with a reduction in energy yield per molecule of carbohydrate used and with increased production of potentially toxic amounts of ethanol and/or lactate (Crawford 1992; Joly and Brändle 1995; Drew 1997). To compensate for the low energy yield, root cells increase their demand for carbohydrate, reducing availability for other plant functions, such as producing new

leaves, shoots and flowers (Lobo and Joly 2000). Nevertheless, some plants can survive long periods of waterlogging, and also grow vigorously in this condition (Kolb and Joly 2009), a capacity associated with a combination of morphological, anatomical, physiological and biochemical adaptations (Joly 1991; Kolb and Joly 2009). Different responses to flooding may determine species composition and/or abundance along a gradient of flooding intensity and duration in natural areas that are flooded seasonally or permanently (Blom and Voesenek 1996; Drew 1997; Silvertown et al. 1999; Van Eck et al. 2004).

All control and flooded plants survived throughout the experimental period. Flooding duration negatively affected the development of the root system, as well as leaf expansion, resulting in lower total dry mass of the plants flooded for 5 months. Nevertheless, using the criteria defined in one of the first studies of flooding tolerance in neotropical trees (Joly and Crawford 1982), it can be classified as flood tolerant, i.e. it is able to grow and increases its biomass while flooded. Using a larger group of species, Lobo and Joly (2000) defined species as flood tolerant if they could at least maintain their total dry weight when experimentally flooded for periods similar to those of their natural environment. Other studies have found similar results in other neotropical species (Andrade et al. 1999; Davanzo et al. 2002; Mielke et al. 2005; Kolb and Joly 2009). After 5 months of flooding, although there was a reduction of total dry mass of flooded plants, when compared to the non-flooded plants, flooded plants showed higher values of total dry mass than the plants at the start of the experiment (T_0), proving that they were able to grow while flooded. This increment can be observed in all three components, roots, stems and leaves, evidence that flooded plants of *C. brasiliense* did not lose their capacity to grow and accumulate dry mass while flooded, which may represent a competitive advantage in the Restinga Forest, and

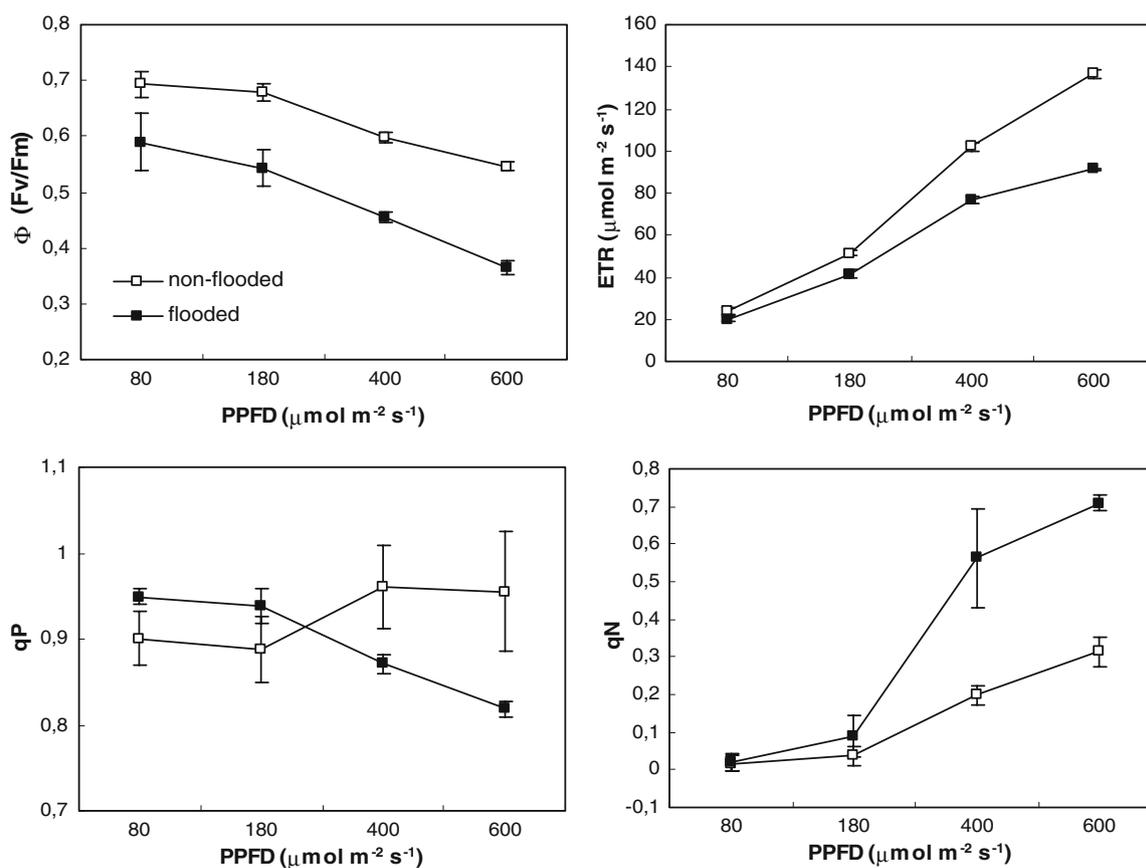


Fig. 3 The maximum quantum efficiency of the photosystem II (F_v/F_m), electron transport rates (ETR), photochemical quenching (qP) and non-photochemical quenching (qN) of flooded for 15 days and non-flooded seedlings of *C. brasiliense*. Symbols represent mean \pm SE ($n = 5$)

Table 3 Chlorophyll concentration of flooded for 15 days and non-flooded seedlings of *C. brasiliense*

	Treatments	
	Non-flooded	Flooded
Chlorophyll <i>a</i> (mg g^{-1})	1.434 ± 0.09	$0.840 \pm 0.11^*$
Chlorophyll <i>b</i> (mg g^{-1})	1.390 ± 0.29	$0.558 \pm 0.11^*$
Total chlorophyll (mg g^{-1})	2.856 ± 0.36	$1.424 \pm 0.22^*$
Chlorophyll <i>a:b</i> (mg g^{-1})	1.063 ± 0.20	$1.522 \pm 0.11^*$

Data are mean \pm SE ($n = 5$)

* $P < 0.05$. Tukey's test

explain why it is one of the most important trees in this environment. Leaf senescence, necrosis or abscission were not observed during the 5 months of flooding, but some plants showed signs of leaf chlorosis, consistent with the observed reduction in chlorophyll content, one of the flooding injuries described by Pezeshki (1994) and Kozłowski (1997).

Schöngart et al. (2002) and Parolin et al. (2004) described the survival of *C. brasiliense* plants under field

conditions in Central Amazonia where the seasonal variation in the river level subjects trees to periods of up to 210 days of continuous flooding per year. During these periods water levels will reach some meters above the soil surface, submerging not only the root system and part of the trunks, but also the whole plant in the case of younger individuals. Other works have described the survival of *C. brasiliense* plants in swamps of southeastern Brazil (Scarano 2002; Duarte et al. 2005; Fischer and Dos Santos 2001). On the other hand, Calvo-Alvarado et al. (2007) reported a high mortality of *C. brasiliense* plants in areas with well defined and extended dry season. So, though surviving, and in some case being the dominant tree, in a variety of neotropical flood-prone areas *C. brasiliense* seems to lose its competitive advantage in drier areas.

The observed reduction in total dry mass of plants flooded was strongly related to the reduction in number and size of leaves, as previously described by Kozłowski (1997), as well as related to the reduction in stem dry mass and substitution of the original root system by a new one, possibly with fewer roots and lower metabolic rates (Mielke et al. 2005). During the substitution of part of the flooded root system the reduction in fully functional roots

contributes to stomatal closure and may be related to the decrease and/or inhibition of cytokinin or gibberellin export (Else et al. 2009).

Lopez and Kursar (1999) working with *C. longifolium* Willd., had similar results to the present results for *C. brasiliense*, with a survival rate of 100% and no evidence of severe injuries in plants flooded for up to 90 days. The gradual changes we observed in flooded *C. brasiliense* suggest that these responses were related to severity and length of the flooded period.

Undoubtedly the morphological changes induced by flooding, such as hypertrophy of lenticels and development of adventitious roots, are strongly associated with the capacity of *C. brasiliense* to survive waterlogging. Parolin et al. (2004) also observed the development of adventitious roots of flooded *C. brasiliense* plants under field conditions in Central Amazonian Floodplain Forest. These morphological/anatomical changes are probably connected with improving oxygen diffusion from the aerial parts to the root system. This strategy to increase aeration of the root system under flooded conditions and to recover its capacity to absorb water and nutrients, as well as to produce hormones such as cytokinins, has been often described (Sena Gomes and Kozłowski 1988; Armstrong et al. 1994; Joly 1996; Núñez-Elisea et al. 1999; Davanzo et al. 2002; Kolb and Joly 2009). However, the conspicuous development of hypertrophic lenticels we observed contrasts with results of Marques and Joly (2000a) that *C. brasiliense* seedlings flooded for 90 days had few such structures. This suggests that different populations of *C. brasiliense* may have different responses to the same stress; i.e. there may be intra-specific genetic variation.

Reductions in A and g_s are commonly observed in flooded plants, and may be due to stomatal or non-stomatal limitations (Kozłowski 1997). Usually, in flooded plants, there is a positive correlation between A and g_s (Pezeshki and Delaune 1990; Pezeshki and Anderson 1997; Lopez and Kursar 1999), but in flooded *C. brasiliense* this correlation only occurred after 90 days of stress, suggesting that the reduction in photosynthesis was not only due to reduced stomatal conductance. The reduction in F_v/F_m , ETR, qP and concentration of chlorophylls and the increase in qN in flooded plants after 15 days of treatment supports the hypothesis that the drop in net carbon assimilation was also caused by non-stomatal factors (Guidi and Soldatini 1997; Haldimann and Strasser 1999; Smethurst et al. 2005). Lower F_v/F_m at the end of the experiment in flooded plants of *Myrica cerifera* was also interpreted by Naumann et al. (2008) as indicative of possible photodamage. In another tropical tree, *Genipa americana*, photosynthesis was reported to be co-limited by stomatal and non-stomatal factors in flooded plants (Mielke et al. 2003). Working with four flood-tolerant tropical tree species, Rengifo et al. (2005) associated a

decrease in A and g_s during flooding with a decrease in maximum quantum yield of PSII, F_v/F_m , and absence of a compensatory increase in qN. According to Herrera et al. (2008) these results suggest that flooding not only reduces A by decreasing g_s but also impairs photosynthetic capacity. Pezeshki (2001) reinforces that in longer periods of flooding the non-stomatal limitations, such as pigment degradation and alteration in enzymes of the Calvin Cycle are the main causes of declines in carboxylation efficiency.

Nevertheless, although growth rate and net carbon assimilation were reduced during flooding, *C. brasiliensis* seedlings were able to survive and accumulate biomass. The rare ability to grow while flooded may explain why *C. brasiliense* is one of the dominant species in naturally flooded areas of southern Brazil to Central America. Based on the data presented we conclude that *C. brasiliense* is flood tolerant, and we recommend its use in restoration programs aiming to recover areas that are naturally subjected to long periods of flooding.

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