Changes in photosynthesis and fluorescence in response to flooding in emerged and submerged leaves of *Pouteria orinocoensis*

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Abstract

In the seasonally flooded forest of the Mapire River, a tributary of the Orinoco, seedlings remain totally covered by flood water for over six months. In order to characterize the physiological response to flooding and submergence, seedlings of the tree *Pouteria orinocoensis*, an important component of the forest vegetation, were subjected experimentally to flooding. Flooding was imposed gradually, the maximum level of flood including submerged and emerged leaves. After 45 d a severe reduction of net photosynthetic rate (P_N) and stomatal conductance (g_s) was observed in emerged leaves, whereas leaf water potential remained constant. The decrease in P_N of emerged leaves was associated to an increase in both relative stomatal and non-stomatal limitations, and the maintenance of the internal/air CO_2 concentration (C_i/C_a) for at least 20 d of flooding. After this time, both P_N and g_s became almost zero. The decrease in photosynthetic capacity of emerged leaves with flooding was also evidenced by a decrease in carboxylation efficiency; photon-saturated photosynthetic rate, and apparent quantum yield of CO_2 fixation. Oxygen evolution rate of submerged leaves measured after three days of treatment was 7 % of the photosynthetic rate of emerged leaves. Submersion determined a chronic photoinhibition of leaves, viewed as a reduction in maximum quantum yield in dark-adapted leaves, whereas the chlorophyll fluorescence analysis of emerged leaves pointed out at the occurrence of dynamic, rather than chronic, photoinhibition. This was evidenced by the absence of photochemical damage, *i.e.* the maintenance of maximum quantum yield in dark-adapted leaves. Nevertheless, the observed lack of complementarity between photochemical and non-photochemical quenching after 12 d of flooding implies that the capacity for photochemical quenching decreased in a non-co-ordinate manner with the increase in non-photochemical quenching.

Additional key words: carboxylation efficiency; chlorophyll; internal CO_2 concentration; oxygen evolution rate; photoinhibition; quantum yield; photochemical and non-photochemical quenching; stomatal conductance; tropical forest.

Introduction

Flooding and subsequent submergence of vegetation in tributaries of tropical rivers is a common phenomenon. The effects of flooding or submergence are multiple and complex, and include mechanical damage, low photon availability, limited gas diffusion, leaching of soil nutrients, and increased susceptibility to pests and diseases (Singh *et al.* 2001). During submergence, gas exchange between plant and its environment is markedly reduced, since the diffusion rates of gases in water are approximately 10 000 times lower than in air. Oxygen deficiency is the main constraint during flooding, but during submergence CO_2 diffusion and high diffusive impedance may also be restrictive to photosynthesis (Nabben *et al.* 1999).

Flood-tolerant species possess metabolic, morphological, and anatomical adaptations to survive to the detrimental effects of the anaerobic soil environment created by continuous flooding. Lenticel hypertrophy, aerenchyma formation, stem hypertrophy, and the formation of adventitious roots are among the morphological and anatomical adaptations caused by flooding (Pimenta *et al.* 1998).

From a physiological point of view, different strategies of flood tolerance have been discerned. They are re-opening of stomata under flood and the speed with which leaf conductance (g_s) is recovered after flooding (Pezeshki 1993, Li *et al.* 2004), the independence of photosynthesis and g_s from flooding (Fernández *et al.* 1999), a high pre-flood root tissue starch content (Gravatt and Kirby 1998), and, on submergence, biochemical tolerance to anoxia (He *et al.* 1999, Schlüter and Crawford 2001), a high initial leaf saccharide content (soluble sugars and starch) (Laan and Blom 1990),

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reduced shoot elongation (Singh *et al.* 2001), and high underwater photosynthetic rates (Nabben *et al.* 1999).

In adult trees of *Acosmium nitens*, *Eschweilera tenuifolia*, and *Psidium ovatifolium* growing in the Mapire forest (Venezuela), submerged leaves that had been under water between four days and four months generally had P_N and g_s similar to those of aerial leaves. Measurements in submerged leaves were done in gas phase, results suggesting maintenance of photosynthetic activity under water (Fernández *et al.* 1999).

Pouteria orinocoensis is a widely distributed species in the seasonal flooded forest of the Mapire river, with a high importance value index in a transect from the Mapire river through the seasonally flooded forest to the non-flooded savannah (Rosales 1988). This distribution determines a great variation in the characteristics of flooding to which the individuals of this species are exposed during an annual flood cycle. I evaluated the changes in leaf gas exchange and fluorescence of chlorophyll (Chl) *a* in response to flood of emerged and submerged leaves of seedlings of *P. orinocoensis* under semi-controlled conditions; photosynthetic rate of submerged leaves was measured as oxygen evolution in the liquid phase.

Materials and methods

Plants: Seedlings of *Pouteria orinocoensis* (Aubr.) Penn. Ined. (Sapotaceae), locally named “Cacho”, were collected from the understory of the Mapire river forest and grown for one year before the experiment in the gardens of the Instituto de Biología Experimental (Caracas). Mean annual minimum/maximum temperature was 20.0/32.2 ºC. Seedlings were transferred to plastic pots (300 cm^3) filled with soil from the Mapire forest, frequently watered, and supplied once a month with 100 cm^3 of Hogland’s nutrient solution. At the beginning of the experiment, plants were 20–45 cm high and were subjected to a previous cycle of flooding. Plants were gradually flooded at the rate of 10 cm d^{-1}, resembling the natural flooding rate at the Mapire River. Flooding was stopped when the water column was 28 cm above soil level; water level was re-established every three days. In this condition, there were submerged and emerged leaves; the duration of the treatment was 45 d. Neither O_2 nor CO_2 concentrations of floodwater were controlled.

Water (Ψ) and osmotic (Ψ_s) potential: Ψ was measured in triplicate in leaf discs placed in *C-52* psychometric chambers connected to *HR-33T* Dew Point Microvoltimeter (*Wescor*, Logan, UT, USA). Ψ_s was determined in the same discs after freezing in liquid N_2. Turgor pressure (P) was calculated as $P = \Psi - \Psi_s$.

Leaf gas exchange: Response curves of P_N to intercellular CO_2 concentration (C_i) and to photosynthetic photon flux density (PPFD) were done using a *CIRAS-1* double CO_2 and H_2O infrared gas analyzer connected to a *PLC* (*B*) assimilation chamber (*PP Systems*, Hitchin, U.K.). Carboxylation efficiency (CE) was calculated as the slope of the linear portion of P_N/C_i curves done in emerged leaves between 08:00 and 12:00 h at a PPFD of 600±100 μmol m^{-2} s^{-1}. Relative stomatal limitation of photosynthesis was calculated as $L_s = 100\,(P_{N0} - P_N)/P_{N0}$, where P_N is the photosynthetic rate measured at ambient [CO_2], $C_a = 350$ μmol mol^{-1}, and P_{N0} is the photosynthetic rate at $C_i = 350$ μmol mol^{-1} (Farquhar and Sharkey 1982). Relative mesophyll limitation was calculated as $L_m = 100\,(P_{NC} - P_{NF})/P_{NC}$, where P_{NC} is the photosynthetic rate in control leaves at saturating C_i (1 600±100 μmol mol^{-1}) and P_{NF} is the rate in leaves of flooded plants at the same C_i (Jacob and Lawlor 1991). Gas exchange was measured *in situ* to avoid aeration of the root system.

Underwater photosynthesis was measured through O_2 evolution using a liquid-phase oxygen electrode (*DW3*, *Hansatech*, Norfolk, UK). Leaf segments of 4 cm^2 area were put in the 20 cm^3 vessel containing the experimental flood water (pH 4.0). The O_2 concentration in the cuvette was lowered before each measurement by flushing with N_2, after Vervuren *et al.* (1999). Average measurement conditions were temperature of 20 °C and PPFD of 400 μmol m^{-2} s^{-1} (saturating, from P_N/PPFD curves). Water temperature was measured with a thermistor connected to a telethermometer (*Yellow Springs Instruments*, Yellow Springs, OH, USA) and PPFD with a *LI-170* quantum sensor connected to a *LI-185* meter (*LI-COR*, Lincoln, NE, USA).

Chl *a* fluorescence was measured in the same leaf used for the gas exchange with a *Mini-PAM* fluorometer (*Walz*, Effeltrich, Germany) after a dark-adaptation of 60 min. Measurements were made *in situ* for emerged leaves and, for the submerged leaves, the fibre optics was adapted to the oxygen electrode chamber. Fluorescence coefficients and related parameters were calculated following Schreiber *et al.* (1994). Maximum quantum yield was determined in dark-adapted leaves as $F_V/F_M = (F_M - F_0)/F_M$. Quantum yield of photosystem 2 was calculated as $\Phi_{PS2} = (F'_M - F_S)/F'_M$ and non-photochemical quenching as $NPQ = (F_M - F'_M)/F'_M$. The quantum yield of photochemical quenching (Y_P) was calculated as $Y_P = F'_M - (F_S/F'_M)$ and the quantum yield of non-photochemical quenching (Y_N) as $Y_N = (F_S/F'_M) - (F_S/F_M)$ (Laisk *et al.* 1997). The parameters Y_P and Y_N were generated through light curves and used to assess the occurrence of chronic photoinbition, which is shown by values of $Y_P + Y_N$ lower than 0.8 (Laisk *et al.* 1997).

Statistical analysis: Results were analysed by a one- or two-way ANOVA followed by Duncan’s multiple range test. When required, data were arcsine transformed previous to the analysis (Φ_{PS2}, Y_P+Y_N, L_s, and L_m).

Results are means ± SE. The significance level for all analyses was set at $p \leq 0.05$.

Results

Between days 0 to 45 of flooding, leaf Ψ did not change, whereas Ψ_s decreased 1.9 times and P increased 1.7 times (Fig. 1*A*). The P_N and g_s of emerged leaves decreased gradually during flooding; stomata were closed after 45 d and P_N was zero (Fig. 1*B,C*). A significant linear decrease in P_N with g_s of zero intercept with the abscissa was found ($r^2 = 0.96$; Fig. 2). Photosynthetic rates of submerged leaves, estimated by oxygen evolution, were one order of magnitude lower after a few days of submergence than emerged leaves, and after 12 d, oxygen evolution rate was almost zero (Fig. 1*B*). Three plants were drained on day 12 of flooding and P_N was measured in the gas phase after three and 30 min of drainage. P_N thus measured was similar to oxygen evolution rates (0.2±0.1 μmol m^{-2} s^{-1}).

In emerged leaves flooding induced a decrease of 70 % in CE and of 74 % in light respiration rate, and an increase of 76 % in CO_2 compensation concentration (Table 1). Operational C_i did not change during the experiment, the average value of C_i/C_a being 0.76±0.01. Relative stomatal limitation of photosynthesis rapidly increased to 71 %, remaining constant until day 20 of flooding. Relative limitation, in contrast, increased gradually during the first 20 d of flooding (Table 1). The measurement of photosynthetic response to PPFD and C_i was not feasible after day 29 due to very low values of g_s.

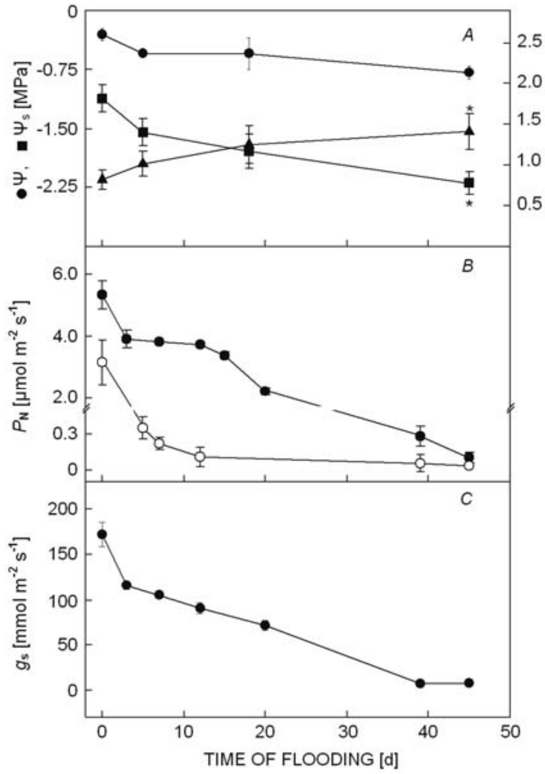

Fig. 1. Time-course of changes in seedlings of *Pouteria orinocoensis* under flooding in (*A*) leaf water potential (●), osmotic potential (■), and turgor pressure (▲); (*B*) photosynthetic rate in emerged leaves measured in gas phase (●) and submerged leaves measured in liquid phase (○), and (*C*) stomatal conductance in emerged leaves. Means ± SE ($n = 6$). An asterisk indicates a significant difference with control values ($p \leq 0.05$).

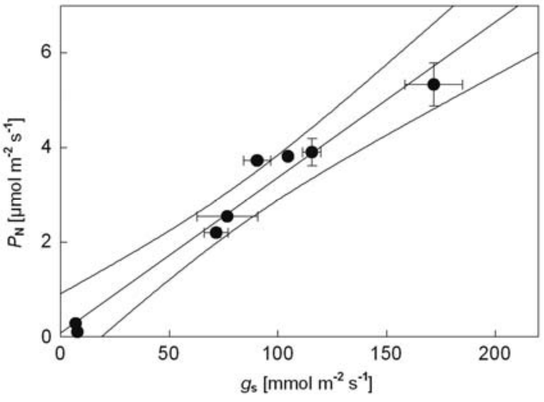

Fig. 2. Relationship between P_N and g_s in emerged leaves of seedlings of *Pouteria orinocoensis* under flooding. Means ± SE.

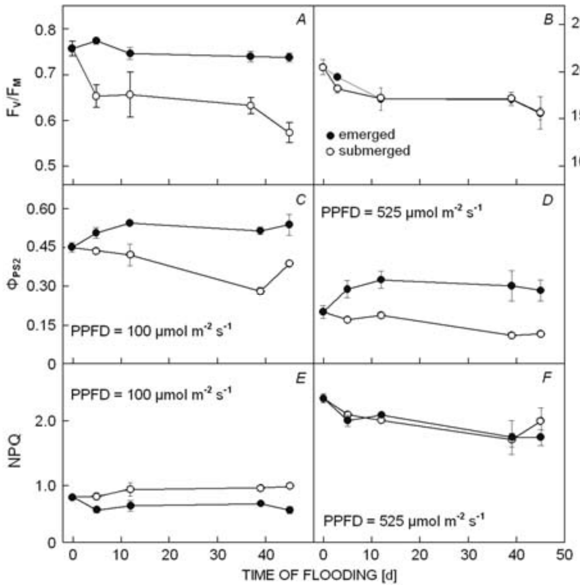

Fig. 3. Time-course of changes under flooding in emerged (●) and submerged (○) leaves of seedlings of *Pouteria orinocoensis* in (*A*) maximum quantum yield of photosystem 2 (PS2) of dark-adapted leaves; (*B*) minimum fluorescence; (*C*) relative quantum yield of PS2 measured at PPFD = 100 μmol m^{-2} s^{-1}; (*D*) non-photochemical quenching (NPQ) measured at PPFD = 100 μmol m^{-2} s^{-1}; (*E*) quantum yield measured at PPFD = 525 μmol m^{-2} s^{-1}; and (*F*) NPQ measured at PPFD = 525 μmol m^{-2} s^{-1}. Means ± SE ($n = 6$).

Table 1. Changes during flooding in parameters calculated from the response curves of photosynthetic rate to intercellular [CO_2] in emerged leaves of seedlings of *Pouteria orinocoensis*. Means (n = 6). *Different letters* by the same column indicate statistically significant differences ($p \leq 0.05$) assessed through a one-way ANOVA and Duncan's post-hoc test.

Time of flooding [d]	Carboxylation efficiency [mol m^{-2} s^{-1}]	Light respiration rate [µmol m^{-2} s^{-1}]	CO_2 compensation concentration [µmol mol^{-1}]	L_s [%]	L_m [%]
0	0.050 a	3.41 a	77.1 a	36 a	0
3	0.041 a	3.69 a	110.7 b	50 b	7 a
7	0.037 b	1.16 b	125.6 b	71 c	16 b
12	0.028 c	1.89 c	128.9 bc	76 c	48 c
20	0.015 d	0.87 d	136.0 c	70 c	61 d

Table 2. Changes during flooding in parameters calculated from the response curves of photosynthetic rate to PPFD in emerged leaves of seedlings of *Pouteria orinocoensis*. Means (n = 6). *Different letters* by the same column indicate statistically significant differences ($p \leq 0.05$) assessed through a one-way ANOVA and Duncan's post-hoc test.

Time of flooding	Saturated rates [µmol m^{-2} s^{-1}]	Apparent quantum yield [µmol $µmol^{-1}$]	Dark respiration rate [µmol m^{-2} s^{-1}]	Compensation irradiance [µmol mol^{-1}]
0	5.62 a	0.043 a	1.28 a	32.3 a
3	3.84 b	0.037 b	1.16 a	35.6 a
7	3.87 b	0.025 c	1.13 a	84.4 b
15	3.84 b	0.020 c	1.80 a	51.7 b
20	3.54 b	0.022 c	1.80 a	119.3 c

Table 3. Statistical significance of the effects on seedlings of *Pouteria orinocoensis* of time under flooding and the leaf condition (emergence or submergence) and the interaction between time under flooding and leaf condition. Significance levels, as calculated by a two-way ANOVA with repeated measurements are shown. Differences were considered significant at $p \leq 0.05$ and were indicated by an asterisk.

	Time under flooding	Condition	Time × condition
F_V/F_M	0.0001^*	0.0001^*	0.0035^*
F_0	0.0071	0.6839	0.6015
Φ_{PS2} at PPFD = 100 µmol m^{-2} s^{-1}	0.0458^*	0.0001^*	0.0017^*
NPQ at PPFD = 100 µmol m^{-2} s^{-1}	0.3377	0.0001^*	0.0060
Φ_{PS2} at PPFD = 525 µmol m^{-2} s^{-1}	0.5604	0.0001^*	0.1567
NPQ at PPFD = 525 µmol m^{-2} s^{-1}	0.0001^*	0.1814	0.0535

Curves of P_N/PPFD of emerged leaves done at different times during flooding (Table 2) showed a reduction of 37 % in photon-saturated P_N, and of 48 % in apparent quantum yield of CO_2 fixation, and an increase in compensation irradiance of 269 % on day 20 of flooding. Dark respiration was not altered by the treatment.

F_V/F_M decreased significantly in submerged leaves and did not change in emerged leaves (Fig. 3*A*). A significant decrease of F_0 with time was observed, with no differences between emerged and submerged leaves (Fig. 3*B*). The Φ_{PS2} at low and high PPFD (100 and 525 µmol m^{-2} s^{-1}) increased in emerged leaves and decreased in submerged leaves during treatment (Fig. 3*C,D*). Non-photochemical quenching at low PPFD decreased in the emerged leaves and did not change in submerged leaves (Fig. 3*E*) but at high PPFD there were no differences between emerged and submerged leaves, values in both decreasing with time (Fig. 3*F*).

The relationship between Y_P and Y_N of emerged leaves during flooding was linear throughout the flooding period but after 12 d of flooding the complementarity was lost, as evidenced by the observation that the interceptions or the confidence intervals did not include 0.8 on one of both axes (Fig. 4). Submergence determined a rapid loss of complementarity, the relationships losing linearity from day 5 (Fig. 4). The sum ($Y_P + Y_N$) decreased with flooding in emerged leaves and decreased even further in submerged leaves; a significant effect of the time of flooding, condition (emerged or submerged), and the interaction between both were found (Table 4).

Discussion

In woody plants subjected to flooding a reduction in root hydraulic conductivity coupled with reduced leaf water potential is the key factor for stomatal closure (Else *et al.* 2001). In seedlings of *P. orinocoensis* leaf Ψ was not reduced in response to flooding, similarly to water-logged seedlings of *Quercus robur, Q. rubra,* and *Q. palustris* (Dreyer *et al.* 1991). In the absence of water deficit, abcisic acid (ABA) accumulated in leaf tissues may induce stomatal closure in response to flooding. Additionally, ABA may be synthesised in root tips submitted to anoxia and transported to leaves *via* the transpiration stream (Jackson 2002), resulting in stomatal closure without decreases in leaf Ψ. The occurrence in seedlings of *P. orinocoensis* during flooding of positive turgor pressures indicates that either leaf water deficit did not occur or osmotic adjustment took place in the leaves. In the present study, if the assumption is made that leaf water content was high, the decrease in Ψ_s without changes in Ψ may be interpreted as evidence of osmotic adjustment. The significance of osmotic adjustment was not clear in *P. orinocoensis* due to the observed decrease in P_N and g_s with flooding. In tropical trees the accumulation of osmotically active metabolites could increase pressure potential and allow the resumption of high P_N and g_s when water falls (Rengifo *et al.* 2005).

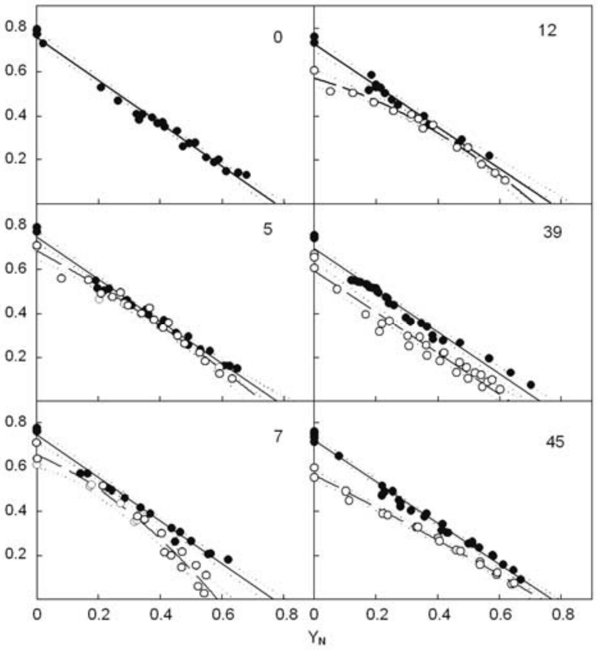

Fig. 4. Relationship between quantum yield of photochemical quenching (Y_P) and quantum yield of non-photochemical quenching (Y_N) of emerged (●) and submerged (○) leaves of seedlings of *Pouteria orinocoensis* after increasing length of time under flooding (*number of days shown in panels*). The linear regression (*solid line*) and non-linear regression (*dotted line*) are distinguished.

Table 4. Changes with flooding in seedlings of *Pouteria orinocoensis* in quantum yields of photochemical and non-photochemical quenching in emerged and submerged leaves. *Different letters* indicate statistically significant differences ($p \leq 0.05$) assessed through a two-way ANOVA and Duncan's post-hoc test.

Time of flooding [d]	Y_P+ Y_N	
	emerged	submerged
0	0.764 c	
5	0.762 c	0.723 c
7	0.754 c	0.698 c
12	0.741 b	0.694 c
39	0.709 a	0.614 d
45	0.742 b	0.657 e

After 45 d of flooding a severe reduction in P_N and g_s was observed in seedlings of *P. orinocoensis*. Flooding induced reductions in gas exchange of a great number of seedlings of tree species, such as *Taxodium distichum*, *Quercus falcata,* and *Quercus lyrata* (Pezeshky *et al.* 1996), *Quercus michauxii*, *Quercus nigra, Quercus nuttallii,* and *Q. lyrata* after 4 months of freshwater flooding (McLeod *et al.* 1999), and some grafts of *Annona* trees (Núñez-Elisea *et al*. 1999). Stomatal closure can be critical for flood tolerance, since it often reduces carbon assimilation and may result in tree mortality (Núñez-Elisea *et al.* 1999).

The relationship between P_N and g_s was a straight line with the interception at zero and the C_i/C_a ratio was constant averaging 0.76±0.01 at least during the first 20 d of flooding. This, together with increases in both relative stomatal and non-stomatal limitations, suggests co-limitation of photosynthesis by stomatal and mesophyll factors during flooding. This is supported by the relatively small daily differences in micro-environmental variables, which, in order to assess the occurrence of co-limitation, should be constant (Schulze and Hall 1982).

A reduction with flooding in the photosynthetic capacity of emerged leaves of seedlings of *P. orinocoensis* was evidenced by the decrease in C_i-saturated P_N and CE, and flood-induced reduction in photon-saturated P_N and apparent quantum yield of CO_2 fixation. This is similar to plants of the flood-tolerant species, *Nyssa aquatica*, subjected to flooding and salinity (Pezeshki 1987).

Submerged leaves of seedlings of *P. orinocoensis* did not exhibit appreciable photosynthetic rates after 5 d under water, measured as oxygen evolution or as CO_2 exchange of leaves brought to the air and measured in the gas phase. The reason for this difference could lie in an age-dependent response to flooding. In *Rumex* species, the responses to flooding and submergence were different between seedlings and mature plants. The differential survival of juvenile and mature plants after 60 d of submergence might have been caused by higher saccharide contents in mature plants (Nabben *et at.* 1999). In *Larix laricina* an age-dependent reduction in P_N and g_s due to flooding was observed which suggested that the absence in young seedlings of development of adventitious roots during flooding determined a lower root hydraulic conductance (Islam and Macdonald 2004). The velocity of submergence development, a gradual transition from normoxia to anoxia provides an opportunity to acclimation. In plants transferred directly from normoxia to anoxia (anoxic shock) the anaerobic respiration collapsed after 1.5 h (Drew 1997) and morphological adaptations that could permit efficient oxygen supply to roots did not develop. Submerged leaves of adult trees of *P. ovatifolium* or *E. tenuifolia* (Fernández *et al.* 1999), in which values of P_N and g_s (measured in the gas phase) were similar to those of emerged leaves and showed daily positive oscillations in glucan content, evidenced the maintenance of photosynthetic capacity under water.

Chl *a* fluorescence is a very sensitive tool to study the stress-induced damage to photosystem 2 (PS2) (Drew 1997). The maximum efficiency of PS2 in dark-adapted leaves, which assesses the functioning of PS2 reaction centres, was considerably reduced in submerged leaves of seedlings of *P. orinocoensis* but did not change with flooding in emerged leaves. In tropical trees, decreases or absence of changes in F_V/F_M have been found in response of submergence. Absence of changes was reported in *Symmeria paniculata* (Rengifo *et al.* 2001, Waldhoff *et al.* 2002), *Psidium ovatifolium* (Rengifo *et al.* 2001), *Nectandra amazonum* (Parolin 2001). Decreases were found in *E. tenuifolia* and *C. laurifolia* (Rengifo *et al.* 2001). In seedlings of the temperate trees *Q. robur* and *Q. rubra*, complete but not partial flooding caused a decrease in F_V/F_M (Dreyer 1994). The decrease in F_V/F_M has been mainly associated to increases in F_0 (Schlüter and Crawford 2001). This was not the case for seedlings of *P. orinocoensis,* in which the observed decreases in F_0 did not explain the differences in F_V/F_M between submerged and emerged leaves.

Φ_{PS2} as an estimate of the rate of linear electron transport in the leaf was considerably reduced in submerged leaves of seedlings of *P. orinocoensis* at low irradiance. In emerged leaves, Φ_{PS2} increased with flooding at both low and high PPFD. The correlation between photochemical and non-photochemical quenching (NPQ) in either emergent of submerged leaves was poor at high PPFD, since NPQ was high in both leaf conditions and did not respond to time under flood. In contrast, in emerged leaves of adult trees of *E. tenuifolia* NPQ increased with flooding, and in submerged leaves a significant decrease relative to the corresponding emerged leaves was found during a long period of flooding (Rengifo *et al.* 2001).

The analysis of Chl *a* fluorescence in seedlings of *P. orinocoensis* indicated that submersion induced a chronic photoinhibition of leaves, viewed as a reduction in F_V/F_M (Osmond 1994). Laisk *et al.* (1997) reported that the sum of $Y_P + Y_N$ is constant and equal to *ca.* 0.8 in a variety of treatments, implying that the capacity for photochemical quenching decreases in a co-ordinate manner with the increase in NPQ. The emerged leaves of *P. orinocoensis* exhibited a dynamic photoinhibition, rather than chronic photoinhibition: even though F_V/F_M did not change with flooding a lack of complementarity between Y_P and Y_N after 12 d of flooding was found, Y_P – Y_N curves being linear but $Y_P + Y_N$ lower than 0.8.

In summary, the decrease in P_N and g_s of emerged leaves of seedlings of *P. orinocoensis* and the maintenance of C_i during flooding could be associated with both stomatal and non-stomatal factor limitations in P_N. The maintenance of F_V/F_M in emerged leaves evidenced the absence of photochemical damage. Submerged leaves were severely affected, underwater P_N decreasing rapidly after the first days of submergence; also, signs of chronic photoinhibition were detected in these leaves. Nevertheless, seedlings of *P. orinocoensis* survived 90 d of complete submersion, new leaves sprouting after 7 d of drainage, and the leaves that had been submerged survived five months after drainage evidencing the absence of post-anoxic injury.

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