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Leaf Pigment Composition and Fluorescence Signatures of Top Canopy Leaves in Species of the Upper Rio Negro Forests

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ABSTRACT

Vegetation of the upper Rio Negro (Amazon region, Venezuela) includes a patchy arrangement of forests of distinct structure and species related to topography, soil fertility and water table fluctuation. The region receives high and non-seasonal rainfall. In all habitats, top canopy leaves are exposed to high light levels year round which saturate photosynthesis and consequently may affect the efficiency of energy conversion. In this study, we assessed top canopy leaves of dominant species in two closed forests to evaluate: 1) if species thriving in contrasting habitats have differing anatomical leaf features and pigment composition and 2) whether species experience fluorescence signature changes at hours of maximal irradiance. Two species from a mixed forest growing in oxisol soil and two species from the Caatinga forest growing in podzol soil were analyzed. Both leaf anatomy and pigment composition of all species were typical of sun-exposed leaves. Additionally, species had comparable chlorophyll a to chlorophyll b ratios ($\mathrm{Chl}_{(a/b)} = \sim 3$) which were independent of habitat soil fertility, suggesting equivalent photochemical capacity across species. Fluorescence signatures declined substantially (12-34%) at midday with small changes in potential quantum efficiency of photosystem II. Thus, the photosynthetic apparatus of these species seems to have adapted to cope with high midday irradiance levels in the respective Amazonian habitats. Although, this effect may result from effective defense mechanisms, it is most likely attributable to xanthophyll cycle activation.

Key words: Amazon forest, chlorophylls, fluorescence, leaf anatomy, high light adaptation

INTRODUCTION

High capacity maintenance for photosynthetic and photorespiratory carbon metabolism is a plant's primary protective mechanism against photoinhibition in sunny environments (Osmond, 1994). Once the dark reactions of photosynthesis are saturated, leaves absorb more photons than can be utilized in photosynthetic electron transport, which leads to potential photoinhibition. Photoinhibition is defined as a reduction in the yield of chlorophyll a variable fluorescence (F_v) of photosystem II (PS II) and is frequently expressed as a decrease in potential quantum efficiency of PSII (F_v/F_m; Krause, 1988), where F_m represents maximum fluorescence. Photoinhibition is reversible in the short-term (within hours) if excess light is harmlessly dissipated in the antennae complexes of PSII as heat (Demmig-Adams and Adams, 1992). In addition, chronic photoinhibition leads to proteolysis of the D1 protein of PSII (Krause and Weis, 1991).

The upper Rio Negro encompasses a patchy arrangement of forests with distinctive structures and species compositions that are related to topography, soil fertility and water table fluctuation. The most diverse, dense and tallest forest is the mixed forest which exists on top of laterite-covered gently rolling hills with soil that consists of well-drained concretionary oxisol with relatively high fertility (Herrera, 1977; Jordan, 1982). The Amazonian Caatinga is found in lower valleys covered by quartz sands and is the most common forest type in the area (Jordan *et al.*, 1982). Within the Caatinga, vegetation is graded from the bottom of the valley to the slopes, with closed forests present in both. The open and low stature sclerophyllous forests are found at the highest elevations (Klinge, 1978). Caatinga soil is generally unfertile and consists of podzolized sand that is relatively more fertile in the valley which becomes waterlogged after heavy rains. The slopes are nutrient poor and the water table fluctuates drastically in the sandy dome areas (Herrera, 1977). Each of the three zones of the Caatinga is home to typical plant species. Leaf traits of dominant tree species become more scleromorphic at the slopes and sandy mounds as the overall fertility declines. This increase in leaf scleromorphy is characterized by greater blade thickness, lower density, higher sclerophyll index (SI; g crude fiber g protein^{-1}), larger carbon to nitrogen ratio and more negative $^{15}\delta$N (Sobrado and Medina, 1980; Sobrado, 2008, 2009, 2010). Regardless of the habitat, top canopy leaves are continually exposed to high light throughout the year which saturates photosynthesis and alters leaf efficiency for energy conversion. Midday dynamic photoinhibition is observed when leaf gas exchange is strongly reduced in dominant tree species of the open sclerophyllous forest which occurs in the most unfertile zone (Sobrado, 2008). Maximum leaf photosynthetic rates are relatively low and comparable across forest types and species (Reich *et al.*, 1994). Therefore, light-saturated net photosynthetic rates may not be high enough to avoid photoinhibition of sunlit leaves in the highest canopy at maximum irradiance. To test this hypothesis, we assessed top canopy leaves of dominant species in two closed forests to evaluate: 1) if leaf anatomical features and pigment composition differ in tree species that thrive in contrasting habitats and 2) if species of different forest types exhibit varying fluorescence signatures at hours of maximal irradiance. We propose possible adaptations that could enable leaves of these tree species to cope with high irradiance in habitats without water supply restrictions and thrive in very contrasting nutritional conditions.

MATERIALS AND METHODS

Study site and plant species: The study site is located near the confluence of the Rio Negro and the Casiquiare rivers in southern Venezuela, 4 km north of San Carlos de Rio Negro (1° 54'N, 67° 3'W; 119 m ASL). The area has an annual rainfall of 3600 mm and a mean temperature of 26°C. The confined area is well-defined with frequently flooded soil (Amazon Caatinga complex) as well as areas of oxisol soils that are never flooded and are covered by a mixed forest (terra firme forest). Both soil types are acidic (pH<4) and organic matter of the poszol soil contains lower phosphorus and nitrogen content than that of the oxisol soil (Medina *et al.*, 1980).

This study is part of a long-term project aimed at using dominant species as ecological indicators to design physiological studies under field conditions. This approach will help elucidate the importance of physiological traits in specific Amazonian habitats within the upper Rio Negro region. Similar to a previous study (Sobrado, 2010), experiments were conducted on species from the two forest types. *Caryocar glabrum* (Aubl.), Pers. (Caryocaraceae), *Ocotea aciphylla* (Nees and Mart. Ex Nees) and Mez (Lauraceae) were selected from the mixed forest (oxisol soil) while *Eperua leucanta* Benth. (Caesalpiniaceae), *Micranda sprucei* (Mull.Arg.) and R.E. Schultes (Euphorbiaceae) were selected from the Amazon Caatinga complex (podzol soil). *E. leucantha* is the dominant species of the Caatinga bottom valley (Klinge, 1978), whereas *M. sprucei* is the dominant species of the ecotone (between the bottom valley and the sclerophyllous forest). Fertility declines and water table fluctuation increases as you move from the top of the oxisol (*C. glabrum* and *O. aciphylla*) to the bottom valley (*E. leucantha*), with the most extreme conditions on the slopes (*M. sprucei*) of the podzolized sands and on the top mounds. Both forests have been previously described in great detail (Klinge, 1978; Uhl and Murphy, 1981). For all measurements, fully exposed top canopy trees were selected and three trees per species were used for field experiments conducted from August to September 2010.

Leaf blade characteristics and pigment composition: Three samples were taken from each tagged tree of each species for subsequent analysis. A subsample of six leaves per species was stored in glycerol and used for anatomical studies; transverse sections of leaf blades were stained with toluidine blue, mounted in glycerol and used to measure the thickness of the leaf and layers as well as palisade and spongy tissue. A second subsample of twenty leaves was used to determine leaf area and dry mass. The third subsample containing ten leaves was frozen for later determination of chlorophyll a (Chl_a), b (Chl_b), and total ($Chl_{(a+b)}$) as well as carotenoid (xantophylls and carotenes; $Car_{(x+c)}$) content. Following the procedures of Lichtenthaler and Wellburn (1983), pigment content was expressed in μm for both leaf area and dry mass basis. The molecular weights of Chl_a, Cl_b and $Car_{(x+c)}$ used for the calculations were 893.5, 906.6 and 545 g moL^{-1}, respectively (Hendry and Price, 1993).

Leaf chl a fluorescence: The Chl_a fluorescence parameters were as follows: Initial (F_o), maximum (F_m), variable (F_m) and F_v/F_m (or F_m-F_o/F_m). Parameters were measured at predawn and midday from the middle of the leaf in order to avoid major veins. These measurements were taken from the sun-exposed adaxial side after leaves were dark-adapted for 30 min. The adaxial side of the leaf has been reported to be highly susceptible to midday photoinhibition (Lichtenthaler *et al.*, 2005). Seventy to ninety measurements were taken per species. A chlorophyll fluorometer (Model OS-30p, OptiSciences, Hudson, USA) was used with white leaf-clips to avoid overheating leaf tissue upon dark-adaptation (Weng, 2006). This instrument uses an adjustable modulated light source to accurately measure F_o which was adjusted to a level that was high enough to measure Fo from leaf antennae but not drive photosynthesis. The OS-30p also has a pre-adjusted saturation pulse light source with a 0.8 sec duration and an intensity of 3000 μmol m^{-2} s^{-1} to reliably measure F_m. Fluorescence parameters were standardized for $Chl_{(a+b)}$. Thus, comparisons of fluorescence signature across species cannot be attributed to differences in $Chl_{(a+b)}$ across species.

Statistical analysis: Measurements were performed on independent replicates and for each parameter, the normal distribution was assessed using the Kolmogorov-Smirnov test and the equal variance was assessed by Levene's test. If data passed the normality and/or equal variance tests, a one-way analysis of variance (ANOVA) was used. Multiple comparisons among the means were made by the Holm-Sidak method. Conversely, if data failed normality and/or equal variance tests, an ANOVA-on-ranks test was used. Statistical differences between means were determined by the Tukey or Dunn's tests for equal or different sample sizes, respectively. The significance level was set at $p<0.05$. Fluorescence parameters at predawn and midday were compared using an independent t-test when data were normally distributed. If the normality test failed, a Mann-Whitney rank sum test was used. All analyses were performed using SigmaStats 3.1 software for Windows.

RESULTS AND DISCUSSION

Leaf blade characteristics: Leaf blade characteristics for each species of both the mixed forest (oxisol) and Caatinga (podzol) are shown in Table 1. The values of leaf area to dry mass ratio (S_w) and leaf thickness were similar to those previously found (Table 1; Sobrado, 2010). *M. sprucei* from the less fertile Caatinga slopes had the highest S_w (161 g m^{-2}, $p<0.05$) and thickest leaf blades (602 µm, $p<0.05$) compared to *E. leucantha* of the Caatinga valley as well as *C. glabrum* and O. *aciphylla* from the mixed forest (Table 1). These patterns were consistent with the fertility decline of the Caatinga slope compared to other habitats analyzed. Leaf anatomy observed in the four species was typically dorsiventral, which is characterized by distinct upper and lower surfaces and stomata located only on the abaxial side (hypostomatous leaves). The chlorenchyma represented a high leaf blade fraction: 83% in *O. aciphylla*, *E. leucantha* and *M. sprucei* and 93% in *C. glabrum* (Table 1). The chlorenchyma was composed of columnar palisade cells and loosely arranged spongy cells on the abaxial side. This leaf anatomy was indeed typical of sun leaves, since shade leaves have diffuse spongy parenchyma. Layers of palisade cells were comparable across species (1-2 layers), whereas the number of spongy cell layers was highly variable (4-12 layers; Table 1). The ration of Palisade to spongy parenchyma (PP/SP) was higher in *C. glabrum* (0.66, $p<0.05$), intermediate in *O. aciphylla*, *E. leucantha* (0.40-0.55) and lowest in *M. sprucei* (0.29, $p<0.05$, Table 1). These values were consistent with those obtained in Borneo tropical forests (0.33-0.66; Cao, 2000). A high PP/SP ratio is indicative of the compact arrangement of cells and high leaf tissue density (Leal and Kappelle, 1994). A low PP/SP ratio indicates larger intercellular space which includes the capacity to scatter light and an increase in the probability of absorption during photosynthesis (Vogelman *et al*., 1996). The four species presented Bundle-Sheath Extensions (BSE) toward both sides of their blades (Table 1). This leaf anatomy is termed heterobaric and is characterized by compartmentalized intercellular gas spaces (Terashima, 1992). A heterobaric anatomy is found in the sun leaves of 96% of emergent top canopy species in tropical forests, whereas a homobaric anatomy is mostly found in shaded environments (Kenzo *et al*., 2007). The BSE creates transparent regions inside the leaf which ultimately improves the light environment, photosynthetic capacity and water use (Liakoura *et al*., 2009). Overall, leaf blade characteristics and anatomical features observed in species of both mixed and Caatinga forests were typical of sun leaves independent of habitat (Cutter, 1971; Cao, 2000; Kenzo *et al*., 2007).

Pigment composition: The Chl_a, Chl_b, $Chl_{(a+b)}$ and $Car_{(x+c)}$ concentrations were determined based on leaf area and dry mass (Table 2). Chl_a and $Chl_{(a+b)}$ were significantly higher ($p<0.05$) in the

Table 1: Mean of leaf blade characteristics

	Mixed forest (Oxisol)		Caatinga forest (Podzol)	
Parameters	*C. glabrum*	*O. aciphylla*	*E. leucantha*	*M. sprucei*
S_w (g m^{-2})	84^c	112^b	87^{bc}	161^a
Leaf blade (µm)	204^b	245^b	222^b	602^a
PP + SP (µm)	191^b	203^b	184^b	499^a
PP/SP (µm µm^{-1})	0.7^a	0.4^b	0.56^{ab}	0.3^c
Layers of PP	2	2	1-2	1-2
Layers of SP	4-5	7-8	5-6	10-12
BSE	Heterobaric	Heterobaric	Heterobaric	Heterobaric

S_w: Leaf dry mass to area ratio, PP: Palisade parenchyma, SP: Spongy parenchyma, BSE: Bundle-sheath extensions. The means followed by different letters were statistically different ($p<0.05$)

Table 2: Mean of leaf pigment compositions

	Mixed forest (Oxisol)		Caatinga forest (Podzol)	
Parameters	*C. glabrum*	*O. aciphylla*	*E. leucantha*	*M. sprucei*
Chl_a (mmol m^{-2})	278^b	436^a	347^{ab}	401^a
Chl_b (mmol m^{-2})	110	156	136	137
$Chl_{(a+b)}$ (mmol m^{-2})	388^b	592^a	473^{ab}	538^a
$Car_{(x+c)}$ (mmol m^{-2})	144	181	170	169
Chl_a (mmol kg^{-1} dm)	3.0^a	3.3^a	2.7^a	1.8^a
Chl_b (mmol g^{-1} dm)	1.2^a	1.2^a	1.1^a	0.6^b
$Chl_{(a+b)}$ (mmol g^{-1} dm)	4.2^a	4.5^a	3.7^a	2.5^b
$Car_{(x+c)}$ (mmol g^{-1} dm)	1.6^a	1.3^a	1.3^a	0.8^b
$Chl_{(a/b)}$	2.6	2.8	2.8	3.0
$Chl_{(a+b)}/Car_{(x+c)}$	2.8	3.4	2.8	3.2

Chl_a, Chl_b $Chl_{(a+b)}$: Chlorophylls a,b and total, $C_{(x+c)}$: Carotenoids. Means followed by different letters were statistically different ($p<0.05$)

mixed forest species *O. aciphylla* (436 and 592 mmol m^{-2}, respectively) and in the Caatinga forest *M. sprucei* (401 and 538 mmol m^{-2}, respectively) compared to the other two species (Table 2). Whereas, Chl_b and $Car_{(x+c)}$ were comparable across species (Table 2), *O. aciphylla* and *C. glabrum* from the mixed forest and *E. leucantha* from the Caatinga showed comparable values in Chl_a, Chl_b, $Chl_{(a+b)}$ and $Car_{(x+c)}$ concentration based on leaf dry mass (Table 2). These parameters were significantly higher than those obtained in *M. sprucei* which were 1.8, 0.6 and 0.8 mmol g^{-1} dm, respectively ($p<0.05$; Table 2). The leaf chlorophyll per unit dry mass is adversely and non-linearly related to leaf thickness, which enhances light penetration within the leaf blades by avoiding the shading effects of the pigment (Cao, 2000). Present results are consistent with this observation; thick leaves of *M. sprucei* had the lowest chlorophyll concentration based on leaf dry mass (Table 2). The Chl_a to Chl_b ratio ($Chl_{(a/b)}$) was found to be approximately 3 which is typical for healthy, sunny leaves and also comparable across species (Sestak, 1985). The values of $Car_{(x+c)}$ were consistent across species when expressed as a function of leaf area. *M. sprucei* (0.8 mmol g^{-1} dm; $p<0.05$) exhibited the lowest content when values were expressed based on leaf dry mass. In sun plants, pigments of the xanthophyll cycle typically make up 30% of the carotenoids and 40% exist in the upper third of the leaf (Robinson and Osmond, 1994). Additionally, the $Chl_{(a+b)}/Car_{(x+c)}$ ratio showed little variation across both forest types analyzed and had a range between 2.6 and 3.4 (Table 2). The $Chl_{(a+b)}/Car_{(x+c)}$ ratio is usually low in sun-exposed leaves when compared to shade leaves (Demmig-Adams and Adams, 1992) due to the involvement of the xanthophyll cycle during the harmless dissipation of excess radiation in leaves exposed to high irradiance (Demmig-Adams *et al.*, 1995).

Leaf Chl a fluorescence: Low leaf fluorescence signatures were observed in both forests measured at midday compared to those at predawn, as shown by the comparatively lower initial (F_o), variable (F_v) and maximum (F_m) fluorescence (Table 3). Differences in fluorescence parameters were species specific and not related to differences in $Chl_{(a+b)}$, since the values for this parameter were standardized according the previous determination. Depression of fluorescence signatures at midday recovered overnight, as shown by the higher values at predawn in all species (Table 3). Thus, F_o was significantly lower in *C. glabrum* ($p<0.001$) as well as *O. aciphylla*, *E. leucantha* and *M. sprucei* at midday ($p<0.01$; Table 2). This decrease in dark-adapted F_o following full sunlight

Table 3: Mean of midday and predawn fluorescence signatures

	Mixed forest (Oxisol)		Caatinga forest (Podzol)	
Parameters	*C. glabrum*	*O. aciphylla*	*E. leucantha*	*M. sprucei*
Midday F_o	107***	107**	99 **	111**
Predawn F_o	131	122	133	131
Midday F_v	389***	373**	398 **	428***
Predawn F_v	587	432	485	499
Midday F_m	496***	480**	496 **	539***
Predawn F_m	718	554	617	630
Midday F_v/F_m	0.78***	0.77	0.80	0.79
Predawn F_v/F_m	0.82	0.78	0.80	0.79

F_o, F_v, F_m: Initial, variable and maximum leaf florescence signatures. Significant differences between midday and predawn measurements: **$p<0.01$, ***$p<0.001$

exposure is likely a result of photoprotection resulting in reduced photon damage (Ball *et al.*, 1994; Osmond, 1994; Guo *et al.*, 2006). Therefore, reaction centers are transformed into highly efficient quenchers that lower F_o (Krause, 1988). F_v and F_m were significantly lower in *C. Glabrum* and *M. sprucei* ($p<0.001$) as well as *O. aciphylla* and *E. leucantha* ($p<0.01$; Table 3) at midday, suggesting that thermal dissipation in the pigment bed is mediated by the xanthophyll cycle (Demmig-Adams *et al.*, 1995). The F_v/F_m ratio (normal value 0.74-0.85) is the most frequent Chl a fluorescence parameter. We determined that all species, with the exception of *C. glabrum* (mixed forest), experienced dynamic changes in their fluorescence parameters without major changes in the maximum quantum yield of PSII (F_v/F_m) at midday (Table 3). Interestingly, a midday F_v/F_m of 0.78 in *C. glabrum* was within the range of a healthy, non-photoinhibited leaf (>0.74). This ratio alludes to inertness and changes in the F_v/F_m ratio are not detected immediately under stress conditions (Lichtenthaler *et al.*, 2005). The F_v/F_o ratio is a more sensitive indicator than F_v/F_m of the maximum efficiency of photochemical processes during PSII (Rohacek, 2002). Although, we observed simultaneous changes in both F_o and F_v, the overall trend of the F_v/F_o ratio (data not shown) was similar to the F_v/F_m ratio. The photosynthetic apparatus of the studied species seems well-adapted to the contrasting nutrient conditions of the study habitats, which allowed adequate coping with the excessive irradiance through an effective defense mechanism that uses xanthophyll cycle activation to optimize and preserve the photosynthetic apparatus function in adaptation to harsh environments (Han *et al.*, 2010). Indeed, continuous sun-exposed leaves can fully compensate for the potential cost associated with preservation and repair of PSII (Krause *et al.*, 2006). Activation of the xanthophyll cycle seems to prevent damage to PSII. Changes in fluorescent parameters in the open sclerophyllous forest (lowest fertility habitat) are more severe, with predawn F_v/F_m ratios remaining slightly depressed (0.74; Sobrado, 2008). This response was also observed in nitrogen-depleted leaves (Verhoeven *et al.*, 1997). Some degree of dynamic photoinhibition (photoprotection) has been observed in a number of tropical sun plants (Krause *et al.*, 1995; Ishida *et al.*, 1999; Kitao *et al.*, 2000). The protective capacity for the xanthophyll cycle is limited by the total pool of xanthins, and sun leaves have lower chlorophyll-to-xanthin ratios than shade leaves (Thayer and Bjorkman, 1990). Low carbon dioxide assimilation rates have been observed in all of the species studied (Reich *et al.*, 1994), suggesting limited photosynthetic capacity for light use. Despite the observation that species thrived in contrasting habitats, the adaptation to high light was remarkably similar. This study adds new insight into leaf adaptation of these forests to high light and shows that top canopy leaves are very well-adapted to cope with high irradiance. Nevertheless, we cannot eliminate the possibility that a greater decrease in the F_v/F_m ratio may occur if the photosynthetic function was further impaired. This would occur through the interaction of high light levels with other environmental factors such as atypical droughts due to the global climate. Future studies should include a complete description of the limitations in observed responses as well as detailed information about the photochemical and biochemical processes involved in both the mixed and Caatinga forests.

CONCLUSION

This study has demonstrated that top canopy leaves of both the mixed and Caatinga forest exhibit an anatomy and contain pigment that is typically associated with sun leaves. Despite species-specific differences in leaf blade characteristics and chlorophyll content, all species had comparable $Chl_{(a/b)}$ ratios. These findings suggest a conserved photochemical capacity in all species regardless of habitat and soil fertility. In addition, similar $Chl_{(a+b)}/Car_{(x+c)}$ values across species suggest an equivalent capability to dissipate excessive light energy. A coordinated quenching of fluorescence signatures was observed at midday in both forests, whereas maximum photochemical efficiency (F_v/F_m) only changed slightly. Furthermore, midday and predawn values were within range for non-photoinhibited leaf tissue of all species.

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