Leaf development and photosynthetic properties of three tropical tree species with delayed greening

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Abstract

Leaf developmental patterns were characterized for three tropical tree species with delayed greening. Changes in the pigment contents, photosynthetic capacity, stomata development, photosystem 2 efficiency, rate of energy dissipation, and the activity of partial protective enzymes were followed in developing leaves in an attempt to elucidate the relative importance of various photoprotective mechanisms during leaf ontogeny. Big leaves of *Anthocephalus chinensis*, a fast-growing light demanding species, expanded following an exponential pattern, while relatively small leaves of two shade-tolerant species *Litsea pierrei* and *Litsea dilleniifolia* followed a sigmoidal pattern. The juvenile leaves of *A. chinensis* and *L. pierrei* contained anthocyanin located below the upper epidermis, while *L. dilleniifolia* did not contain anthocyanin. Leaves of *A. chinensis* required about 12 d for full leaf expansion (FLE) and photosynthetic development was delayed 4 d, while *L. pierrei* and *L. dilleniifolia* required 18 or 25 d for FLE and photosynthetic development was delayed 10 or 15 d, respectively. During the leaf development the increase in maximum net photosynthetic rate was significantly related to changes in stomatal conductance and the leaf maturation period was positively related to the steady-state leaf dry mass per area for the three studied species. Dark respiration rate of leaves at developing stages was greater, and pre-dawn initial photochemical efficiency was lower than that of mature leaves. Young leaves displayed greater energy dissipation than mature leaves, but nevertheless, the diurnal photoinhibition of young *L. dilleniifolia* leaves was higher than that of mature leaves. The young red leaves of *A. chinensis* and *L. pierrei* with high anthocyanin contents and similar diurnal photoinhibition contained more protective enzymes (superoxide dismutase, ascorbate peroxidase) than mature leaves. Consequently, red leaves may have higher antioxidant ability.

Additional key words: *Anthocephalus*; ascorbate peroxidase; chlorophyll fluorescence; intercellular CO_2 content; leaf dry mass per area; *Litsea*; net photosynthetic rate; stomatal conductance; superoxide dismutase.

Introduction

The leaves of many rainforest plants exhibit an unusual form of development in which the expanding leaves contain little chlorophyll (Chl), are often brightly coloured, and leaf greening and photosynthetic capacity develop after full leaf expansion (Richards 1952, Kursar and Coley 1991, 1992). Developing young leaves in species with delayed greening have leaf colours varying from red to blue and sometimes even white. In particular, red juvenile leaves have been noted in many shade-tolerant species (Kursar and Coley 1992). Such red to blue colouration is caused by anthocyanin pigmentation. The ecophysiological role of this pigment in juvenile leaves is, however, poorly understood, although photoprotective (Gould *et al*. 1995, Smillie and Hetherington 1999, Manetas *et al*. 2002), UV screening (Lee and Lowry 1980), and anti-herbivory (Coley and Kursar 1996, Numata *et al.* 2004) functions have been attributed to anthocyanins. For developing young leaves, irradiance may be saturating and in excess of the capacity for photosynthetic utilization at much lower irradiance than in older leaves, making them more susceptible to photoinhibition (Krause *et al*. 1995). Opposed to this, Dodd *et al*. (1998) and Manetas *et al*. (2002) found a lower sensitivity to photoinhibition of younger leaves.

In the present study, we examined leaf developmental patterns of one light demanding and two shade tolerant broad-leaved tropical species with delayed greening and we focused on photosynthetic leaf properties. We followed changes in leaf area, Chl and anthocyanin contents, photosynthetic capacity, photosystem 2 (PS2) efficiency, rate of energy dissipation, and activity of partial protective enzymes in developing leaves of the three species in an attempt to elucidate the relative importance of the various photoprotective mechanisms during leaf ontogeny.

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Materials and methods

Study site and plants: The study was carried out from June to July 2002 in the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences (21°56′N, 101°15′E, 560 m altitude), which is situated in the south of Yunnan Province, SW China. Three broadleaved species were studied: a fast-growing, short-lived light demanding species *Anthocephalus chinensis* (Lam.) Rich. *ex* Walp, and two shade tolerant species *Litsea pierrei* var. *semois* Liou and *Litsea dilleniifolia* Py. Pai *et* Ph. Huang. Plants were grown in a nursery from seeds collected in a nearby forest. They were transplanted to an open site at least 1 y before the start of the research. The seedlings (2–3-y-old) were grown in 53 pots in non-limiting nutrient conditions and were watered two to three times per week to maintain the soil near field capacity. The maximum irradiances (photosynthetic photon flux density, PPFD) at the top of the canopy reached 1 900 μmol m^{-2} s^{-1} during the experimental period. Daytime temperatures reached 32 °C, while night-time temperatures dropped to 17 °C.

Three to four sprouting shoots per species were used to characterise changes in morphological and physiological variations (see below). Leaf pairs were tagged and one of the leaves was measured with a leaf area meter (*LI-3000A*, *Li-COR*, Lincoln, NE, USA). The other leaf was left to reach full leaf expansion.

Pigment analysis and LMA: For determination of the anthocyanin content, leaves of known mass were ground in a mortar and pestle in HCl/methanol (1 %, v/v). This extract was incubated at room temperature for 2 h and subsequently centrifuged at 5 000×*g* for 5 min. The absorptance of the supernatant was measured spectrophotometrically at 530 nm and expressed as relative absorbance per fresh mass, FM [A_{530} g^{-1}(FM); Underhill and Critchley 1994]. Chls were extracted in 80 % acetone and the Chl content was determined by measuring absorbances at 663 and 640 nm, using an UV-B spectrophotometer (*UV-B 2501*, Shimadzu, Japan). Chl content was then calculated according to Arnon (1949). After the measurements, the matured leaves of the selected leaf pairs were dried at 80 °C for 48 h and leaf dry mass per area was determined (LMA, g m^{-2}).

Rates of net photosynthesis (P_N) and dark respiration (R_D) were measured using a portable gas exchange system (*LI-6400*; *Li-Cor*, Lincoln, NE, USA). After bud break when leaves were large enough till leaf full expansion, selected leaves were regularly sealed in the leaf chamber for measurement of photosynthesis at 25 °C with an initial PPFD of 600 μmol m^{-2} s^{-1}. Irradiation was provided by a voltage-controllable halogen lamp. Once P_N had reached a steady state, the PPFD was increased to about 1 800 μmol m^{-2} s^{-1} and measurements were repeated for 3 min. PPFD was then reduced in seven steps to darkness and at each step steady-state P_N was measured. In the last step (darkness) R_D was determined. All values were then analysed using non-linear regressions to fit a rectangular hyperbolic function. P_{max}, the maximum saturated photosynthetic rate, was calculated.

Dependence of Chl fluorescence on irradiance was measured using a portable fluorescence system (*FMS-2.02*, *Hansatech*, King's Lynn, UK) equipped with a leaf clip and a purpose-designed lamp controller to provide PPFD up to 2 000 μmol m^{-2} s^{-1}. Initial (F_0) and maximal (F_m) fluorescence were measured on leaves maintained in the dark for about 15 min and the PPFD was then increased to about 50 μmol m^{-2} s^{-1}. Saturating flashes were then given at 2-min intervals to measure the steady-state fluorescence and the maximum fluorescence during irradiation (F_m'). Then, by briefly darkening the leaf prior to irradiation with far-red (FR) beams, the initial fluorescence (F_0') was measured. The PPFD was then increased in six steps using the same cycle of flashes and FR irradiation at each step. The actual quantum efficiency of electron transport to PS2, $\Delta F/F_m'$, non-photochemical quenching, NPQ, and the Q_A reduction state, $1-q_P$, were calculated according to van Kooten and Snel (1990). The diurnal change in initial photochemical efficiency (F_v/F_m) was measured pre-dawn (06:00) and at the middle of the day (13:00). The diurnal change was calculated as:

$$\%(\text{diurnal photoinhibition}) = \\ = 100 - (F_v/F_{m13:00})/(F_v/F_{m06:00})] \times 100.$$

Determination of enzyme activities and total soluble protein content: For the determination of total superoxide dismutase (SOD) activity, 0.4 g fresh leaf tissue from the leaves was ground in a chilled mortar in 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA. For the extraction of total ascorbate peroxidase (APX), phyllode tissue was ground in 50 mM phosphate buffer (pH 5.0) containing 0.1 mM EDTA, 5 mM ascorbate, 0.5 % (m/v) polyvinylpyrrolidone, 0.1 % (v/v) *Triton X-100*, and 0.05 % (v/v) β-mercaptoethanol. After centrifuging at 12 000×*g* at 4 °C for 15 min, the supernatant of each extract was used as the crude enzyme extract for determination of soluble protein concentration and the activities of SOD and APX. SOD activity was assayed by determining the ability of the extracted enzymes to inhibit the photochemical reduction of nitro-blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (1971). One unit [U] of SOD was defined as the amount of SOD that caused 50 % inhibition of the photo-reduction of NBT. APX activity was determined according to Nakano and Asada (1987) by monitoring the rate of ascorbate oxidation at 290 nm (extinction coefficient = 2.8 mM^{-1} cm^{-1}). The 3 cm^3 of reaction mixture contained 50 nM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.3 mM H_2O_2, and 40 mm^3 of

the enzyme extract. One U of APX was defined as the amount of enzyme that oxidized 1 μmol of ascorbate per min at room temperature. Soluble protein content was determined using a spectrophotometer at 595 nm according to Bradford (1976) with bovine serum albumin as a standard.

Statistical analysis: Multiple analysis of variance (Tukey test, $p \leq 0.05$) was conducted to analyse the significance of differences in the physiological parameters determined.

Results

Leaf flushing and pigment changes: The growth of large leaves of *A. chinensis* followed an exponential pattern, full leaf expansion (FLE) being reached in approx. 12 d. In contrast, the growth of *L. pierrei* and *L. dilleniifolia* followed a sigmoidal pattern, with FLE being reached in 17–19 d and 24–26 d, respectively (Fig. 1).

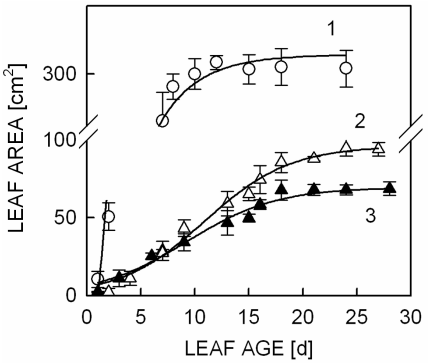

Fig. 1. Changes in leaf area during leaf development in *Anthocephalus chinensis* (○), *Litsea pierrei* (▲), and *L. dilleniifolia* (Δ). Regressions: $1 = -107.27 + 422.68\ [1 - e^{(-0.27\ \text{leaf age})}]$; $2 = 97.78/\{1 + e^{[-(\text{leaf age} - 11.11)/3.95]}\}$; $3 = 69.45/(1 + e^{[-(\text{leaf age} - 9.25)/4.15]})$.

The young leaves of *A. chinensis* and *L. pierrei* were coloured red, whereas those of *L. dilleniifolia* were light green (Table 1). Red colouration was a reliable indicator of anthocyanin presence. Microscopic observations in leaf cross-sections revealed that anthocyanins were located below the upper epidermis of both *A. chinensis* and *L. pierrei* (data not shown). On a FM basis, anthocyanin content decreased gradually as leaves expanded (Fig. 2*A*). After full leaf expansion the leaves of *A. chinensis* and *L. pierrei* lost their red colour and became progressively greener (Table 1). During this period, anthocyanin contents decreased until they were almost undetectable in mature leaves, suggesting their complete catabolism after full leaf expansion. Chl content was very low in young leaves and increased throughout development in all three species (Fig. 2*B*). Even when FLE was reached, Chl content continued to increase till a steady-state Chl content was reached in mature leaves.

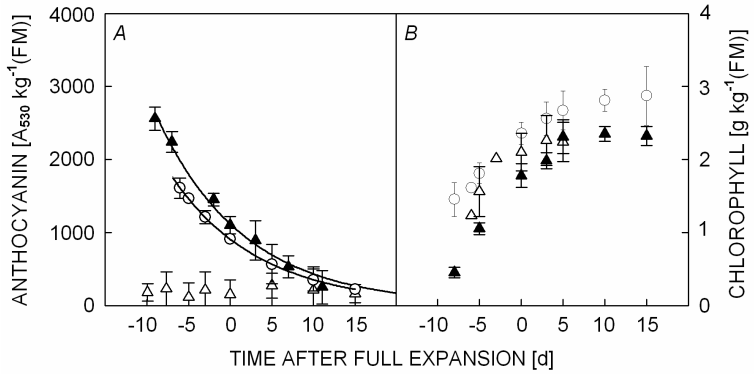

Fig. 2. Anthocyanin content (*A*) in relative absorbance per fresh mass and chlorophyll content (*B*) during leaf expansion in *A. chinensis* (○), *L. pierrei* (▲), and *L. dilleniifolia* (Δ). Error bars indicate SD (n = 3–4).

P_{max}, R_D, and stomatal development: The highest P_{max} values were obtained not before FLE in all three species. About 4 d after FLE, P_{max} of *A. chinensis* leaves was the highest, *ca*. 12 μmol m^{-2} s^{-1}, suggesting a fairly rapid maturation of the photosynthetic apparatus. The climax species *L. pierrei* and *L. dilleniifolia*, on the other hand, needed more time for the complete development of the photosynthetic apparatus, as the highest P_{max} values were reached 15 and 10 d after FLE, respectively (Fig. 3*A*). In all plant species, R_D was highest at the beginning of leaf expansion and decreased gradually to a steady-state level. In the developing leaves of *A. chinensis*, R_D was higher than in the more slowly expanding leaves of *L. pierrei* and *L. dilleniifolia*, and high R_D was retained after full leaf expansion (Fig. 3*B*).

L. pierrei and *L. dilleniifolia* showed a stomatal conductance (g_s) generally less than 100 mmol m^{-2} s^{-1}, whereas mature leaves of *A. chinensis* had mean g_s of

Table 1. Changes in leaf colour during leaf development in three tropical rainforest tree species. 'Mature' category represents leaves 4–6 d after leaf highest P_{max} was reached.

Leaf stage [% FLE]	Leaf colour *A. chinensis*	*L. pierrei*	*L. dilleniifolia*
15	Red	Dark red	Pink-green, soft
24	Red-blue	Red	Light green
56	Green-red	Pink-red	Light green
100	Light green	Green, soft	Green
Mature leaves	Green	Dark green	Green, hardened

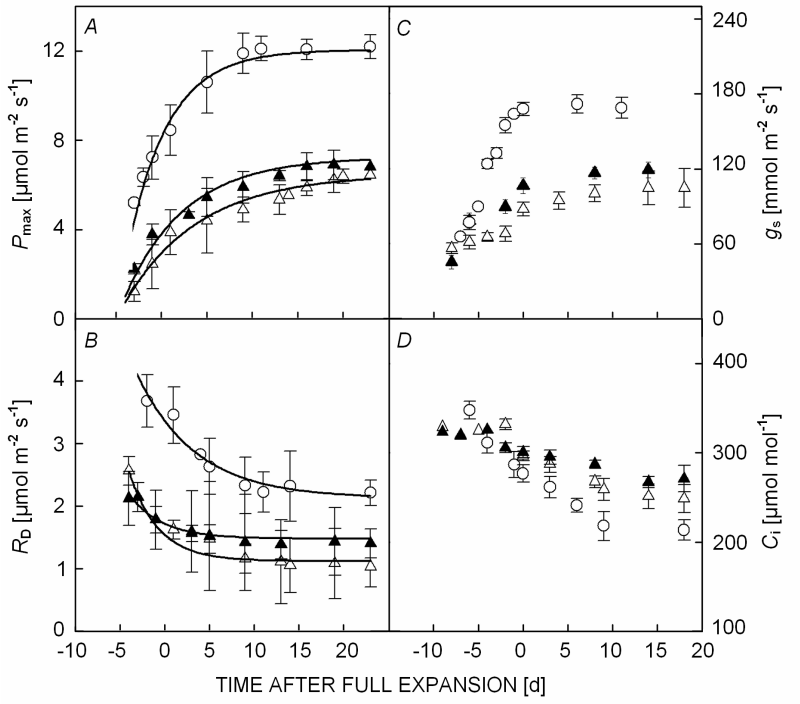

Fig. 3. Changes in maximum net photosynthetic rate (P_{max}) (*A*), dark respiration (R_D) (*B*), stomatal conductance (g_s) (*C*), and intercellular CO_2 content (C_i) (*D*) during leaf development in *A. chinensis* (○), *L. pierrei* (▲), and *L. dilleniifolia* (Δ). Error bars indicate SD (n = 3–4).

150 mmol m^{-2} s^{-1} (Fig. 3*C*). Stomatal conductance was lowest in expanding leaves and gradually increased during leaf expansion to reach maximum after maturation. Similarly, the intercellular CO_2 content (C_i) in young, developing leaves of the three species was higher than that of mature leaves (Fig. 3*D*). Low g_s and high R_D in young developing leaves likely contributed to the high C_i. During leaf development the increase in P_{max} was significantly related to changes in g_s (Fig. 4).

Irradiance response curves and diurnal photoinhibition during leaf development: At any given incident PPFD, the actual PS2 efficiency ($\Delta F/F_m'$) was markedly lower in the younger than older leaves for all three species (Fig. 5*A*). Lower PS2 efficiency was associated with greater energy dissipation as measured by non-photochemical quenching (NPQ) of Chl fluorescence (Fig. 5*B*).

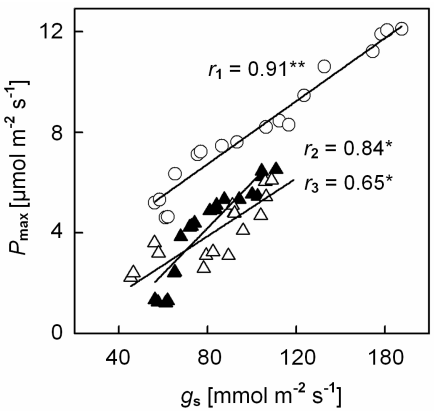

Fig. 4. Relationships between maximum net photosynthetic rate (P_{max}) and stomatal conductance (g_s) with leaf development in *A. chinensis* (○), *L. pierrei* (▲), and *L. dilleniifolia* (Δ).

Table 2. Dark-adapted predawn photochemical efficiency and diurnal photoinhibition measured in developing leaves of three tropical rainforest tree species on sunny days. Means ± SD (n = 4–6). Different letters within one column indicate significantly different means.

Leaf stage [% FLE]	Predawn F_v/F_m			% diurnal photoinhibition		
	A. chinensis	*L. pierrei*	*L. dilleniifolia*	*A. chinensis*	*L. pierrei*	*L. dilleniifolia*
15	0.665 a	0.672 a	0.694 a	23.5 a	23.1 a	35.6 a
24	0.683 a	0.692 a	0.723 b	23.2 a	22.3 a	32.6 ab
56	0.735 b	0.704 a	0.754 b	21.7 a	23.4 a	28.7 b
100	0.784 b	0.752 b	0.782 bc	22.1 a	22.8 a	24.5 bc
Mature leaves	0.847 c	0.826 c	0.835 c	20.5 ab	23.2 a	22.5 c

Younger leaves displayed higher energy dissipation than the older ones, implying that photons were excessive to a greater extent in the younger leaves. In correspondence with this, young leaves displayed a higher Q_A reduction state at any given incident PPFD below photon saturation (Fig. 5*C*), implying constraints in electron transport in young leaves. With increasing leaf age these constraints apparently decreased as the reduction state declined. However, at photon saturation the reduction state of leaves of different ages did not vary markedly.

The dark-adapted pre-dawn F_v/F_m increased with leaf aging and the difference between juvenile and mature leaves was greatest in *A. chinensis* (21.5 % difference) and least in *L. dilleniifolia* (16.9 % difference). On sunny days (maximum PPFD 1 900 μmol m^{-2} s^{-1}), juvenile leaves of *L. dilleniifolia* had the greater diurnal photoinhibition compared to mature leaves. Diurnal photoinhibition of *A. chinensis* and *L. pierrei* on the other hand, was not significantly different for juvenile and mature leaves (Table 2).

Enzymatic activities: SOD and APX were present in all leaves (Fig. 6). Activities of SOD and APX were consistently higher in the young, red leaves of *A. chinensis* and *L. pierrei* than in the older leaves. For leaves of *L. dilleniifolia* the SOD and APX activities were much lower than for the other two species and no significant age-dependent decrease was found.

Discussion

Changes in photosynthetic characteristics during leaf development: Photosynthetic development varied between the three species studied. Optimum Chl content and P_{max} were delayed after FLE in all three studied species. Delayed greening exists in other evergreen trees and shrubs (Kursar and Coley 1992, Woodall *et al.* 1998, Hieke *et al*. 2002, Miyazawa *et al*. 2003, Numata *et al.* 2004). The increase of both Chl content and P_{max} with leaf expansion indicates that total photochemical capacity as well as light-harvesting capacity increased. Therefore the proportion of absorbed photons, that was not actually used in photochemistry decreased, resulted in a generally higher F_v/F_m in more fully expanded leaves. In most herbaceous species, R_D is very high in newly unfolded leaves and then decreases dramatically to a low steady-state level until the completion of leaf area expansion (Šesták *et al*. 1985). However, our three tropical tree species showed high R_D even at FLE (Fig. 3*B*). High R_D in the early stages of leaf development is attributed to the metabolism associated with the contribution of new leaf tissue (Amthor 1989). The high R_D of *A. chinensis* at FLE is consistent with the marked increase in P_{max} and F_v/F_m at this stage (Fig. 3*A*, Table 2).

The development of g_s depends on the plant species and environmental conditions (Čatský *et al*. 1985). Our studied species with delayed leaf greening also had a delayed stomata development and low g_s during leaf expansion, and g_s was strongly related with P_{max} during leaf development. This is consistent with earlier observations that g_s largely limits photosynthesis during leaf expansion (Schaffer *et al*. 1991).

Leaf maturation period: The period needed for full leaf maturation varied among species. The large leaves of *A. chinensis* expanded more quickly than the small ones of *L. pierrei* and *L. dilleniifolia*. Although this is in contrast with Moles and Westoby (2000) who suggested that leaves of large-leaved species usually need longer time to expand than the leaves of small-leaved plants, it is not so surprising, knowing that *A. chinensis* is a fast growing light demanding species that needs fast development for survival. Also, *A. chinensis* is a fast growing light demanding species with a low LMA, for which Miyazawa *et al*. (1998) hypothesized that these would have shorter maturation periods, compared to species with larger LMA, because of the extra time needed to build their heavier construction. Consistent with this idea we found a positive relation between maturation period (from emergence to photosynthetic maturation of a leaf) and steady-state LMA among our three species (Fig. 7). The difference between the two *Litsea* species was in correspondence with finding of Woodall *et al.* (1998) of a large variation in leaf development within the genus *Syzygium*.

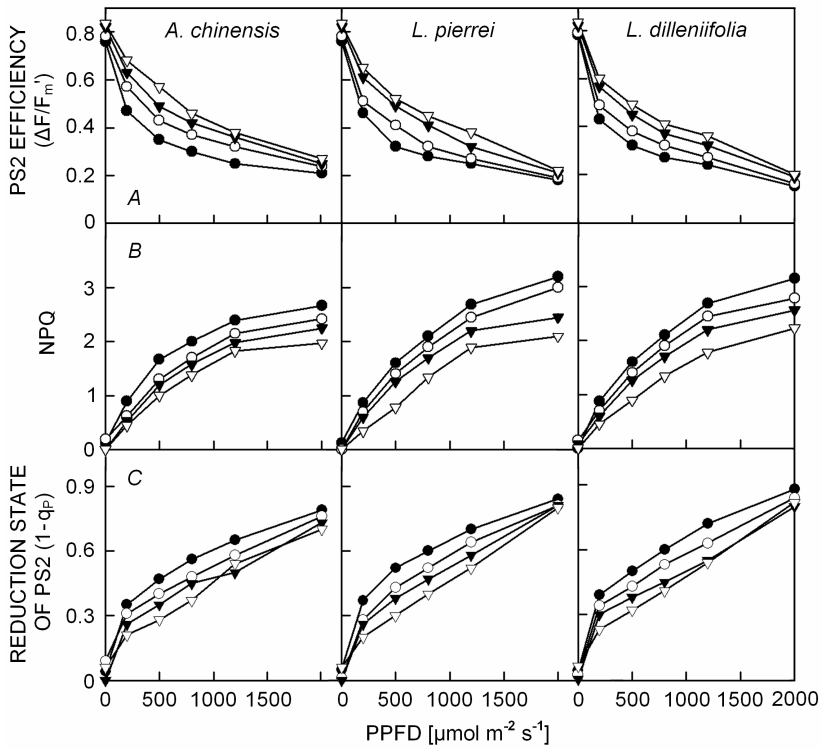

Fig. 5. Photon response curves of actual efficiency of photosystem 2, PS2 ($\Delta F/F_m'$), non-photochemical quenching (NPQ), and the reduction state of PS2 centres ($1 - q_P$) in three tree species at different stages (● 24, ○ 56, ▼ 100 % FLE, ▽ mature leaves).

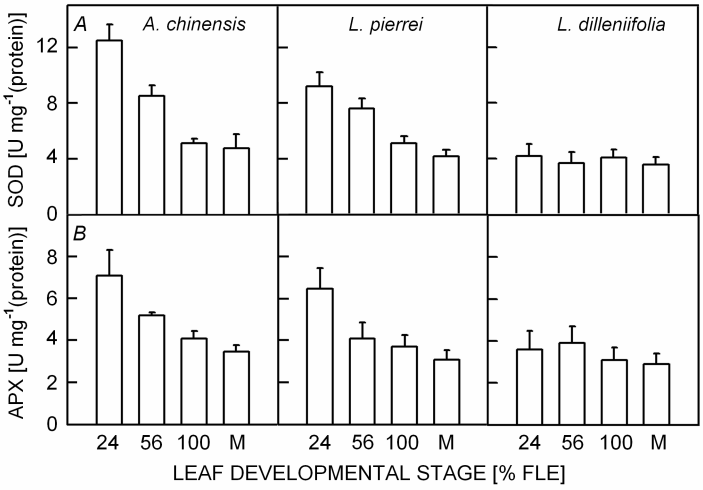

Fig. 6. The activities of protective enzymes during leaf expansion in *A. chinensis*, *L. pierrei*, and *L. dilleniifolia*. 'M' represents mature leaves (4–6 d after highest P_{max} value was reached).

Photoinhibition and photo-protective mechanisms: The pre-dawn F_v/F_m values increased with leaf age (Table 2), which reveals a sustained depression in the efficiency of PS2 in the younger leaves. As leaves aged, F_v/F_m increased from 0.665–0.694 in the youngest leaves to 0.826–0.847 in mature leaves, thus approaching the value for healthy, unstressed C_3 plants (Björkman and Demmig 1987). The diurnal photoinhibition on sunny days was clearly more pronounced in green juvenile leaves of *L. dilleniifolia* than in red juvenile leaves of *A. chinensis* and *L. pierrei*, and consequently the green leaves had the lowest photosynthetic capacity (Fig. 3*A*). Leaves of rainforest trees may thus show different patterns in sensitivity to photoinhibition with leaf aging. Young expanding leaves may be more sensitive and this sensitivity then decreases during leaf aging, as is the case in *L. dilleniifolia*, and as was previously found by Krause *et al*. (1995), or the sensitivity of young leaves is similar

to that of mature leaves and does not change during aging. The latter was shown in this study for *A. chinensis* and *L. pierrei* and is in correspondence with results of Greer (1996) and Dodd *et al.* (1998).

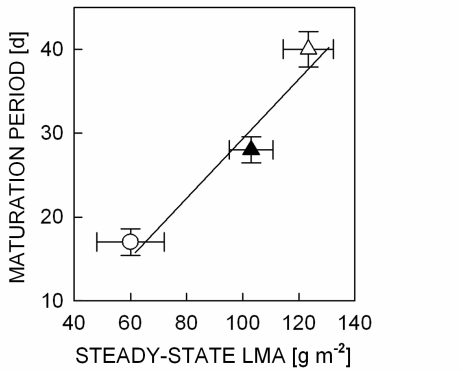

Fig. 7. Relationship between leaf maturation period and steady-state leaf dry mass per area (steady-state LMA) for *A. chinensis* (○), *L. pierrei* (▲), and *L. dilleniifolia* (Δ).

Protective mechanisms against photo-oxidative damage from increased excitation pressure include an increased non-photochemical quenching of excitation energy *via* xanthophylls and counteracting the formation of singlet oxygen and subsequent detoxification of reactive reduced oxygen species (Foyer *et al.* 1994). Leaf ontogeny strongly influences the photochemical and dissipation properties of leaves. Despite the low PS2 efficiency in developing leaves, younger leaves of our three species possessed markedly larger non-photochemical dissipation (Fig. 5*A,B*). Thus the youngest leaves were able to dissipate a greater percentage of the absorbed photon energy thermally than the older leaves. The proportions of energy dissipation shifted from non-photochemical to photochemical quenching as the leaves developed. The reduced non-photochemical dissipation in the mature leaves suggests that mature leaves are better able to utilise the excitation energy photosynthetically, and hence to reduce the need for thermal energy dissipation. This view is supported by the apparently age-dependent decrease in PS2 reduction state at irradiances below saturation (Fig. 5*C*). The reduction state of leaves of different ages was similar at photon saturation, which implies that Q_A in the younger leaves was maintained at a reduction state comparable to that of the mature leaves presumably as a result of thermal energy dissipation.

The red leaves of *A. chinensis* and *L. pierrei* had higher activities of SOD and APX than their older, better developed green leaves (Fig. 6). Clearly, red leaves are better equipped to scavenge reactive oxygen species than green leaves (*e.g.* Neill *et al.* 2002). The size and composition of the antioxidant pool changed during leaf development as Chl content increased and the capacity for photochemical energy quenching reached maximum values. These changes probably reflect developmental shifts in antioxidant requirements.

While enzymes play a role in reducing adverse effects of exposure to excessive radiation, the presence of anthocyanin should also be considered. Although anthocyanins occur in the mesophyll layers of plant genera including *Mahonia* and *Viburnum* (Katu *et al*. 1998), they are often found in or just below the upper epidermis of leaves (this research and Woodall *et al.* 1998). The functions of anthocyanins in plants are numerous and apparently depend on environment and species. For example, shade plants accumulate anthocyanins in their lower epidermis, perhaps to assist in photon capture (Lee *et al.* 1987), while photoprotective, antioxidant, UV screening, and anti-herbivory functions of anthocyanins have also been proposed (see review by Chalker-Scott 1999). Anthocyanins may have contrasting properties when located in the upper epidermis as opposed to location in the lower epidermis. In the present study the high amounts of anthocyanin in the young red leaves with relatively low diurnal photoinhibition of *A. chinensis* and *L. pierrei* were accompanied by high activities of the protective enzymes SOD and APX. In other studies, leaves with anthocyanin showed protection against photoinhibition as a result of associated large pools xanthophyll cycle pigments (*e.g.* Krause *et al.* 1995). Apparently, there is an indirect role of anthocyanins in optimising the balance between photon energy harvested and energy required for photosynthesis. Although further research is required to unravel the precise function, anthocyanins in developing leaves of delayed greening species serve to reduce photoinhibition of photosynthesis and their presence might be a result of species specific genotypic adjustment to high irradiances.

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