NITROGENOUS COMPOUNDS, PHENOLIC COMPOUNDS AND MORPHOLOGICAL ASPECTS OF LEAVES: COMPARISON OF DECIDUOUS AND SEMIDECIDUOUS ARBOREAL LEGUMES

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ABSTRACT: In general, perennial species contain higher concentrations of certain secondary compounds, such as phenolics, lower levels of nitrogenous compounds, and greater specific leaf mass (SLM) than deciduous species. The aim of this study was to verify whether the comparative data reported in the literature regarding deciduous and evergreen species, also applies to four selected species of a semideciduous tropical forest (a remnant of the Atlantic Forest). The four species chosen for this study, each with apparently different leaf life spans, were two semideciduous (*Hymenaea courbaril* L. var. *stilbocarpa* (Hayne) Lee et Lang., and *Lonchocarpus guilleminianus* (Tul.) Malme, and two deciduous (*Enterolobium contortisiliquum (Vell.)* Morong., and *Peltophorum dubium* (Spreng.) Taub.). Plants were grown in a greenhouse in plastic pots containing soil taken from their natural habitat. Mature leaves were harvested for determination of the SLM and contents of phenolic compounds, tannins, nitrate, protein, free amino acids, chlorophyll, and nitrogen. Data registered are in agreement with those of the literature. Leaves with the longest life span had the highest content of phenolics and the lowest levels of tannins, nitrate, protein, amino acids, chlorophyll and nitrogen, as well as presenting the greatest SLM.
Key words: leaf life span, specific leaf mass, total nitrogen, tannins, Atlantic forest

COMPOSTOS NITROGENADOS, FENÓLICOS E ASPECTO MORFOLÓGICO EM FOLHAS: COMPARAÇÃO ENTRE LEGUMINOSAS ARBÓREAS SEMIDECÍDUAS E DECÍDUAS

RESUMO: Em geral as espécies perenifólias possuem maior concentração de alguns compostos secundários, como por exemplo fenóis, menor concentração de compostos nitrogenados e maior massa foliar específica (MFE), quando comparadas a espécies decíduas. O objetivo deste trabalho foi verificar se as observações encontradas na literatura, comparando espécies perenifólias e decíduas aplicam-se também a quatro espécies de uma mata semidecídua, remanescente da Mata Atlântica. Das quatro espécies usadas neste estudo, que aparentemente apresentavam longevidades foliares diferentes, duas eram semidecíduas (*Hymenaea courbaril* L. var. *stilbocarpa* (Hayne) Lee et Lang. e *Lonchocarpus guilleminianus* (Tul.) Malme) e duas decíduas (*Enterolobium contortisiliquum (Vell.)* Morong. e *Peltophorum dubium* (Spreng.) Taub.). As plantas foram cultivadas em vasos plásticos, contendo solo da mata. O cultivo foi feito em casa de vegetação. Folhas maduras foram coletadas para análise de fenóis totais, taninos condensados, nitrato, proteínas totais, aminoácidos livres totais, clorofila total, nitrogênio total e MFE. Os resultados obtidos corroboram os da literatura, já que as espécies com folhas mais longevas apresentaram concentrações maiores de fenóis totais, menores de taninos condensados, nitrato, proteínas solúveis totais, aminoácidos livres totais, clorofilas totais, nitrogênio total e maior MFE.
Palavras-chave: longevidade foliar, massa foliar específica, nitrogênio total, taninos, floresta Atlântica

INTRODUCTION

Comparative studies of the nutrient content of leaves from perennial and deciduous species have revealed that the lowest levels occur in species which leaves have the longer life span (Wright & Westoby, 2003). The longer life span of leaves of perennial species and their lower nitrogen content are important factors regarding the economy of this nutrient. Such plants benefit from leaves that function for a longer period using less nitrogen (Carrera et al., 2000; 2003).

The perennials have a low photosynthetic rate due to their low levels of Rubisco and chlorophyll (Ingestad & Lund, 1979). Such species invest more in secondary compounds and tissues for structural support (Chabot & Hicks, 1982), which may explain the lower nitrogen content of their leaves. By contrast, species that have high photosynthetic rates invest more in organic compounds, especially nitrogenous (Field & Mooney, 1986 in Cordell et al., 2001). One explanation for the fact that perennials have lower nitrogen content in their leaves is that such species have greater concentrations of phenolic compounds than do leaves with shorter life span (Coley et al., 1985), and the synthesis of these compounds competes with that of the nitrogenous compounds (Kouki & Manetas, 2002).

One possible reason for a higher concentration of phenolic compounds in perennial leaves could be the greater need for a defense mechanism against herbivorous predators due to their longer life span (Aerts, 1990). Phenolic compounds are known to participate in the defense against herbivores (Hudgins et al., 2003) and pathogenic attack (Haukioja et al., 1985). On the other hand, tannins are often present in higher concentration in deciduous species (Aerts, 1995).

Species that have leaves with a longer life span generally have a lower specific leaf area (SLA) (Eamus & Prichard, 1998), or higher specific leaf mass (SLM), due to the leaf blades and cuticles being thicker, as well as a larger lignified area (Read et al., 2000). This results in an increased mass to area ratio, which permits such leaves to resist adverse conditions for a longer time (Gates, 1914 in Chabot & Hicks, 1982).

The Atlantic Forest has been reduced to only 7% of its original area (Araújo et al., 2004). However little is known of the relationship between leaf life span and conservation of nutrients for species of this ecosystem, even though such knowledge is important for the recovery of degraded areas with native species.

The aim of this study was comparing two semideciduous with two deciduous species, taken from a semideciduous forest (a remnant of the Atlantic Forest), regarding leaf longevity in relation to certain metabolic components (total nitrogen, nitrogenous and secondary compounds), and morphological characteristics (SLM) of mature leaves.

MATERIAL AND METHODS

Species and habitat characterization

Seeds of arboreal legume species were taken from a semideciduous tropical forest, a remnant of the Atlantic Forest, denominated the Santa Genebra Municipal Reserve, region of Campinas, São Paulo State, Brazil (22°49'45" S, 47°06'33" W; altitude 670 m). The Santa Genebra forest is classified as semideciduous because it has several arboreal species with seasonal lost of leaves, that occurs in the dry and cold period of the year (Morellato, 1995). The species used in this study were: *Hymenaea courbaril* L. var. *stilbocarpa* (Hayne) Lee et Lang., *Lonchocarpus guilleminianus* (Tul.) Malme, *Enterolobium contortisiliquum (Vell.)* Morong., and *Peltophorum dubium* (Spreng.) Taub., the first two classified as semideciduous and the last two as deciduous (Lorenzi, 1992). The semideciduous species lose their leaves to a greater extent during a period of the year but never remain totally without leaves. Deciduous species lose their leaves in a specific period of the year, when they remain totally, or almost totally, without leaves (Morellato et al., 1989).

Seed germination

Seeds of *H. courbaril* and *E. contortisiliquum* were scarified with concentrated sulphuric acid for 1 hour and those of *P. dubium* for 30 minutes, followed by thorough washing with water. Seeds from the four species were then left to germinate at 25°C, 12 hours photoperiod.

Growth conditions

The experiments were carried out from January, 2001 to March, 2003. Plants were grown under natural light and temperature conditions in a greenhouse, in plastic pots (5 L capacity) containing soil taken from the Santa Genebra Reserve. Plastic saucers were used under the pots and irrigation carried out using a sprinkler system.

To ensure nodulation in species associated with rhizobia (*L. guilleminianus* and *E. contortisiliquum*), inoculation was carried out with slow growth rhizobia strains (2 mL per pot), previously isolated from root nodules (Vincent, 1970), from the same species grown in the Santa Genebra forest soil.

Evaluation of leaf development and leaf characterization

Growth curves were constructed based on leaf rachis length in order to determine when leaf expansion was completed for harvest and analysis. The mean length of fully expanded leaves (mature-green) of each of the studied species was: *H. courbaril* 8.8 ± 2.2 cm; *L. guilleminianus* 17.8 ± 3.5 cm; *P. dubium* 6.7 ± 0.4 cm; and *E. contortisiliquum* 13.9 ± 2.9 cm. For the determination of leaf longevity, the same leaves used for measurement were date-tagged with strips of plastic around the petioles.

Determination of SLM

SLM was calculated using the formula: SLM $= DM_{mature-green\ leaves}/A_{mature-green\ leaves}$ (g cm^{-2}), where DM = dry mass, and A = leaf area. Dry mass was obtained after leaves were kept in oven at 60°C for 7 days. Leaf area was obtained using a LI-COR instrument, model LI-300.

Determination of total phenolics, condensed tannins, nitrate, soluble protein and free amino acids

Leaves (500 mg fresh mass) were macerated using liquid N$_2$ and extracted with methanol, chloroform and water (12:5:3 v/v/v), according to Bieleski & Turner (1966). After standing for 24 hours, the extract was centrifuged at 2000 g for 30 minutes. Aliquots of the supernatant were stored in freezer for posterior determination of phenolic compounds (Swain & Hillis, 1959), and condensed tannins (Porter, 1989). Further aliquots (4 mL) were taken from the supernatant to which chloroform (1 mL) and water (1.5 mL) were added, and shaken. After 24 hours in a refrigerator, when phase separation occurred, the lower phase was removed and discarded. The upper aqueous phase was concentrated on water bath at 45°C for 8 hours, and kept in a freezer until the determination of amino acids (Cocking & Yemm, 1954) and nitrate (Cataldo et al., 1975).

The residue obtained after centrifugation of the extract was suspended in 0.1 N NaOH, left to rest for 24 hours, and then centrifuged at 2000 g for 30 minutes. The supernatant was used for the determination of total proteins (Bradford, 1976).

Determination of chlorophyll

Chlorophyll was extracted according to Hiscox & Israelstam (1979). Portions of leaves (20 mg fresh weight) were immersed in dimethyl-sulphoxide (DMSO) in sealed tubes kept in the dark. The tubes were incubated in a water-bath at 65°C for 30 minutes and cooled to room temperature. Total chlorophyll content was calculated according to Arnon (1949) and Lichtenthaler & Wellburn (1983).

Determination of total tissue nitrogen

Samples of 100 mg of the leaves dried at 65ºC for 7 days were placed in digestion tubes (Folin-Wu type), containing 1 g of catalyst (100 g of K$_2$SO$_4$, 10 g of CuSO$_4$.5H$_2$O, and 1 g of Se), 3 mL of concentrated sulphuric acid and 1 mL of hydrogen peroxide (H$_2$O$_2$). The samples were digested at 360°C and nitrogen determined by the Kjeldahl method (Nelson & Sommers, 1973).

Experimental design and statistical analysis

Ten replicates (each represented by one leaf for each plant) were used for leaf life span and leaf growth curves and five replicates were used in biochemical analysis in a completely randomized design. Statistical analysis was performed using the software SAS/STAT (1990). The data were analysed for normal distribution using the Kolmogorov-Smirnov test. The data were subjected to an analysis of variance and means compared for significant differences by Duncan´s Multiple Range test ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Leaf longevity and SLM values for the four species are shown in Table 1. Semideciduous species (*H. courbaril* and *L. guilleminianus*) presented leaf life spans at least twice as long as those of the deciduous species (*P. dubium* and *E. contortisiliquum*). Greater leaf longevity in some species is *per se* a mechanism of nutrient economy, since it reduces mineral loss (Carrera et al., 2000; 2003).

The semideciduous species also revealed significantly greater values of SLM compared to deciduous, which is consistent with the findings of Peeters (2002) that a correlation exists between morphological characteristics of a leaf and the duration of its life cycle, as well as with the earlier observations that species showing greater leaf longevity present a greater SLM (Castro-Díez et al., 2000; Prior et al., 2003; Wright et al., 2002). It is believed that a reciprocal relationship exists between SLM and the life span of the leaf in that a smaller SLM indicates a greater potential for the rapid production of biomass in view of the proportionally greater area for harvesting light, while a longer leaf life span indicates a longer duration of biomass yield (Westoby, 1998), and nutrient conservation (Aerts & Chapin, 2000; Navas et al., 2003).

Table 1 - Leaf life span and specific leaf mass (SLM) of mature green leaves of semideciduous (*Hymenaea courbaril* and *Lonchocarpus guilleminianus*) and deciduous (*Peltophorum dubium* and *Enterolobium contortisiliquum*) arboreal species.

Species	Leaf life span	SLM
	days	g DM cm^{-2}
H. courbaril	419 ± 54.8 a	0.0065 ± 0.0003 a
L. guilleminianus	340 ± 9.7 b	0.0072 ± 0.0015 a
P. dubium	152 ± 23.1 c	0.0046 ± 0.0006 b
E. contortisiliquum	139 ± 11.9 c	0.0053 ± 0.0003 b

± = mean standard error. Means followed by a different letters differ by Duncan's Multiple Range test ($\alpha = 0.05$). DM = dry mass

Coley (1988) relates leaf longevity to the concentration of secondary compounds. Species with greater leaf life spans (*H. courbaril* and *L. guilleminianus*) presented higher concentrations of phenolic compounds than the shorter life span species (Figure 1A). Although the concentration of total phenolics produced a direct relationship to leaf longevity, this did not occur with tannin content (Figure 1B). The semideciduous species showed lower concentrations of these compounds than the deciduous (*P. dubium* and *E. contortisiliquum*), which is in agreement with the work of Aerts (1995), who registered when comparing perennial and deciduous species, that tannins are generally found in lower concentrations in species with longer-lasting leaves. Differences in the concentration of diverse types of phenolics can be explained by the fact that their synthesis has high energy cost, and this cost will determine the amounts produced (Aerts et al., 1999).

Some authors suggest that the formation of leaves with long life spans is a costly process in view of the greater investment required for defense compounds and structural components (Kikusawa, 1991). The building cost per unit of leaf mass has been proposed to be the result of a negative correlation between costly compounds involved in rapid metabolic activity, such as proteins, and those related to leaf persistence, such as phenolics (Martínez et al., 2002). Our data are consistent with this proposal, since the species with greater leaf life spans presented lower contents of soluble proteins (Figure 2). Similar data have been reported by Haddad et al. (2004). Takashima et al. (2004) proposed that species with greater leaf life spans invest more in cell wall (insoluble) proteins, in detriment of those (soluble) proteins related to photosynthetic activity, whereas for deciduous species the opposite is found.

Besides proteins, other nitrogenous compounds were related to leaf life span. Our data (Figures 3 and 4) are in agreement with literature report showing that, for species with longer life span leaves, a lower level was found for nitrate (Figure 3) (Schimidt et al., 1998), chlorophyll (Haddad et al., 2004; Prior et al., 2003), and total N (Figure 4) (Haddad et al., 2004; Prior et al., 2003) compared with species with a short foliar life span.
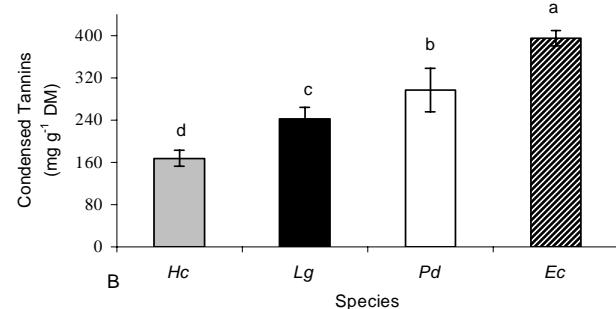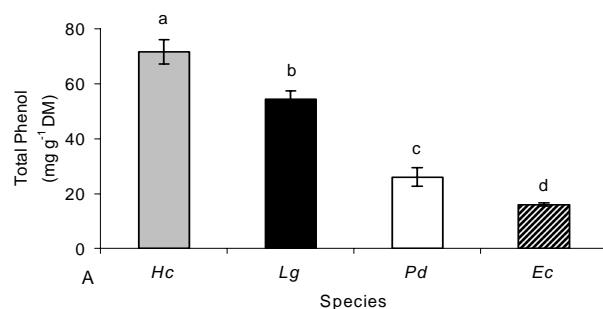

Figure 1 - Concentration of phenolic compounds (A – total phenolics and B – condensed tannins) expressed on a dry mass (DM) basis in mature green leaves of arboreal legumes: *Hc - Hymenaea courbaril* (semideciduous), *Lg - Lonchocarpus guilleminianus* (semidecídua), *Pd - Peltophorum dubium* (deciduous) and *Ec - Enterolobium contortisiliquum* (deciduous). Vertical bars represent standard error of the mean (in some cases the error bar was smaller than the symbol). Means followed by different letters differ by Duncan's Muliple Range test ($\alpha = 0.05$).
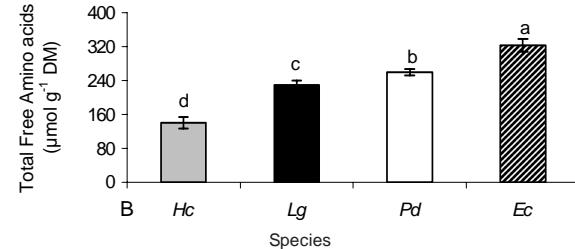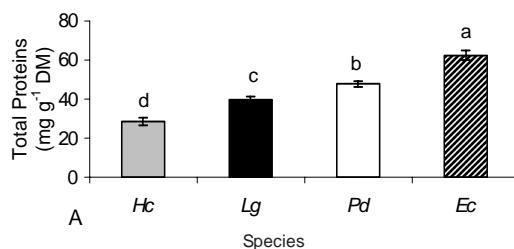

Figure 2 - Concentration of nitrogenous compounds (A – total soluble proteins, B – free amino acids) expressed on a dry mass (DM) basis in mature green leaves of the arboreal legumes: *Hc – Hymenaea courbaril* (semideciduous), *Lg – Lonchocarpus guilleminianus* (semideciduous), *Pd - Peltophorum dubium* (deciduous) and *Ec - Enterolobium contortisiliquum* (deciduous). Vertical bars represent standard error of the mean (in some cases the error bar was smaller than the symbol). Means followed by different letters differ by Duncan's Muliple Range test ($\alpha = 0.05$).
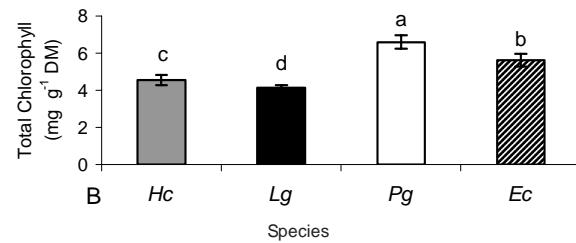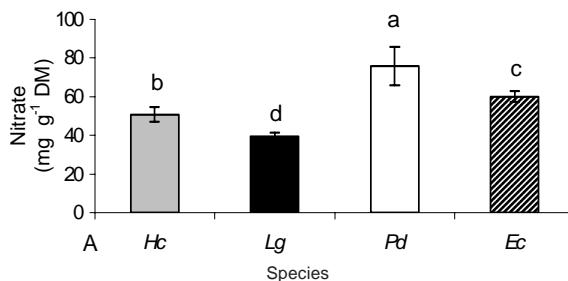
Figure 3 - Concentration of nitrogenous compounds (A – nitrate, B – total chlorophyll) expressed on a dry mass (DM) basis in mature green leaves of the arboreal legumes: *Hc – Hymenaea courbaril* (semideciduous), *Lg – Lonchocarpus guilleminianus* (semideciduous), *Pd - Peltophorum dubium* (deciduous) and *Ec - Enterolobium contortisiliquum* (deciduous). Vertical bars represent standard error of the mean (in some cases the error bar was smaller than the symbol). Means followed by different letters differ by Duncan's Muliple Range test ($\alpha = 0.05$).
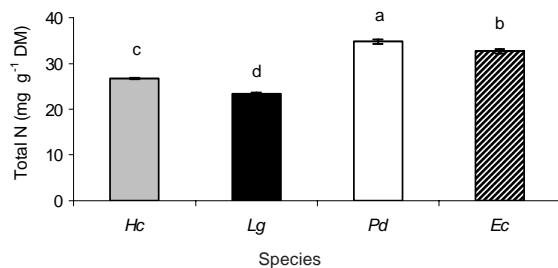

Figure 4 - Total N expressed on a dry mass (DM) basis in mature green leaves of the arboreal legumes: *Hc - Hymenaea courbaril* (semideciduous), *Lg - Lonchocarpus guilleminianus* (semideciduous), *Pd - Peltophorum dubium* (deciduous) and *Ec - Enterolobium contortisiliquum* (deciduous). Vertical bars represent standard error of the mean (in some cases the error bar was smaller than the symbol). Means followed by different letters differ by Duncan's Muliple Range test ($\alpha = 0.05$).

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