Year season on epicuticular waxes in leaves of *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli (Alismataceae)

Walderez Moreira Joaquim[2], Elizabeth Orika Ono[3], Maria Luiza Faria Salatino[4], João Domingos Rodrigues[3]

[2] Universidade do Vale do Paraíba - UNIVAP, 11244-000, São José dos Campos, SP, Brazil.
[3] Instituto de Biociências, UNESP - Univ Estadual Paulista, Departamento de Botânica, , C.P. 510, 18618-000, Botucatu, SP, Brazil. E-mail: eoono@ibb.unesp.br
[4] Universidade de São Paulo – USP, Departamento de Botânica, Instituto de Biociências, C.P. 11461, 05422-970, São Paulo, SP, Brazil.

ABSTRACT

The paper aimed to study the influence of different seasons of the year on epicuticular waxes in *Echinodorus grandiflorus* plants and to analyze the profile of n-alkanes in epicuticular waxes. The experiment used adventitious plantlets from the flower stem and approximately 10-cm-tall adventitious plantlets with an average of 4 to 5 leaves, planted in 10-liter pots containing a 40% soil, 40% rice hulls, and 20% vermicompost mixture. *E. grandiflorus* presented an intermediary profile between terrestrial and aquatic plants, with a main homologue C27 (heptacosane), followed by C25 (pentacosane) and C29 (nonacosane). The studies presented here provide information about ecological aspects and chemical and mechanical defenses of *Echinodorus grandiflorus.*

Key words: phenology, *n*-alkanes, aquatic plants

RESUMO

Este trabalho objetivou estudar a influência das estações do ano sobre o conteúdo de ceras epicuticulares e a análise dos perfis dos n-alcanos da cera em folhas de *Echinodorus grandiflorus*. No experimento foram utilizadas mudas adventícias com aproximadamente 10 cm de altura e 4 a 5 folhas, que foram plantadas em vasos de 10 L, tendo como substrato a mistura de 40% de terra, 40% de palha de arroz e 20% de húmus de minhoca. *Echinodorus grandiflorus* apresentou perfil intermediário entre plantas terrestres e aquáticas, apresentando como principal homólogo C27 (heptacosano), seguido de C25 (pentacosano) e C29 (nonacosano). Os estudos realizados fornecem informações sobre os aspectos ecológicos e as defesas químicas e mecânicas de *Echinodorus grandiflorus*.

Palavras-chave: fenologia, *n*-alkanos, planta aquática

INTRODUCTION

Echinodorus is a genus that belongs to Alismataceae, which is found exclusively in the Americas, with a center of dispersion in South America, occurring

in several regions of Brazil. Popularly known as "chapéu-de-couro", it is frequently used by the population as diuretic, antirheumatic, antiinflammatory, and against uric acid and skin problems (Teske and Trentini, 2001).

With regard to ecological aspects, *Echinodorus grandiflorus* (Cham. & Schltdl.) Micheli is important in aquatic ecosystems because it provides refuges for aquatic fauna reproduction, feeding, and because it prevents erosion on the banks of those systems (Rego, 1988).

The passage of plants from aquatic to terrestrial ecosystems about four hundred million years ago led them to adaptations for their survival on the continent. One of the most important adaptations was the acquisition of a protective lipid layer that covers the epidermal cells of all terrestrial plants, the cuticle (Gülz, 1994; Riederer and Schreiber, 1995; Kunst and Samuels, 2003). For Gray and Boucout (1977), the presence of a cuticle is an adaptation in both terrestrial and emersed aquatic plants.

The cuticle functions in a number of ways, which allows the adaptation and survival of plants in adverse environments. Among the functions attributed to the cuticle are prevention against desiccation, avoiding excessive water loss during transpiration, action as a barrier against pathogens and herbivory, plant protection against loss of ions and nutrients by leaching, plant protection against damage caused by wind and cooling, and also by the effect of UV radiation (Edwards et al., 1982; Harborne and Turner, 1984).

The main constituents of the cuticle are cutin, suberin, and waxes, which may cover the cuticle surface (Bianchi, 1995; Taiz and Zeiger, 2009). According to Baker (1982), when waxes are deposited on the cuticle matrix they are called epicuticular waxes, and when they are embedded in it they are termed intracuticular waxes. The intracuticular wax is defined as an amorphous mixture of lipids embedded in the cutin, which binds the cuticle to the cell wall, while the epicuticular wax refers to a layer of lipids that form crystallizations or a smooth film on the cuticle surface (Kunst and Samuels, 2003).

The epicuticular waxes cover the more external portion of the cuticle, and present as constituents a complex mixture of linear- or ramified-chain alkanes, esters, ketones, aldehydes, alcohols, carboxylic acids, terpenoids, phenolic substances, usually flavonoids, coumarins, cinnamic acid, at various concentrations and relative percentages, with greater predominance of alkanes, esters, alcohols, and ketones (Bianchi, 1995; Shepherd et al., 1999; Kunst and Samuels, 2003). The occurrence of *n*-alkanes, the most apolar constituents of epicuticular wax, is almost universal, and for this reason they are considered by some authors as good taxonomic markers, although there is a line of thinking that opposes this idea because it considers *n*-alkanes extremely influenced by environmental factors (Salatino, 1986).

It can be seen from the literature that several studies have been carried out focusing on the roles of cuticle and epicuticular wax in terrestrial plants. However, according to Amaral et al. (1990), although the cuticle in aquatic plants also has an

important ecological function, studies dealing with epicuticular waxes in these plants are very limited. Some authors have conducted researches on epicuticular wax in some species of aquatic plants, highlighting aspects of their nature. However, there is a total lack of researches focusing on the epicuticular wax of *Echinodorus grandiflorus*.

The objective of this work was to investigate the influence of the different seasons on the production of epicuticular waxes in the leaf blade of *E. grandiflorus*, and also to identify the profile of *n*-alkanes, the most apolar constituents of epicuticular wax in the leaves of plants originated from adventitious plantlets of this species, and the influence on phenological aspects. The importance of these studies in *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli plants can be justified by a lack of studies with the objectives here proposed and because of the action of epicuticular waxes against herbivory.

MATERIAL E METHODS

The experiment was conducted at the Medicinal Plants Garden, at Universidade do Vale do Paraíba - UNIVAP Campus, São José dos Campos, 23°14' S, 45°51' W (SP, Brazil).

The *Echinodorus grandiflorus* experiment was conducted in a nursery covered with a black polypropylene shading (50% shade cloth), up to a height of 30 cm above the ground (Joaquim, 2000), thus providing the most suitable light intensity for the development of *E. grandiflorus* adventitious plantlets.

The *E. grandiflorus* adventitious plantlets used in the experiment came from plants grown in a lowland area near the experiment site. After adventitious plantlets were emitted, they were removed from the plant and placed in Styrofoam® trays, which remained in a greenhouse until transplanted to the final pots used during the experiment.

The water-draining holes in 10-L white plastic pots were sealed with Durepoxi® resin. The pots were filled with substrate consisting of 20% vermicompost, 40% rice hulls, and 40% dirt, and fertilized according to a soil chemical analysis performed by the Soils Sector of the Natural Resources Department of Faculdade de Ciências Agronômicas de Botucatu (Botucatu Agricultural Sciences College), of Universidade Estadual Paulista (São Paulo State University) – UNESP, Brazil. *Echinodorus grandiflorus* plantlets 10 cm in height with 4 to 5 leaves were transplanted to the pots containing substrate, which was kept saturated with water up to the edge of the pot; the water was replaced as the level decreased, according to Joaquim (2000).

A completely randomized experimental design was used, with 5 replicates of 5 pots each.

The collection of leaf from *E. grandiflorus* plants was made in the seasons half, for chemical analysis of epicuticular waxes contents. All leaf blades collected were packaged in paper bags and placed to dry in a model 315SE - FANEM drying oven at

60ºC, at the Chemistry Laboratory of Instituto de Engenharia, of Universidade do Vale do Paraíba – UNIVAP.

The dry material was taken to the Department of Botany's Phytochemistry Laboratory, of Instituto de Biociências at Universidade de São Paulo (São Paulo University) – USP, São Paulo, Brazil, where phytochemical analyses of the leaves of *E.grandiflorus* were performed. The extraction and analysis of epicuticular waxes was performed according to Silva Fernandez et al. (1964).

The epicuticular wax content ($\mu g\ cm^{-2}$) per unit surface area was calculated by the ratio between the amount of wax obtained (μg) and the leaf area (cm^2) (multiplied by two, the abaxial and adaxial surfaces).

$$\text{Wax content} = \frac{\text{amount of wax}}{\text{leaves area } (cm^2) \times 2}$$

The leaf area (cm^2) was determined with a LI-3100 Area Meter digital device from Li-Cor at the Department of Botany of Instituto de Biociências de Botucatu - UNESP.

In order to analyze the profile of the n-alkanes in the epicuticular waxes, these were separated from the crude wax by aluminum oxide column chromatography, using hexane as the mobile phase. The fraction thus obtained was dried in a double boiler at 60°C and redissolved in hexane.

The n-alkane fraction was analyzed at the Phytochemistry Laboratory of the Department of Botany of Instituto de Biociências at Universidade de São Paulo – USP. We injected 1μL of the sample in a model HP 5890 series II Plus gas liquid chromatograph. The concentration varied according to the amount of alkane obtained; a HP-5 (30 m × 0.25 mm) column was used, at an initial temperature of 100°C for 5 minutes, with an increment of 10°C per minute until a temperature of 300°C was reached. Helium was used as carrier gas, at a constant flow of 1.56 mL $minute^{-1}$. The temperature for both the injector and the detector was 300ºC.

The peaks obtained in the chromatogram were identified with a HP 5989 B mass spectrophotometer, attached to the 5890 series II Plus gas liquid chromatograph operated in the split mode. The mass spectra were obtained using electron impact ionization, with an ionization energy of 70eV. Alkane identification was achieved by the mass spectra obtained, which were compared with the mass spectra of standards injected in the chromatograph and also with spectra found in the Wiley 275L library.

Each component of the fraction was quantified by the integration of the peaks obtained, and the percentages of substances identified in the analyzed fractions were calculated.

The relationship between alkanes with even and odd carbon numbers, mentioned in the discussion regarding this topic, were performed by the sum of the percentages of odd numbers of carbon atoms obtained then divided by the sum of the percentages of the even numbers of carbon atoms.

Phenological observations such as flowering period and emission of adventitious plantlets were made every fifteen days, before collecting the leaf blades of *Echinodorus grandiflorus*.

RESULTS AND DISCUSSION

Flowering and emission of adventitious plantlets of *E. grandiflorus*

During the experiment, it was observed in all treatments that the *Echinodorus grandiflorus* plants began flowering in the spring (end of October), ending practically in the beginning of March (summer), with a significantly higher intensity from November to January. It is suggested that translocation of metabolites occurs from the leaves into the flowers with a defensive function and for attraction of pollinators.

Emission of adventitious plantlets was also observed in the months from November to January and leaf senescence was verified in the winter.

In Rio Grande do Sul (southern of Brazil), Rego (1988) reported that an interruption of *E. grandiflorus* flowering was observed in the winter, followed by significant senescence of leaves; the same was verified by Vieira and Lima (1997) in Viçosa (State of Minas Gerais), at southeastern of Brazil. The latter authors reported that in Viçosa, *E. grandiflorus* flowered from October to January, during the rainy season, reaching the flowering peak in November.

Pimenta (1993) reported that the cyclic behavior of *E. grandiflorus* showed a marked difference between winter and summer; the same was verified in other perennial monocotyledons with the presence of rhizomes. In these plants, leaf development occurs in the summer, while in the winter there is accumulation of reserves in the rhizome, with new regrowth in the spring.

In recent studies, Pimenta (2003) observed a higher amount of inflorescences in *E. grandiflorus* grown in the full sun and at 50% shading in Rio de Janeiro, in the Brazilian Southeast; he also verified that the population of plants with smaller leaves suffered greater interference from light than the population that showed larger leaves.

In general, the phenological aspects of E*chinodorus grandiflorus* addressed in the present work, under the conditions to which plants were submitted, corroborate the data described above.

Epicuticular wax contents

The smallest epicuticular wax contents in leaves of *E. grandiflorus* plants were observed in the summer (10.3 μ cm^{-2}), without significant change in the fall (11 μ cm^{-2}) (Table 1). Contents increased in the winter (14 μ cm^{-2}) and peaked during the spring (17.8 μ cm^{-2}). There was significant difference between the different seasons of the year, suggesting its influence in the synthesis and deposition of epicuticular waxes (Table 1).

Table 1. Mean epicuticular wax contents (μg cm^{-2}) in the leaf blades of *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli plants in different seasons of the year.

Season	Epicuticular wax contents
Fall	11.1±1.1 B
Winter	14.0±1.9 A
Spring	17.8±3.9 A
Summer	10.3±2.7 B

Means followed by the same letter are not significantly different by Tukey test ($p < 0.05$).

The epicuticular wax content analysis performed in this work is pioneer for *E. grandiflorus*. In general, it was verified that the quantities found were similar to those found by Amaral et al. (1990) in studies involving several aquatic plants; however, *E. grandiflorus* showed higher contents. Amaral (1985) obtained 14.4 μg cm^{-2} of epicuticular wax for *Pistia stratiotes*; the author mentioned that these values are near the values found in terrestrial plants; the same can be stated with reference to *E. grandiflorus*.

With regard to the structural characteristics of leaves in emergent hydrophyte plants, Sculthorpe (1967) reported that they are similar to those in terrestrial plants, and added that both in plants with emerging leaves and in those with floating leaves, the cuticle also has the function of restricting water loss, in addition to a protective function against fungi and epiphytic plants that may become installed on the leaf.

The difference in contents observed in the epicuticular wax of *Echinodorus* perhaps could be explained by the change in the typical temperature for each season of the year. In studies that evaluated the influence of seasonality on epicuticular wax content, Jenks et al. (2002) verified in three species of *Hosta* (Liliaceae) that the highest contents were obtained during the spring. The wax content in *Quercus robur* leaves per dry mass or per cm^2 of leaf surface doubled in the months of May and June (therefore, in the spring). The leaflets that involve the buds showed different wax contents and composition when compared with mature leaves. In addition, tetracosanol was the dominant compound found in the epicuticular wax during leaf development (Gulz and Muller, 1992).

E. grandiflorus showed intensive flowering from November to January (spring/summer), suggesting that the secondary metabolites produced in the leaf may have been translocated from the leaf to the flowers, with a defensive function or even for the attraction of pollinators (Joaquim, 2004). To make up for the low levels of these metabolites in the leaf, wax production was intense, especially in the spring, thus playing its role as a barrier against insects, pathogens, and herbivory; these functions have been mentioned, among others, by Edwards et al. (1982) and Harborne and Turner (1984).

Wiermann (1981) mentioned that there is a number of cases indicating that the plant developmental stage influences in the secondary metabolite synthesis. Generally, it seems that the onset of flowering significantly influences alkaloid biosynthesis, decreasing or even inhibiting their synthesis. In *Lupinus,* the synthesis of alkaloids does not start until two weeks after germination and continues until the plant initiates flowering (Wiewiorowski et al., 1966 apud by Bell, 1981).

Profile of n-alkanes

Amaral (1985) and Amaral et al. (1990) conducted studies with several aquatic species and highlighted that, in general, researches on the profile of the *n*-alkanes of leaf epicuticular waxes in aquatic angiosperms are very limited.

The profile analysis results for the epicuticular wax *n*-alkanes of *E. grandiflorus* are presented in Table 2. The chromatogram obtained by gas liquid chromatography of the mixture of *n*-alkane standards is represented in Figure 1 e 2, in addition to example chromatograms of the *n*-alkane profiles obtained from *E. grandiflorus* epicuticular wax in different seasons of the year.

From Table 2 it can be noted that the distribution of *n*-alkanes along the seasons of the year showed variation, with slight predominance of homologues with an odd number of carbon atoms in relation to homologues with an even number. It was also verified that homologues C_{24} (tetracosane), C_{25} (pentacosane), C_{26} (hexacosane), C_{27} (heptacosane), C_{28} (octacosane) and C_{29} (nonacosane). In several analyses, there was no predominance of homologues with an odd number of carbon atoms in relation to even-number homologues.

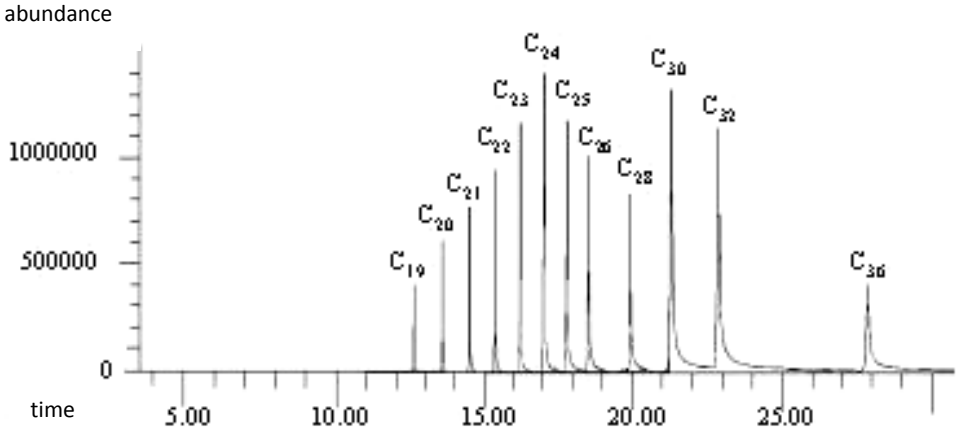

Figure 1. Chromatogram obtained from a mixture of *n*-alkane standards. The upper case letter C followed by subscript numbers corresponds to the number of carbon atoms in the chain. Numbers on the x-axis correspond to retention times.

In the fall, the main homologues found in the paraffin of plants were C_{27} and C_{29} (13%), followed by C_{25}, C_{26}, and C_{30} (12%), and C_{24} and C_{28} (10%). In the winter, plants showed C_{27} (19%), followed by C_{25} (17%), C_{26} (15%), C_{29} (11%) and C_{28} (8%) as main homologues. In the spring, the main homologues found in the

paraffin of plants were C_{27} and C_{29} (13%), followed by C_{25} (12%), C_{26} (11%), C_{28} (9%), and C_{23} and C_{24} (8%). In the summer, plants showed paraffins with C_{27} (19%), followed by C_{29} (17%), C_{31} (15%), C_{25} (12%), and C_{26} and C_{24} (9%) as main homologues.

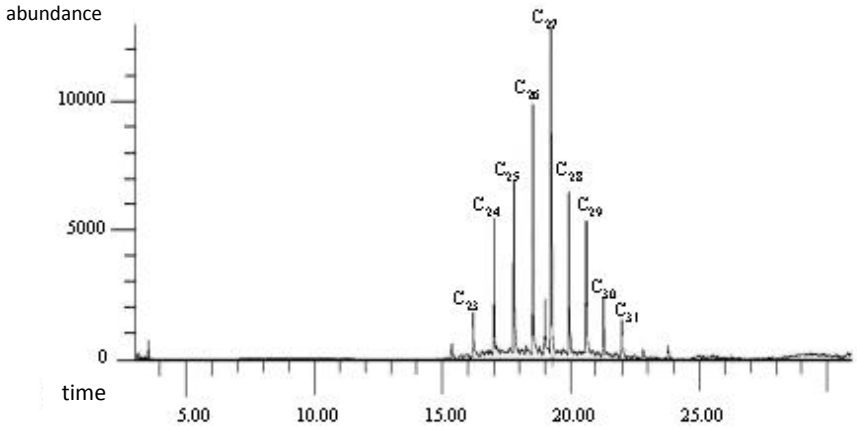

Figure 2. Chromatogram obtained from the profiles of *n*-alkanes in the epicuticular wax of leaf blades of *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli plants in the winter. The upper case letter C followed by subscript numbers corresponds to the number of carbon atoms in the chain. Numbers on the x-axis correspond to retention times.

According to Harborne and Turner (1984), in the homologue series the number of odd carbons are predominant over even carbons, most times at a 10:1 ratio; however, Herbin and Robins (1968) mentioned that a predominance of odd over even carbons, in the alkane homologue series of leaf epicuticular wax is not universal.

Table 2. Distribution of *n*-alkanes (%) in the leaf blades of *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli plants in different seasons of the year.

	Percentage of *n*-alkanes													
Season/Treatment	C_{20}	C_{21}	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}
Fall	2	2	3	6	7	12	12	13	10	13	12	7	1	1
Winter		1	2	6	11	17	15	19	8	11	4	6		
Spring	1	2	5	8	8	12	11	13	9	13	7	9	1	1
Summer	1	2	3	6	9	12	9	19	5	17	2	15		

In general, the percentages of *n*-alkane homologues, especially from C_{24} to C_{29}, were distributed in a practically homogeneous way. Therefore, it was observed that long-, linear-, and saturated-chain hydrocarbons, especially those having between 21 and 31 carbon atoms, were found in the epicuticular wax of *Echinodorus grandiflorus*.

Sculthorpe (1967) reported that emergent hydrophyte plants present structural leaf characteristics similar to those of terrestrial plants, and that aquatic angiosperms are derived from terrestrial plants. The author also added that plants with emersed leaves live in the same environment as the leaves of the terrestrial vegetation, showing both structural and anatomical characteristics related to the mechanical and physiological problems that result from exposure to the air. According to Baker (1982), the *n*-alkanes of higher plants consist of homologues containing chains from C_{17} to $C_{35,}$ with odd numbers of carbon atoms. Frequently, the main homologues found are nonacosane (C_{29}), hentriacontane (C_{31}), and tritriacontane (C_{33}), as reported by Bianchi (1995).

Amaral (1985) and Amaral et al*.* (1990) performed the extraction of epicuticular wax from emersed and submerged plants, and verified, via *n*-alkane hydrocarbon analysis, that emersed (*Myriophyllum brasiliense*, *Sphagnum cuspidatum*) and floating plants (*Eriocaulon aquatile*, *Eichornia crassipes*, *Nymphaea mexicana*, *Pistia stratiotes*, *Salvinia herzogii*) showed alkane profiles with quite significant similarity, and were therefore very much alike the profile of terrestrial plants. Emersed plants, such as *Myriophyllum brasiliense*, showed C_{29} as main homologue (47.7%), followed by C_{27} (10.5%); *Sphagnum cuspidatum* showed C_{23} (7.0%), and C_{29} (6.9%), followed by C_{27} (6.0%); floating plants like *Eriocaulon aquatile* showed C_{27} (11.4%) as main homologue; *Eichornia crassipes* showed C_{31} (28.8%), followed by C_{29} (26.3%) and C_{27} (18.6%) as main homologues; *Nymphaea mexicana* showed C_{29} (41.2%) as main homologue; *Pistia stratiotes* showed C_{31} (22.6%) as main homologue, while *Salvinia herzogii* showed C_{23} (9.1%). The authors called the attention to the fact that the *Sphagnum* and *Salvinia* plants showed C_{23} as main homologue and that submerged plants also showed a higher proportion of constituents smaller than C_{27}, while emersed and floating plants showed a higher proportion of constituents containing more than 27 atoms of carbon. They emphasized that the emersed aquatic plant *Myriophyllum* showed a hydrocarbon composition very similar to that of most terrestrial plants, while in submerged plants the alkane carbon atom chains are smaller than in emersed plants (C_{23} – C_{29}). Studies conducted by Ficken et al*.* (2000) revealed that submerged or floating species show a marked abundance of *n*-alkanes C_{23} and C_{25}, and therefore present medium-sized carbon chains. On the contrary, emergent aquatic plants have an *n*-alkane distribution similar to terrestrial plants, with special predominance of long-chain homologues greater than C_{29}.

In the present study, the leaves of *E. grandiflorus* plants showed a *n*-alkane profile intermediate between terrestrial and aquatic plants. For this reason, some

authors refer to it as "amphibian", having C_{27} (heptacosane) as main homologue, followed by C_{25} and C_{29} (the latter being typical of terrestrial plants). These homologues have an odd number of carbon atoms, and these results corroborate those obtained by Amaral (1985), Amaral et al. (1990), and Ficken et al. (2000).

CONCLUSIONS

Under the study conditions and considering the proposed objectives, it can be concluded that:

- Phenology of *Echinodorus grandiflorus* (Cham. & Schldl.) Micheli showed that the flowering peak and the emission of adventitious plantlets occurred between November and January; besides, leaf senescence was detected in the winter.

- the production of epicuticular wax in leaf blades of *E. grandiflorus* was intense in the spring;

- with regard to the profile analysis of *n*-alkanes in epicuticular waxes of *E. grandiflorus*, the plant showed an intermediate profile between terrestrial and aquatic plants, having C_{27} as main homologue.

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